Genetic Analysis of Bacteriophage P22 Lysozyme Structure

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ABSTRACT

The suppression patterns of 11 phage P22 mutants bearing different amber mutations in the gene encoding lysozyme (*19*) were determined on six different amber suppressor strains. Of the 60 resulting single amino acid substitutions, 18 resulted in defects in lysozyme activity at 30°; an additional seven were defective at 40°. Revertants were isolated on the "missuppressing" hosts following UV mutagenesis; they were screened to distinguish primary- from second-site revertants. It was found that second-site revertants were recovered with greater efficiency if the UV-irradiated phage stocks were passaged through an intermediate host in liquid culture rather than plated directly on the nonpermissive host. Eleven second-site revertants (isolated as suppressors of five deleterious substitutions) were sequenced: four were intragenic, five extragenic; three of the extragenic revertants were found to have alterations near and upstream from gene *19*, in gene *13*. Lysozyme genes from the intragenic revertant phages were introduced into unmutagenized P22, and found to confer the revertant plating phenotype.

THE basic protein structure problem is easy to state, though it has proven hard to solve: the precise three-dimensional structures of proteins are determined by their amino acid sequences; understanding of the rules by which this happens would permit us to solve protein structures by sequencing, as well as to design new proteins. Some of the most promising approaches to the protein structure problem have made extensive use of mutant proteins. High resolution X-ray crystallographic studies of thermosensitive mutant phage T4 lysozymes have probed the structural determinants of protein stability (reviewed by MATTHEWS 1987). The study of series of mutant proteins in which defined stretches of polypeptide have had their amino acid sequences randomized provides a view of the limits of the sequence plasticity of specific structural elements in proteins (REIDHAAR-OLSON and SAUER 1988). In this paper, we describe a genetic approach to the protein structure problem that centers on the isolation of intragenic second-site revertants of mutations that inactivate a protein, in this case the lysozyme of bacteriophage P22. A related approach has been employed by SHORTLE and LIN (1985) in the study of thermostability determinants in staphylococcal nuclease.

P22 lysozyme is essential for lysis of the host cell in the phage's lytic growth cycle (BOTSTEIN, CHAN and WADDELL 1972; RENNELL and POTEETE 1985). It is a small protein, with an activity profile that closely resembles that of phage T4 lysozyme (RAO AND BURMA 1971). The sequence of its gene, *19*, indicates that it is a basic polypeptide of 145 residues and a molecular mass of 16 kilodaltons (RENNELL and POTEETE 1985); its gel filtration characteristics suggest that it is monomeric (RAO and BURMA 1971). P22 and T4 lysozymes exhibit significant sequence homology; the details of this homology strongly suggest that the two enzymes are closely related in their "core" structures, including the active site clefts (WEAVER *et al.* 1985).

The genetic scheme detailed below entails the following steps: (1) Amber mutations are introduced into the P22 lysozyme gene, and amino acid substitutions are effected by growing the mutant phage on a set of amber suppressor-bearing hosts. (2) Deleterious substitutions caused by the insertion of amino acid residues that do not work at the position in question (an outcome hereafter referred to as "missuppression") are identified by the resulting deficiencies in plaque formation by the mutant phage. (3) Following mutagenesis, revertants are selected as plaque-forming variants of the mutant phages on missuppressing hosts. (4) Second-site revertants are distinguished from primary-site revertants by their inability to form plaques on a non amber suppressor-bearing host. (5) Lysozyme genes of the candidate double mutant phages are sequenced. (6) Sequenced double mutant lysozyme genes are subcloned, then reintroduced into wild-type P22 to verify that the second-site mutation(s) in the lysozyme gene, rather than an extragenic mutation in the revertant phage, is responsible for suppression of the primary mutant phenotype.

The goal of our study is to generate, by a model-free approach, mutant proteins that have significant structural alterations, which can be attributed to a

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limited number of amino acid substitutions. Such mutants might be exceptionally informative in structural studies: mutants that have been examined to date generally cause only small alterations of the protein structure in the immediate vicinity of the changed residue (MATTHEWS 1987). Results presented below lead us to believe that the scheme outlined above can produce such structural variants.

MATERIALS AND METHODS

Enzymes, buffers and media: T4 DNA ligase, restriction endonucleases and *E. coli* DNA polymerase I large fragment were purchased from New England Biolabs. AMV reverse transcriptase, *Hpa*I and *Sal*I restriction endonucleases were purchased from Boehringer-Mannheim. P22 tail protein was a gift from Peter Berget. Hen egg white lysozyme was purchased from Sigma; it was used at concentrations of 20–50 μg/ml to lyse induced cultures of lysis-defective prophage-bearing strains.

Restriction, kinase, and ligase buffers were as recommended by the suppliers. Precipitation buffer was 20 mM Tris HCl (pH 8.0), 10 mM NaCl, 2 mM EDTA, 0.5 M ammonium acetate. Buffer C was 200 mM Tris HCl (pH 7.6), 100 mM $MgCl_2$, 500 mM NaCl, 10 mM dithiothreitol (DTT). Buffer D was 200 mM Tris HCl (pH 7.6), 100 mM $MgCl_2$. DNA buffer was 10 mM Tris HCl (pH 7.4), 5 mM NaCl, 1 mM EDTA. Buffered saline was 41 mM Na_2HPO_4, 22 mM KH_2PO_4, 130 mM NaCl (pH approximately 7).

LB broth contained 10 g tryptone, 5 g yeast extract, 5 g NaCl, and 1 ml 1 M NaOH per liter. LB agar contained, in addition, 15 g agar per liter. LB medium was supplemented with appropriate antibiotics at the following concentrations: 10–20 μg/ml tetracycline, 50–100 μg/ml ampicillin, or 20 μg/ml kanamycin. Soft agar contained 10 g tryptone, 2.5 g NaCl and 9 g agar per liter. Minimal medium was M9CAA (SMITH and LEVINE 1964) with the casein hydrolysate omitted, and 1% glucose added. Lambda agar contained 10 g tryptone, 2.5 g NaCl and 11 g agar per liter.

Plasmids: Plasmid constructions were done by standard methods (MANIATIS, FRITSCH and SAMBROOK 1982). Transformation of *Salmonella* was carried out as described by Lederberg and Cohen (1974). Plasmids constructed for these studies are derivatives of pBR322 (BOLIVAR *et al.* 1977); details of their constructions are given in Table 1. Plasmids pGFIB:phe and pGFIB:cys (NORMANLY *et al.* 1986); pDR105, pDR118, pDR110, pDR100, and pDR116 (RENNELL and POTEETE 1985); pTP352 (KNIGHT *et al.* 1987); pTP30 (BERGET, POTEETE and SAUER 1983); pZ152 (ZAGURSKY and BERMAN 1984); and pKM101 amp^R (YOUDERIAN, BOUVIER and SUSSKIND 1982) were previously described. Plasmid pPB20::Tn*5*-13 was supplied by Peter Berget. Plasmid pBR322 *bla-oc* contains an ochre mutation in its β-lactamase-encoding gene (D. BOTSTEIN, personal communication).

