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# Efficient site-directed mutagenesis by simultaneous use of two primers

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## ABSTRACT

A rapid and efficient procedure for site specific mutagenesis is described. A double primed synthesis with a 17-mer mismatch primer and a "universal" 15-mer M13 sequencing primer was used to introduce a T to A transversion into an ompF signal peptide gene cloned in the M13mp8 vector. The two primers were annealed to the circular single stranded M13 template. After a short extension and ligation reaction, a double stranded restriction fragment containing the mismatch (ompF*/EcoR1-SalI) was cut out of the partly single stranded circular DNA and inserted into pBR322. 42% of the E.coli transformants harboured plasmid with the desired mutation, which could be detected by the appearance of a new restriction site (MboII) and by dot blot hybridization of plasmid DNA with the $^{32}$P-labeled 17-mer.

## INTRODUCTION

Synthetic oligonucleotides are useful tools for directed site specific mutagenesis of DNA inserted into circular single stranded bacteriophage vector (1,2) or plasmid (3,4). Even phenotypically silent mutations can be detected by using the oligonucleotide as a probe (5).

Most of the published methods depend on the enzymatic extension of the mutagenic oligonucleotide "all the way round" the circular template, followed by T4-ligation, to produce a covalently bonded double stranded circular molecule with a mutation in the newly synthesized strand. Due to a generally low efficiency in this process additional purification steps are necessary before the DNA is introduced into a host organism and mutants identified.

We describe a modified procedure in which a chemically synthesized 17-mer mismatch primer and a "universal" 15-mer M13 dideoxy sequencing primer (6) are used simultaneously on a circular

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single stranded M13 template. After a brief extension and ligation a double stranded fragment containing the mismatch was excised by restriction enzymes and cloned in a plasmid.

The procedure was used to introduce a T to A transversion with high efficiency into DNA coding for the OmpF signal peptide.

OmpF, one of the major outer membrane proteins of *E. coli* K12, is synthesized as a precursor molecule containing a signal peptide, which is required for the transport of the protein across the inner membrane (7).

One or more positively charged amino acids near the amino-terminal region seems to be a common feature for prokaryotic signal peptides. Recently oligonucleotide directed site specific mutagenesis has been used to study the effect on protein secretion of reducing the number of positively charged amino acids at the amino-terminal end of the prolipoprotein of the *E. coli* outer membrane (8).

We have constructed an *ompF* mutant with an additional positive charge in the amino-terminal end of the OmpF precursor. The effect of the mutation on the transport of the OmpF protein is under investigation.

## MATERIALS AND METHODS

15-mer M13 dideoxy sequencing primer d(TCCCAGTCACGACGT), T4 DNA ligase and restriction enzymes were obtained from New England Biolabs. DNA Polymerase I "Klenow fragment" and T4 polynucleotide kinase were purchased from P-L Biochemicals. [$\gamma$-$^{32}$P]-ATP (7500 Ci/mmol) was obtained from New England Nuclear. The support for oligonucleotide synthesis was 5'-O-dimethoxytritylthymidine bound via a 3'-O-succinyl group to aminomethylated 1% crosslinked polystyrene beads from Bachem. *E. coli* LaC1 is an *ompB101* (9) derivation of MC1000 (10).

### Construction of M13mp8::ompF hybrid phage

The M13mp8 derived phage M13mp8/8A3 carrying the *ompF* promoter and coding sequences for the OmpF signal peptide and the first 12 amino acids of the mature protein, was constructed by introducing a 355bp EcoRI-BglII fragment containing the *ompF* sequences into EcoRI-BamHI cut M13mp8 RF (11). The EcoRI site of the 355 bp fragment was introduced by addition of an EcoRI linker upon exo-

nucleolytic removal of DNA upstream to the ompF promoter in a previous set of experiments (unpublished). The BglII site is located in the 12th codon of the mature OmpF protein. The sequence of the fragment is in full accordance with the one recently published (7). Single stranded M13mp8/8A3 was prepared as described (11).

Oligodeoxyribonucleotide synthesis

The 17-mer mismatch primer d(TATTGCGCTTCTTCATT) was synthesized by the triester method on a 1% crosslinked polystyrene support (12). The polymer was packed in a short column, and solvents and reagents were delivered semi-automatically by means of an HPLC pump and a control module. The oligonucleotide was purified after deprotection by HPLC on a Partisil-10 SAX column (Whatman) (13) and rechromatographed on a LiChrosorp RP18 column (Merck) (14).

5'-$^{32}$P-labeling of oligodeoxyribonucleotide

The 17-mer was labeled at the 5' end in a 10 µl reaction mixture containing 50 mM Tris-HCl at pH 9.5, 10 mM $MgCl_2$, 5mM DTT, 0.4% glycerol,20 pmole ATP, 15 µCi of [$\gamma$-$^{32}$P]-ATP (2 pmole), 20 pmole of oligonucleotide, and 5 units of T4 polynucleotide kinase. The reaction was carried out at 37$^{o}$ C for 20 min, and terminated by heating at 100$^{o}$ C for 2 min. The labeled oligonucleotide was separated from unreacted [$\gamma$-$^{32}$P]-ATP by chromatography on a column (1x8 cm) of Sephadex G50, superfine in 0.05 M triethylammonium bicarbonate at pH 7.5.

For dot blot hybridization the oligonucleotide was labeled without the addition of "cold" ATP as described (15).