Plasmid pTP399 (unpublished) was used to construct a deletion-substitution allele of gene *19*. It contains the following DNA sequences: (1) a *Pvu*II-*Eco*RI origin of replication-containing fragment from pZ152, with the *Eco*RI end filled in and joined via a *Sal*I linker (GGTGCACC) to (2) a fragment of P22 DNA extending from the *Rsa*I site in gene *13* to the first *Hin*fI site in gene *19* (including the first eight codons of gene *19*), with the *Hin*fI end joined via a synthetic linker sequence (5′ AATCTAAGC 3′ + the partially complementary 5′ TAAGCTTAG 3′) to (3) the *Dde*I site immediately upstream from phage T4 gene *e*, which is followed by all of gene *e* and 41 bp downstream, then a *Bgl*II linker, then (4) P22 DNA including the last 70 bp of gene *19* and additional sequences to the *Hpa*I site 384 bp downstream from gene *19*, with the *Hpa*I end joined via a *Bam*HI linker to the *Pvu*II end of the pZ152 fragment.

TABLE 1

Plasmid Construction

pDR281	Ligation of the *Eco*RI-*Bam*HI gene *19*-containing fragment of pDR118 to the *Eco*RI-*Bam*HI origin-containing fragment of pZ152
pTP289	Ligation of the *Hin*dIII-*Bam*HI replication origin-containing fragment of pBR322 with the *Bam*HI-*Nae*I fragment of pDR100 containing the carboxy-terminal portion of gene *19*, and a fragment containing the amino-terminal portion of gene *19* generated by digesting pDR110 with *Eco*RI, filling in the ends, and digesting with *Hin*dIII. A *Bgl*II linker (5′ CAGATCTG 3′) is inserted between the *Nae*I and filled-in *Eco*RI ends
pDR321	Ligation of the large internal *Hpa*I fragment of Tn*5* (from pPB20::Tn*5*-13), with linearized pTP289 generated by digesting with *Bgl*II and filling in the ends
pDR392	Ligation of the gene *19*-containing fragment generated by digesting pDR116 with *Cla*I, filling in the ends, then digesting with *Bam*HI, with the *Pst*I-*Pvu*II P_{lac}-containing fragment of pTP30, and the *Pst*I-*Bam*HI origin of replication-containing fragment of pTP352
pDR401	Ligation of the gene *19*-containing fragment of pDR392 generated by digesting with *Eco*RI + *Bam*HI and filling in the ends, with linearized pBR322 *bla-oc* generated by digesting with *Eco*RI and filling in the ends.
pDR463	Ligation of linearized pGFIB:Phe generated by digesting with *Cla*I and filling in the ends, with the tetracycline resistance gene-containing fragment of pBR322 generated by digesting with *Eco*RI + *Pvu*II and filling in the ends
pDR464	Same construction as pDR463, starting with pGFIB:Cys
pDR469	Ligation of the large internal *Hpa*I fragment of Tn*5* (from pPB20::Tn*5*-13) with the replication origin-containing fragment of pTP399 generated by digesting with *Bgl*II and *Hin*dIII and filling in

Bacteria: *Escherichia coli* W3110 *lacI*^{q} *L8* (BRENT and PTASHNE 1981) was used for propagation of plasmids and for growth of lambda phage. W3110 (prototroph *sup*^{o}) and MM294 (*endo*I$^-$ *thi* $r_K^-m_K^+$ *su*II) were used as hosts to prophages P22 *Kn469 sieA44 m44* and P22 *Kn321 sieA44 m44* respectively. Strain GM1675 (*dam-4* Δ(*lac-pro*) *thi-1 supE relA1*/F′ *lacI*^{q} ΔM15 pro$^+$, used for propagating phage f1 *IR1* and generating single-strand plasmids, was provided by MARTIN MARINUS. All other strains are derivatives of *Salmonella typhimurium* LT2. Strains MS1362, MS1363, MS1364, MS1365 (all *leuAam414*, bearing the amber suppressor alleles *supD*, *supE*, *supF*, *supG*, respectively), DB7000 (*leuAam414*), MS1868 (*leuAam414* r^- m^+), MS2310 (MS1868 bearing the plasmid pKM101 amp^R) and MS1387 (*supQ pro* (Δ *attP22*) *cysB his leuD fol101*/F′ *lac pro*) were

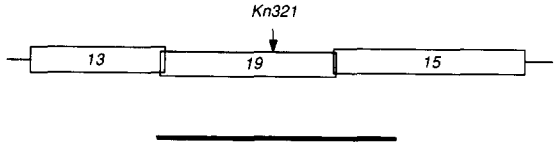

FIGURE 1.—Map of the P22 chromosome in the vicinity of gene *19*, drawn approximately to scale. The point of insertion of *Kn321* is indicated. The bar underneath indicates the P22 sequences borne by plasmids used for oligonucleotide-directed mutagenesis of gene *19*. The three genes shown have functions in bacterial lysis. Gene *13* is a homolog of the phage λ *S* gene; it encodes a protein that promotes bacterial lysis, probably by making pores or lesions in the cytoplasmic membrane through which lysozyme (the product of gene *19*) gains access to the peptidoglycan (RENNELL and POTEETE 1985). Gene *15* is a newly identified homolog of the λ *RZ* gene; it is required for bacterial lysis in the presence of some divalent cations (CASJENS *et al.* 1989).

from MIRIAM SUSSKIND. Strain CV112 (*polAts*) was provided by ANDREW WRIGHT. TP278, TP279, TP280, TP282 are MS2310 with *supE*, *supF*, *supG*, and *supD*, respectively. TP284 is MS1868 lysogenized with P22 *sieA44 Ap2 Δ7283*. TP308 and TP309 are MS2310 bearing pDR463 and pDR464, respectively.

Suppressor-bearing strains used for determination of suppression patterns and reversion of missuppressed amber mutants were maintained and cultured by the following procedures. Following construction by transduction or transformation, purified clones were grown in minimal medium (supplemented with ampicillin, and, where appropriate, tetracycline), and portions of the cultures were stored with cryoprotectant at −80°. In addition, working stocks were made by addition of glycerol to 50% (v/v) and storage at −20°. Cultures were grown by streaking from the glycerol stocks on LB agar; after incubation at 30° overnight, isolated colonies were restreaked on minimal agar plates supplemented with antibiotics, which were incubated at 30° for 24 hr. Single colonies were used to inoculate 5 ml of minimal medium with antibiotics; the cultures were aerated at 30° until they reached a density of approximately 1 × 10^8/ml, and used the same day for plating. These procedures were especially important for TP308 and TP309, because their suppressor-bearing plasmids were readily lost if selection was not maintained. Plating of TP308 and TP309 for plaque assays was done on LB-tetracycline agar; the other strains were plated on lambda agar.

Phage: λ *Ram5* cI^- was used for complementation tests. Phage f1 *IR1* was used to package single stranded plasmids. The remainder of the phages used were P22. Synthetic amber mutations were introduced into P22 *sieA44 m44*. P22 *HT* was used for transductions. A lysogen of P22 *Ap2 sieA44 Δ7283* (deletion of the *imm*I region) was used to titer defective oversized P22, as described by WEINSTOCK, SUSSKIND and BOTSTEIN (1979). Phage bearing amber mutations in gene *19* were P22 *19amH1162* (codon 64) *h21 c1–7*, P22 *19amH1164* (codon 113) *h21 c1–7*, P22 *19amH1239* (codon 122) *h21 c1–7*, P22 *19amN111* (codon 80) $c2^-$, P22 *19am12* (codon 12) *sieA44 m44*, P22 *19am22* (codon 22) *sieA44 m44*, P22 *19am39* (codon 39) *sieA44 m44*, P22 *19am61* (codon 61) *sieA44 m44*, P22 *19am65* (codon 65) *sieA44 m44*, P22 *19am82* (codon 82) *sieA44 m44*, and P22 *19am83* (codon 83) *sieA44 m44*; hereafter, these phages will be designated by their gene *19* alleles only; the allele names will have the form "*amN*," where *N* is the number of the amber codon. The phage used in immunity tests were P22 *vir3*, P22 *c2–5*, and P22 *c1–7*.

P22 *Kn321 sieA44 m44* and P22 *Kn469 sieA44 m44* were used to transfer mutant alleles of gene *19* from plasmids to phage. They were constructed by crossing P22 *sieA44 m44* with pDR321 and pDR469, respectively. MS1868 bearing pDR321 or pDR469 was infected with P22 *sieA44 m44* at a multiplicity of 10. After 2 hr of growth at 37°, chloroform was added, and debris was removed by centrifugation at 5900 × *g* for 5 min; the resulting lysate was titered on TP284, then used to infect CV112, grown to a density of 1 × 10^8/ml, at a multiplicity of 10. Kanamycin resistant transductants were selected at 37°, at which temperature the *polAts* host is unable to support the replication of pDR321. Colonies were screened for ampicillin sensitivity, immunity to P22 infection, and inability to yield plaque-forming phage when induced by UV-irradiation.

P22 *Kn469 sieA44 m44* was transferred to *E. coli* W3110 as follows. CV112 bearing the defective prophage was grown in LB broth supplemented with kanamycin to a density of 1 × 10^8/ml at 37°; mitomycin C was added (2.4 μg/ml final concentration) and the culture was aerated for 3 hr at 37°. A lysate was obtained after treating the cells with chloroform and hen egg white lysozyme and then centrifuging at 5900 × *g* for 5 min to remove cell debris. The lysate was used to infect the Salmonella F′-bearing strain MS1387, in which the only P22 attachment site is episomal. Lysogens were selected by spreading the infected cells on kanamycin plates. The colonies were screened for P22 immunity and inability to yield plaque-forming phage. A colony that passed these tests was used to mate with *E. coli* strain W3110. The *E. coli* lysogen was selected on minimal plates supplemented with kanamycin. A similar procedure was used to transfer P22 *Kn321 sieA44 m44* to *E. coli* strain MM294.

Primer-directed mutagenesis: The primers were 15 base oligonucleotides synthesized by the University of Massachusetts DNA synthesis facility. They were designed to introduce amber codons into P22 gene *19*. The sequences of the primers were: am12(Lys) 5′ AGATTATAGCGTGAA 3′; am22(Tyr) 5′ GCCTAGTCAGATAGC 3′; am39(Val) 5′ GGAAAATAGGATGGT 3′; am61(Glu) 5′ CTGCTTAAATAGGAT 3′; am65(Trp) 5′ TTGCAGTAGGTTGAA 3′; am82(Gln) 5′ CTAAATTAGTATGAT 3′; am83(Tyr) 5′ AACCAGTAGGATGCG 3′. (Bases that differ from the wild-type sequence are underlined.)

Strain GM1675 bearing plasmid pDR281 was infected with f1 *IR1* and single stranded template was isolated according to ZAGURSKY and BERMAN (1984). *In vitro* extension-ligation reactions were done by mixing 0.6–1 pmol of single strand target plasmid with 15–17 pmol of mutagenic primer, 10 pmol of a second primer designed to anneal to pBR322 sequences in the plasmid (5′ GTATCACGAGGCCCT 3′, purchased from New England Biolabs), 1 μl of buffer C, and 5 μl of water. The mixture was incubated at 65° for 5 min, then placed at room temperature for 30 min. To this annealing reaction, 10 μl of an enzyme mixture which contained 1 μl buffer D, 1 μl of a solution containing 2.5 mM each of dATP, dGTP, dTTP, dCTP, 1 μl 100 mM ATP, 1 μl 100 mM DTT, 2 μl (20 units) *E. coli* DNA polymerase I large fragment and 2 μl T4 DNA ligase was added. The mixture was incubated at 15° overnight. In addition to pDR281, two other plasmids were used as targets for primer-directed mutagenesis. Plasmid pDR392 is closely related to pDR281; in it, gene *19* is transcribed from P_{tac}*UV5* instead of P_{tac}. Plasmid pDR401 was used in a mutagenesis procedure in which the target molecule was a gapped circular heteroduplex. In all cases, the segment of P22 DNA contained in the target plasmid was the same.

Screen for lysozyme mutants: Ampicillin-resistant colonies that arose from transforming W3110 $lacI^q$ L8 with the

in vitro extension-ligation reaction mixtures were screened for the introduced amber mutation by a complementation test. Approximately 1×10^8, 1×10^6, and 1×10^4 plaque-forming units of λ *Ram5 cI* were streaked in lines on a lambda plate and the transformants were streaked across the lines from most dilute to most concentrated using a toothpick. Transformants that did not lyse, due to a failure of the mutagenized plasmid to complement the lysis-defective λ, were found at a frequency of 1–40%.

Transfer of amber alleles to P22: Plasmid DNA prepared from colonies that were scored as lysozyme minus was used to transform strain MS1868 (P22 *Kn321 sieA44 m44*) or MM294 (P22 *Kn321 sieA44 m44*) to ampicillin resistance. Cultures were grown with aeration at 37° to a density of approximately 2 X 10^8/ml. Mitomycin C was added to a concentration of 2.4 μg/ml and the cultures were aerated for an additional 4 hr. Chloroform and hen egg white lysozyme were added to lyse the cultures. The debris was spun out by centrifugation at 5900 × *g* for 5 min, 1×10^{11} phage tails were added per ml of lysate, and portions were plated on suppressor-bearing Salmonella strains.

Sequencing phage DNA: In the preparation of P22 stocks, phage were pelleted from lysates by centrifugation, and resuspended in buffered saline. DNA was extracted from 0.4 ml of phage stock in a microfuge tube by adding 2 μl diethylpyrocarbonate, 10 μl 10% SDS and 50 μl 2 M Tris-HCl 0.2 M Na_2EDTA (pH 8.5), and incubating uncovered at 70° in a fume hood for 5 min. Protein and SDS were precipitated by addition of 50 μl 5 M potassium acetate, followed by incubation on ice for 30 min, and centrifugation in a microfuge for 10 min. The supernatant was transferred to a new tube, and the DNA was precipitated with 2.5 volumes of ethanol. The DNA was spun onto a sealed glass pasteur pipet and transferred to a clean microfuge tube containing 200 μl precipitation buffer. Following extraction with phenol and ether the DNA was precipitated with ethanol, pelleted by centrifugation in a microfuge, dried under vacuum, and redissolved with 50 μl DNA buffer. The usual yield from this procedure was about 50 μg DNA. The phage DNA was digested with *Hin*dIII, heat denatured, and used without further purification in the sequencing reactions. The sequencing primers were: (1) 5′ CTCGATTGGTTCGCT 3′; (2) 5′ GTTGGGCATACCGGA 3′; (3) 5′ AGTAGTCTTGTTCGC 3′; (4) 5′ GCTTTCCTGTTATGG 3′. The primers were phosphorylated in a reaction mixture that contained [γ-^{32}P]ATP. Sequencing reactions were carried out as described (KNIGHT *et al.* 1987). All revertants were sequenced from 32 bases 5′ of the start site of gene *19* to 25 bases 3′ of gene *19*. The original amber codon was confirmed in all cases.

Revertant isolation: Revertants of missuppressed amber mutations were isolated by two methods. (1) UV-generated revertants were isolated by direct plating of irradiated phage on missuppressing hosts. UV mutagenesis was performed by irradiating 1×10^{10} phage in 1 ml buffered saline in an open glass Petri dish with 3500 erg/mm^2 UV light at 254 nm as measured by a dosimeter (Ultra Violet Products). The phage were transferred to foil-wrapped glass tubes and stored at 4°. This treatment typically reduced the titer of the phage by a factor of 10^3 to 10^5, with 1–4% clear plaque formers among the survivors, when the UV-irradiated phage were plated on UV-stimulated (300 erg/mm^2) permissive suppressor strains bearing pKM101 amp^R. (Strains bearing pKM101 ampR were UV-stimulated before infection, for selection of revertants as well as determination of survivor titers, to induce SOS repair and mutagenesis; the induced cells reactivate UV-damaged phage, producing 10–40-fold higher titers than unstimulated, non-pKM101 amp^R strains–data not shown). (2) UV-generated revertants were isolated by plating on missuppressing hosts after passaging the irradiated phage through a partially permissive suppressor strain. Passaging was done by infecting 1 ml cultures of pKM101 amp^R-bearing, UV-stimulated suppressor cells at a density of 1×10^9/ml, with 1×10^9 UV-irradiated phage, allowing 20 min for adsorption, then diluting into 50 ml of LB broth and growing at 30° or 37° for 2–6 hr. Chloroform and hen egg white lysozyme were added and debris was pelleted by centrifugation at 5900 × *g* for 5 min. Plating for revertants was done on lambda agar at 40°, or on LB-tetracycline agar at 37° when TP308 or TP309 was used as host. (These strains grow poorly at 40°; well enough for scoring suppression patterns, but not for selection of revertants.)

Reconstruction of revertants: Alleles of gene *19* from revertant phages were subcloned and then reinstalled into unmutagenized P22. Gene *19*-containing fragments of 1.1 kb (band 6) were purified from *Hpa*I-digested phage DNA by electrophoresis in 5% bis-acrylylcystamine-crosslinked polyacrylamide gels (HANSEN 1981). Each *Hpa*I fragment was ligated with *Eco*RV-digested pBR322. The ligation reaction mixture was used to transform W3110 to ampicillin resistance. Plasmid DNA was isolated from tetracycline-sensitive transformants and screened by agarose gel electrophoresis. Candidates of the correct size were then used to transform W3110 (P22 *Kn469 sieA44 m44*) to ampicillin resistance. A clone was selected and grown in 50 ml of LB to a density of 2×10^8/ml; the culture was then induced with mitomycin C (40 μl at 0.4 mg/ml). After an additional 3 hr at 37°, the culture was lysed by addition of chloroform and hen egg white lysozyme. Debris was removed by centrifugation at 5900 × *g* for 5 min. P22 tails were added to 1×10^{10}/ml, and recombinant phage that had acquired the plasmid-borne gene *19* allele were recovered by plating on an appropriate Salmonella suppressor strain.

RESULTS

Collection and characterization of gene *19* amber mutants: A collection of P22 strains bearing amber mutations in eleven codons of the lysozyme gene was assembled. Identification of amber mutations in codons 64, 113, and 122 has been described (RENNELL and POTEETE 1985). Another amber mutation generated by random chemical mutagenesis was found by pairwise crosses and sequencing to affect codon 80 (data not shown). An additional seven amber mutations, in codons 12, 22, 39, 61, 65, 82 and 83, were transferred to P22 from plasmids following oligonucleotide-directed in vitro mutagenesis, as described in MATERIALS AND METHODS.

The relative plaque-forming abilities of the 11 amber mutants on a set of six amber suppressor strains are shown in Table 2. Of the 66 combinations tested, 60 represented amino acid substitutions; at 30°, 18 of these resulted in a significant diminution of the plaque-forming ability of the phage; seven of the 18 led to defects apparently as severe as those of unsuppressed amber mutants. In a screen for temperature-sensitive phenotypes, the amber mutants were plated on suppressor strains at 40° (Table 2). No mutants formed plaques better at 40° than at 30°, whereas 15

TABLE 2

Lysozyme amber mutant suprression patterns

Allele (wild-type residue)	Temp. (°C)	Amino acid inserted by suppressor strain[a] Gln	Tyr	Leu	Ser	Phe	Cys
am12 (Lys)	30	++	++	++	++	+	+/−
	40	+	++	++	++	+/−	−
am22 (Tyr)	30	+	++	++	++	++	−
	40	+	++	+	+	++	−
am39 (Val)	30	++	++	++	++	++	+
	40	++	++	++	++	++	+/−
am61 (Glu)	30	++	++	++	++	++	++
	40	++	++	++	++	++	+
am64 (Gln)	30	++	++	++	++	++	++
	40	++	++	++	++	++	++
am65 (Trp)	30	++	++	++	++	++	+
	40	++	++	++	++	++	+
am80 (Gln)	30	++	−	++	++	−	+/−
	40	++	−	++	++	−	−
am82 (Gln)	30	++	−	++	+	−	−
	40	++	−	++	+/−	−	−
am83 (Tyr)	30	++	++	+	+/−	++	−
	40	+	++	−	−	++	−
am113 (Gln)	30	++	++	++	++	++	+
	40	++	++	++	++	+	+
am122 (Trp)	30	++	++	++	++	++	+
	40	++	++	++	++	+	−

The entries represent the plaque-forming ability of the amber mutant phage on the indicated strain, relative to its plaque-forming ability on the non amber suppressor strain MS1868 with and without the lysozyme-producing plasmid pDR105. Key: ++ normal plaque size and efficiency of plating (e.o.p.) 0.5–1; + pinpoint plaques, e.o.p. 0.01–0.5; +/− e.o.p. 0.0001–0.01; − plaque-forming ability no greater than on non-suppressor, non plasmid-bearing strain.

[a] Strains: Gln-TP278; Tyr-TP279; Leu-TP280; Ser-TP282; Phe-TP308; Cys-TP309.

did noticeably worse; of these 15, none displayed a "clean" temperature-sensitive phenotype (*i.e.*, healthy at 30°, highly defective at 40°).

Isolation of second-site revertants: Revertants of missuppressed amber mutants were selected from stocks of UV-mutagenized phage, as described in MATERIALS AND METHODS. Primary and secondary-site revertants were distinguished by a simple test: primary-site revertants, which by definition do not have the original gene *19* amber codon, usually form plaques on a non-amber suppressor-bearing host; presumptive second-site revertants were identified by their failure to lyse the nonsuppressing host. One relatively uninteresting class of mutant that passed this test—primary site revertants that have acquired an amber mutation in some other essential gene as a consequence of mutagenesis—was eliminated by testing the revertant phage for ability to form plaques on a nonamber suppressor host bearing a lysozyme-expressing plasmid; phages unable to do this were discarded.

TABLE 3

Isolation of second-site revertants

Amber	Passaged through	Reverted on	Revertants screened	Second-sites	Sequenced alleles
am22	TP278	TP278	150	0	
	TP278	TP280	150	0	
	TP282	TP278	18	0	
	TP282	TP280	50	16	*sr517, sr520*
am39	(not passaged)	TP309	50	14	*sr575, sr581*
am82	TP278	TP279	50	0	
	TP278	TP282	50	0	
	TP279	TP282	50	0	
	TP282	TP279	150	0	
	TP282	TP282	100	5	*sr512-516*
am83	TP278	TP278	50	18	
	TP278	TP280	200	29	*sr542*
	TP280	TP278	50	7	
	TP280	TP280	50	12	*sr532*

Revertants from combinations of amber mutations and suppressors amounting to 18 defective substitutions were screened. These represented all cases in which plaque-forming ability is scored as less than "++" in Table 2, except that revertants of *am113* and *am122* were not isolated on strain TP308, and revertants of *am61*, *am65*, *am83*, *am113* and *am122* were not isolated on strain TP309. The number of revertants screened in each case varied, depending upon the ease with which they could obtained, from 32 to 750; a total of over 4000 revertants were screened. Only one of the 18 combinations, *am39* on the cysteine-inserting strain TP309, yielded second-site revertants; in this case, 14 out of 50 revertants tested.

To obtain a variety of second-site revertants, it was necessary to passage UV-mutagenized phage through a permissive or semipermissive host before selection of revertants. Table 3 shows the results of screening following this procedure: four out of six deleterious substitutions tested gave rise to second-site revertants.

Candidate second-site revertants were chosen for sequencing, one isolate for each distinct plaque morphology type among five independently isolated groups, for a total of 11. All of these retained their primary amber mutations; five had sequence changes implying other amino acid substitutions in gene *19* (two of these, *am82sr513* and *am82sr514*, had the same gene *19* sequence, but differed in plaque morphology, presumably due to mutation(s) elsewhere in the phage); four had base substitutions in the C-terminal-encoding end of gene *13*; and two had no changes in the region sequenced (including all of gene *19*). The amino acid alterations in the intragenic revertants are discussed below. Of the four mutations in gene *13* three were the same, changing Ala_{103} to Thr, while the fourth merely changed the arginine codon at position 97 to another arginine codon. Second-site mutant sequences are shown in Table 4.

TABLE 4

Sequence changes of second-site revertants

Mutant	Altered sequence	Location
am22sr517	None found	
am22sr520	AGA CTA to AGT TTA Arg Leu Ser Leu 18 19	gene *19*
	TCA to AAA Ser Lys 23	gene *19*
am39sr575	CGC to CGT Arg Arg 97	gene *13*
am39sr581	GCC to ACC Ala Thr 103	gene *13*
am82sr512	AAT TCT to AAA CCT Asn Ser Lys Pro 42 43	gene *19*
	TCA to TTA Ser Leu 46	gene *19*
am82sr513	TCA to TTA Ser Leu 46	gene *19*
am82sr514	TCA to TTA Ser Leu 46	gene *19*
am82sr515	None found	
am82sr516	TCA to TTA Ser Leu 23	gene *19*
	TCT to ACT Ser Thr 56	gene *19*
	GAC to GTC Asp Val 128	gene *19*
am83sr532	GCC to ACC Ala Thr 103	gene *13*
	TTA to TTG Leu Leu 11	gene *19*
am83sr542	GCC to ACC Ala Thr 103	gene *13*

The suppression patterns of the second-site revertant phages are shown in Table 5. The revertants all exhibited simple improvements over the host ranges of their parents: none lost the ability to grow on any suppressor; two (*am82sr513* and *am82sr514*) gained the ability to grow on one additional suppressor (the serine-inserting suppressor on which they were selected); the others all improved their plating efficiencies on more than one host.

TABLE 5

Second-site revertant suppression patterns

	Amino acid inserted by suppressor strain					
Phage	Gln	Tyr	Leu	Ser	Phe	Cys
am22	+	++	+	+	++	−
am22sr517	++	++	++	++	++	+/−
am22sr520	++	++	++	++	++	+/−
am39	++	++	++	++	++	+/−
am39sr575	++	++	++	++	++	+
am39sr581	++	++	++	++	++	++
am82	++	−	++	+/−	−	−
am82sr512	++	+/−	++	++	−	+/−
am82sr513	++	−	++	+	−	−
am82sr514	++	−	++	+	−	−
am82sr515	Not determined					
am82sr516	++	−	++	+	−	−
am83	+	++	−	−	++	−
am83sr532	++	++	++	−	++	−
am83sr542	++	++	++	−	++	−

Suppression patterns for *am82*-bearing strains were determined at 30°, the others 37°; otherwise as in Table 2.

Reconstruction of double mutants: We sought to determine whether the sequence changes in gene *19* described in Table 4 were in fact responsible for suppression of the plating defects of their parent phages. The alternative possibility to eliminate in each case was that the observed suppression was actually accomplished by some other mutation outside the sequenced region of the double mutant phage. *Hpa*I fragments of the revertant phage chromosomes containing gene *19* (Figure 2) were purified and cloned in plasmids. (In the course of doing this, it was noted that all of the revertant phages produced the same pattern of *Hpa*I fragments as wild-type phage; thus none bore extensive chromosomal rearrangements—data not shown.) The double mutant lysozyme genes were then transferred by recombination from the plasmids to unmutagenized P22, and the resulting recombinants were tested to determine whether they exhibited parental or revertant suppression patterns. The cloned *Hpa*I fragments included sequences on both sides of gene *19* that were not determined for each mutant. For this reason, it was necessary to determine the linkage of the suppressing mutation to the parental amber. Interpretation of the reconstruction crosses was as follows (Figure 2): The phage into which the mutant lysozyme genes were transferred, P22 *Kn469 sieA44 m44*, bears a deletion-substitution that removes most of gene *19* (codons 9–123) and replaces it with a large fragment of Tn*5* (described in MATERIALS AND METHODS). Plaque-forming progeny generated by recombination events between this phage and the gene *19*-bearing plasmids necessarily acquire both the original amber mutation and the second-site gene *19* mutations (all except for the Asp_{128}-Val of *am82sr516*, but this should be very

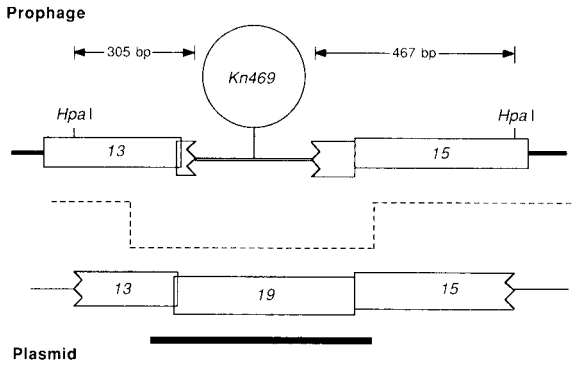

FIGURE 2.— Diagram of the cross used to reconstruct revertant phages. The extents of homology between the defective prophage and the plasmid on either side of the *Kn469* deletion-substitution are indicated. The bar below the plasmid map indicates the plasmid DNA that was sequenced in each case. P22 bearing *Kn469* is oversized; plaque-forming recombinants can be formed by recombination as indicated by the dashed line.

tightly linked nevertheless). In contrast, hypothetical mutations outside the sequenced region, which extends more than 50 bp beyond both boundaries of the *Kn469* deletion-substitution in each case, should be transferred to less than 100% of the progeny. In crosses involving all four of the plasmids made from phages bearing different gene *19* intragenic second-site revertants, recombinants were isolated by plating on a host permissive for both the original amber mutant and the revertant; in each case, 50 were tested, and all exhibited the revertant suppression pattern. The linkage test was validated by reconstructing P22 *am83sr532*. This phage's second-site suppressing mutation lies 46 bp to the left of the left endpoint of *Kn469*, and in its case, 46 out of 50 recombinants had the revertant suppression pattern, while the remaining four had the suppression pattern of the original amber. We conclude that the 100% observed linkage in the crosses involving gene *19* secondary mutations implies that these mutations suppress the parental ambers.

DISCUSSION

Interpretation of amber mutant suppression patterns: The method employed in these studies for the generation of structural variants of P22 lysozyme makes extensive use of the properties of phage amber mutants. Particularly useful properties include: (1) a predictable phenotype—loss of function in a non-amber suppressor-bearing host; (2) the ability to make several lysozyme variants with the same phage strain, by infecting hosts that insert different amino acid residues in response to the amber codon; (3) an extremely simple and efficient method for distinguishing between primary and (potentially rare) second-site revertants.

A possible drawback to the use of amber mutations involves interpretation of suppression patterns: the inability of an amber mutant to form plaques on a given amber suppressor host could be due either to a defect resulting from the insertion of an inappropriate amino acid residue, or to inefficiency of the amber suppressor. The efficiencies of amber suppressors are generally lower than those of other amino acyl tRNAs, and vary with the context of the amber codon in question (BOSSI 1983; MILLER and ALBERTINI 1983). While we cannot rule out a role of amber suppressor efficiency in determining the suppression patterns described above, we suspect that their contribution is minimal, as suggested by several observations. The amber suppressors employed in these studies are all relatively efficient (WINSTON, BOTSTEIN and MILLER 1979; NORMANLY *et al.* 1986). Every suppressor suppressed at least one amber mutant effectively; conversely, every amber mutant was suppressed effectively by at least one suppressor. Moreover, in each of six cases in which an amber suppressor inserted the wild-type amino acid residue, the mutant phage made plaques as well as wild-type.

The great majority of amino acid substitutions characterized in this study were phenotypically silent, or else produced apparently mild defects. However, plaque size is not a sensitive measure of lysozyme activity; other observations (L. HARDY and A. POTEETE, unpublished results) indicate that wild-type P22 makes at least 30 times as much lysozyme as it needs to form a plaque of normal size. For this reason, we suspect that the reversion studies described above were confined to mutations with relatively strong effects on lysozyme activity.

Isolation of second-site revertants: The frequency with which second-site revertants were isolated following direct plating of UV-mutagenized P22 was extremely low; it was much higher when the phage were first passaged through an intermediate host in liquid culture. We do not understand this effect. Multiplication of the irradiated phage through several lytic cycles can be expected to increase the population of viable phage relative to inactivated phage in the stock. If the intermediate host were nonpermissive or semipermissive for the mutant in question, there might additionally be a mild selection for revertants. However, the effectiveness of this passaging procedure did not appear to depend in any obvious way on the permissiveness of the intermediate host; moreover, it is not obvious that such a selection would favor second-site revertants over primary site revertants.

Extragenic second-site mutations: Both intragenic and extragenic second-site suppressor mutations were isolated. The extragenic revertants included two with no sequence changes within 30 bp of gene *19* as well as four with sequence changes in the gene *19* proximal end of gene *13*. Gene *13*, a homolog of the phage λ *S* gene, encodes a protein that promotes bacterial lysis,

FIGURE 3.— Sequence alterations of second site mutations upstream from gene *19*. The sequences are represented as RNA base-paired with the 3′ end of 16S rRNA (top), and in a stem-loop structure (bottom). The translational initiation codon of gene *19* is underlined in the top drawing.

probably by making pores or lesions in the cytoplasmic membrane through which lysozyme gains access to the peptidoglycan (RENNELL and POTEETE 1985). The gene *13* mutations include one in codon 97 that does not change its sense, and one in codon 103 that changes it from Ala to Thr; one of three independent isolates of the latter mutation bore in addition a presumably silent mutation in gene *19*, TTA to TTG in codon 11. That the Ala_{103}-Thr mutation in gene *13* suppresses defects in lysozyme is shown by the reconstruction test described in RESULTS. Its suppressing effects cannot be highly allele-specific, as it suppresses three substitutions in two different positions (Table 5). Its location is quite close to, though not in, sequences upstream from gene *19* that could pair with the 3' end of 16S rRNA (Figure 3).

It is likely that one or both of the sequenced second site mutations in gene *13* suppress defects in gene *19* by increasing expression of gene *19*. Genes *13* and *19* overlap; the ribosome binding site of *19* is embedded in the coding sequence of *13*. Such an overlap suggests the possibility of translational coupling (GATENBY, ROTHSTEIN and NOMURA 1989). Indeed, it is possible to include the entire sequence shown in Figure 3 in a stem and loop in the mRNA, with a stability of −11.4 kcal/mol, calculated according to TINOCO and coworkers (1973). In this structure, the core of the putative gene Shine-Dalgarno sequence (GGAG) is entirely base-paired, and, conceivably, inaccessible to ribosomes. The mutation *sr575*, which is silent in

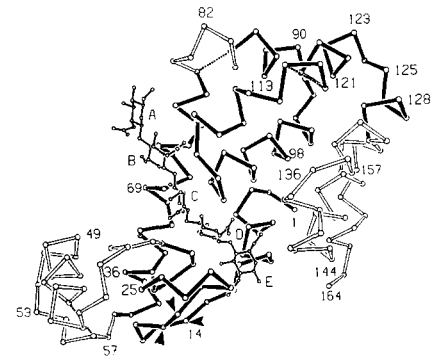

FIGURE 4.— Structural relationship between T4 and P22 lysozymes. The drawing shows α carbon atoms in T4 lysozyme, which are connected by solid bars in regions of high homology with P22 lysozyme. The dotted lines indicate surface loops in T4 lysozyme that are evidently not found in P22 lysozyme. The *N*-acetylglucosamine-*N*-acetylmuramic acid copolymer backbone of peptidoglycan is shown docked in the active site cleft (from WEAVER *et al.* 1985). Arrows indicate positions corresponding to the residues altered in the P22 lysozyme mutant *am22sr520*.

terms of the amino acid sequence of gene *13* protein, destabilizes this structure; the base in question is a C that would be paired directly with the third G of the Shine-Dalgarno sequence. A scheme that accounts for the effect of *sr575* would propose that the ribosome binding site of gene *19* is relatively inaccessible unless the mRNA secondary structure that occludes it is disrupted. This disruption, which is normally caused by translation of gene *13*, would be increased by the *sr575* mutation, leading to an increased rate of lysozyme synthesis. While such a scheme is plausible, there is no direct evidence for translational coupling of genes *13* and *19*. On the contrary, amber mutations in genes *13* and *19* complement each other; if *19* translation were tightly coupled to *13* translation, one would expect amber mutations in gene *13* to be highly polar on *19*. The failure to observe polarity, however, does not rule out partial translational coupling; as discussed above, a tenfold decrease in the production of wild-type lysozyme would probably have no noticeable effect on cell lysis. The translational coupling hypothesis does not account for the activity of the gene *13* suppressor allele *sr581* (Ala_{103}-Thr); the affected base is in the loop part of the postulated mRNA structure.

Intragenic second-site revertants: Intragenic second-site revertants were isolated for two amber mutations. In one case, *am82*, three different suppressing mutations were isolated, all on the serine-inserting strain. The simplest of these, represented by *am82sr513* and *am82sr514*, was Ser_{46}-Leu. From the suppression patterns shown in Table 5, we conclude that this change makes a lysozyme that is better able to tolerate a substitution of serine for glutamine at position 82, but has little apparent effect on the sensitivity of the protein to inactivation by substitutions of tyrosine, phenylalanine, or cysteine there. A more

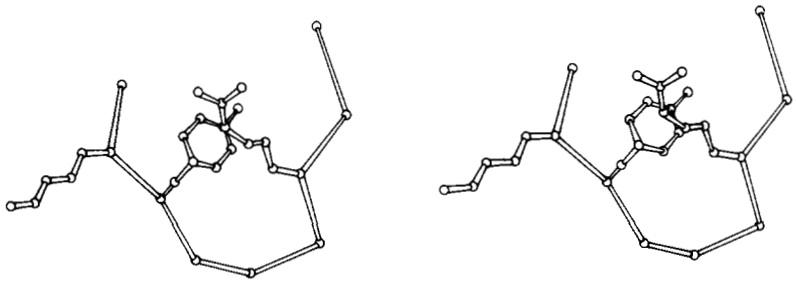

FIGURE 5.—Residues in T4 lysozyme corresponding to residues altered in the P22 lysozyme variant *am22sr520*. The stereo pair shows α carbons of residues 12 to 20, with the side chains of (from right to left) Arg_{14}, Tyr_{18}, and Lys_{19}.

complex revertant, *am82sr512*, bears this same Ser_{46}-Leu substitution, along with two other changes, Asn_{42}-Lys and Ser_{43}-Pro. These additional changes further improve the ability of lysozyme to tolerate serine at position 82, as well as tyrosine and cysteine (but not phenylalanine). We do not know whether one or both of the two additional residues altered in *am82sr512* is responsible for its greater tolerance relative to the two simpler revertants. The third intragenic revertant of *am82*, *am82sr516*, contains three single amino acid substitutions: Ser_{23}-Leu, Ser_{56}-Thr, and Asp_{128}-Val. The suppression pattern of this revertant is barely distinguishable from that of the simple revertant bearing Ser_{46}-Leu, although the structural basis for its effects must clearly be different. It should noted that we do not know whether any of the mutations described here affect lysozyme's activity or stability, or both. Interpretation of the possible structural effects of mutations in P22 lysozyme is not straightforward, due to the lack of a determined structure.

The single intragenic revertant of missuppressed *am22*, *am22sr520*, is the most nearly interpretable as a structural variant. In this case, the secondary mutations, Arg_{18}-Ser and Ser_{23}-Lys, as well as the primary mutation, alter residues in a part of P22 lysozyme that has a high degree of homology to T4 lysozyme (Figure 4). Indeed, the residues corresponding to Arg_{18} and Tyr_{22} of P22 lysozyme in T4 lysozyme, Arg_{14} and Tyr_{18}, respectively, are in contact (Figure 5). Moreover, the other altered residue in *am22sr520*, Lys_{23} (serine in the wild type), corresponds to Lys_{19} in T4 lysozyme. From the data in Table 5, it is clear that the second-site mutations in *am22sr520* allow P22 lysozyme to tolerate a number of substitutions for Tyr_{22} that the wild type does not.

The T4 lysozyme structure gives us a precise description of the spatial arrangement of Arg_{14}, Tyr_{18} and Lys_{19}. All are part of a surface loop that subtends the active site cleft in the amino-terminal domain of the protein. Arg_{14} and Lys_{19} are relatively solvent-exposed, while Tyr_{18} is relatively buried. However, little information is available concerning the effects of single amino acid substitutions at these positions. Most of the temperature-sensitive mutant lysozymes studied in detail have alterations in solvent-inaccessible positions in the carboxy-terminal domain (ALBER *et al.* 1987). We are thus unable to guess what structural rearrangement is brought about by these mutations. However, two aspects of the revertant encourage further exploration of this approach as a model-free way to generate protein structure variants. First, we could apparently fix a defect in lysozyme structure with untargeted mutations that affect residues in contact with the originally altered residue. Second, the mechanism by which the secondary alterations compensate for the primary is completely unclear; with a model-driven, protein engineering approach, we presumably would not have constructed such a mutant.

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LITERATURE CITED

ALBER, T., S. DAO-PIN, J. A. NYE, D. C. MUCHMORE and B. W. MATTHEWS, 1987 Temperature-sensitive mutations of bacteriophage T4 lysozyme occur at sites with low mobility and low solvent accessibility in the folded protein. Biochemistry **26:** 3754–3758.

BERGET, P. B., A. R. POTEETE and R. T. SAUER, 1983 Control of phage P22 tail protein expression by transcription termination. J. Mol. Biol. **164:** 561–572.

BOLIVAR, F., R. L. RODRIGUEZ, P. J. GREENE, M. C. BETLACH, H. L. HEYNECKER, and H. W. BOYER, 1977 Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene **2:** 95–113.

BOSSI, L., 1983 Context effects: translation of UAG codon by suppressor tRNA is affected by the sequence following UAG in the message. J. Mol. Biol. **164:** 73–87.

BOTSTEIN, D., R. K. CHAN, and C. H. WADDELL, 1972 Genetics of bacteriophage P22. II. Gene order and gene function. Virology **49:** 268–282.

BRENT, R., and M. PTASHNE, 1981 Mechanism of action of the *lexA* gene product. Proc. Natl. Acad. Sci. USA **78:** 4204–4208.

CASJENS, S., K. EPPLER, R. PARR, and A. R. POTEETE, 1989 Nucleotide sequence of the bacteriophage P22 gene *19* to *3* region: identification of a new gene required for lysis. Virology **171:** 588–598.

GATENBY, A. A., S. J. ROTHSTEIN and M. NOMURA, 1989 Translational coupling of the maize chloroplast *atpB* and *atpE* genes. Proc. Natl. Acad. Sci. USA **86:** 4066–4070.

HANSEN, J. N., 1981 Use of solubilizable acrylamide disulfide gels for isolation of DNA fragments suitable for sequence analysis. Anal. Biochem. **116:** 146–151.

KNIGHT, J. A., L. W. HARDY, D. RENNELL, D. HERRICK and A. R. POTEETE, 1987 Mutations in an upstream regulatory se-

quence that increase expression of the bacteriophage T4 lysozyme gene. J. Bacteriol. **169:** 4630–4636.

LEDERBERG, E. M., and S. N. COHEN, 1974 Transformation of *Salmonella typhimurium* by plasmid deoxyribonucleic acid. J. Bacteriol. **119:** 1072–1074.

MANIATIS, T., E. F. FRITSCH and J. SAMBROOK, 1982 *Molecular Cloning. A Laboratory Manual.* Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

MATTHEWS, B. W., 1987 Genetic and structural analysis of the protein stability problem. Biochemistry **26:** 6885–6888.

MILLER, J. H., and A. M. ALBERTINI, 1983 Effects of surrounding sequence on the suppression of nonsense codons. J. Mol. Biol. **164:** 59–71.

NORMANLY, J., J.-M. MASSON, L. G. KLEINA, J. ABELSON and J. H. MILLER, 1986 Construction of two *Escherichia coli* amber suppressor genes: $tRNA^{Phe}_{CUA}$ and $tRNA^{Cys}_{CUA}$. Proc. Natl. Acad. Sci. USA **83:** 6548–6552.

RAO, G. R. K., and D. P. BURMA, 1971 Purification and properties of phage P22-induced lysozyme. J. Biol. Chem. **246:** 6474–6479.

REIDHAAR-OLSON, J. F., and R. T. SAUER, 1988 Combinatorial cassette mutagenesis as a probe of the informational content of protein sequences. Science **241:** 53–57.

RENNELL, D., and A. R. POTEETE, 1985 Phage P22 lysis genes: nucleotide sequences and functional relationships with T4 and λ genes. Virology **143:** 280–289.

SHORTLE, D., and B. LIN, 1985 Genetic analysis of staphylococcal nuclease: identification of three intragenic "global" suppressors of nuclease-minus mutations. Genetics **110:** 539–555.

SMITH, H. O., and M. LEVINE, 1964 Two sequential repressions of DNA synthesis in the establishment of lysogeny by phage P22 and its mutants. Proc. Natl. Acad. Sci. USA **52:** 356–363.

TINOCO, I., P. N. BORER, B. DENGLER, M. D. LEVINE, O. C. UHLENBECK, D. M. CROTHERS and J. GRALLA, 1973 Improved estimation of secondary structure in ribonucleic acids. Nature New Biol. **246:** 40–41.

WEAVER, L. H., D. RENNELL, A. R. POTEETE and B. W. MATTHEWS, 1985 Structure of phage P22 gene *19* lysozyme inferred from its homology with phage T4 lysozyme: implications for lysozyme evolution. J. Mol. Biol. **184:** 739–741.

WEINSTOCK, G. M., M. M. SUSSKIND and D. BOTSTEIN, 1979 Regional specificity of illegitimate recombination by the translocatable ampicillin-resistance element Tn*1* in the genome of phage P22. Genetics **92:** 685–710.

WINSTON, F., D. BOTSTEIN and J. H. MILLER, 1979 Characterization of amber and ochre suppressors in *Salmonella typhimurium*. J. Bacteriol. **137:** 433–439.

YOUDERIAN, P., S. BOUVIER and M. M. SUSSKIND, 1982 Sequence determinants of promoter activity. Cell **30:** 843–853.

ZAGURSKY, R. J., and M. L. BERMAN, 1984 Cloning vectors that yield high levels of single-stranded DNA for rapid DNA sequencing. Gene **27:** 183–191.

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