Oligodeoxyribonucleotide primed DNA synthesis

Single stranded M13 mp8/8A3 with ompF insert (0.2 pmole) was incubated with a mixture of 17-mer 5'-($^{32}$P)-labeled oligodeoxyribonucleotide primer (20 pmole) and unlabeled 15-mer universal primer for dideoxy sequencing (12 pmol) in 19.4 µl of 77 mM NaCl, 30 mM Tris-HCl, pH 7.5 and 18 mM $MgCl_2$ for 5 min. at 55$^{o}$ C and annealed for 20 min. at 23$^{o}$ C. Then 8 µl of d-NTP-mix consisting of 38 mM β-mercaptoethanol, 188 µM of each dATP, dCTP, dGTP, dTTP and 188 µM ATP was added followed by 2.5 units of E. coli DNA polymerase I (Klenow). The mixture was kept for 60 min. at 20$^{o}$ C, then 720 units of T4 DNA ligase and 3µl 10 mM ATP were added. The

final volume of the mixture was 33 µl. After incubation for 60 min. at room temperature 10 µl 4M sodium acetate was added, and the volume adjusted to 100 µl with TE-buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA).

The mixture was extracted twice with phenol/chloroform. 1 µg (0.4 pmole) of the purified large fragment of pBR322 cut with EcoRI and SalI was added as carrier DNA. After ether extraction of the aqueous phase, the DNA was isolated by ethanol precipitation.

Endonuclease digestion

The DNA, prepared as described above, was cut with 12 (60 min.) units each of restriction endonucleases SalI and EcoRI in a total volume of 13 µl of buffer (6 mM $MgCl_2$,6mM Tris-HCl, pH 7.9, 150 mM NaCl, 1 mM β-mercaptoethanol) for 40 min. at 37° C. The mixture was extracted with phenol/chloroform followed by ether and the DNA isolated by ethanol precipitation and then dissolved in 12 µl $H_2O$. 1 µl was used for electrophoresis on a 7 M urea - 5% polyacrylamide gel.

Ligation

The main portion of the DNA (11 µl) was ligated at 16° C for 16 hrs. in a total volume of 41 µl containing 66 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 1 mM ATP, 10 mM DTT, 40 µg/ml gelatine and 400 units of T4 DNA ligase.

Transformation

20 µl of the ligation mixture was used to transform calcium chloride treated *E. coli*, strain LaC1 (MC1000, *ompB101*) (16). The bacteria were plated on LB-agar plates and selected for resistance to ampicillin (50 µg/ml).$6.7 \cdot 10^5$ colonies per pmole of M13 mp8/8A3 were obtained. All transformants were sensitive to tetracyclin (25 µg/ml), as expected.

Dot blot hybridization

2 µl of each of 24 plasmid preparations (17) was mixed with 1 µl 0.3 N sodium hydroxide and applied as a spot on Whatman 540 filter paper, 8 cm diameter. The paper was "baked" at 80° C for two hours and then prehybridized at 67° C for one hour in a sealed plastic bag with 3 ml of 6xSSC, 10X Denhardt (18), 0.2% SDS. Hybridization was performed with $3 \cdot 10^6$ cpm of $^{32}P$-labeled 17-mer in 3 ml 6xSSC, 10 x Denhardt at room temperature for one hour.

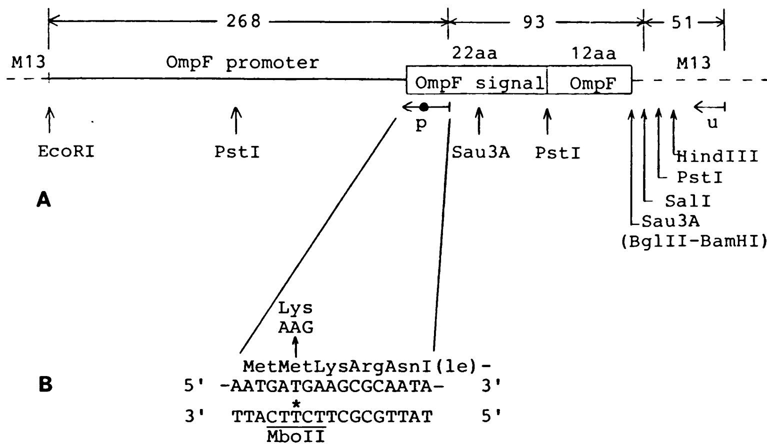


Fig. 1.A: Schematic illustration of the ompF gene fragment inserted into M13mp8. p= 17-mer mismatch primer, u= universal dideoxy sequencing primer. B: Nucleotide sequence of the region corresponding to the N-terminal part of the OmpF signal peptide and 17-mer complementary primer with one mismatch (*).

After washing three times with 50 ml of 6xSSC at room temperature for a total of 10 min. autoradiography was performed for 1 hour. The filter was then washed with 50 ml 6xSSC for 5 min. at increasing temperatures (10° C intervals) and autoradiographed after each wash (1). After washing at 52° C identification of the colonies containing mutated plasmid was possible (fig. 4).

DNA sequence analysis

3 µg of mutated plasmid was cut with SalI under standard conditions, phenol extracted and ethanol precipitated. The "filling out" of the SalI sticky ends was performed with Klenow DNA polymerase I, dATG, dGTP, dTTP and $\alpha$-$^{32}$P-dCTP. After phenol extraction and ethanol precipitation the DNA was cut with EcoRI. The $^{32}$P-labeled fragment containing the ompF sequence was purified by electrophoresis on a 6% polyacrylamide gel. After electroelution and ethanol precipitation the DNA fragment was sequenced by the Maxam Gilbert method (19).

## RESULTS AND DISCUSSION

A 17-mer primer complementary - except for one mismatch - to DNA coding for the N-terminal part of the OmpF signal peptide

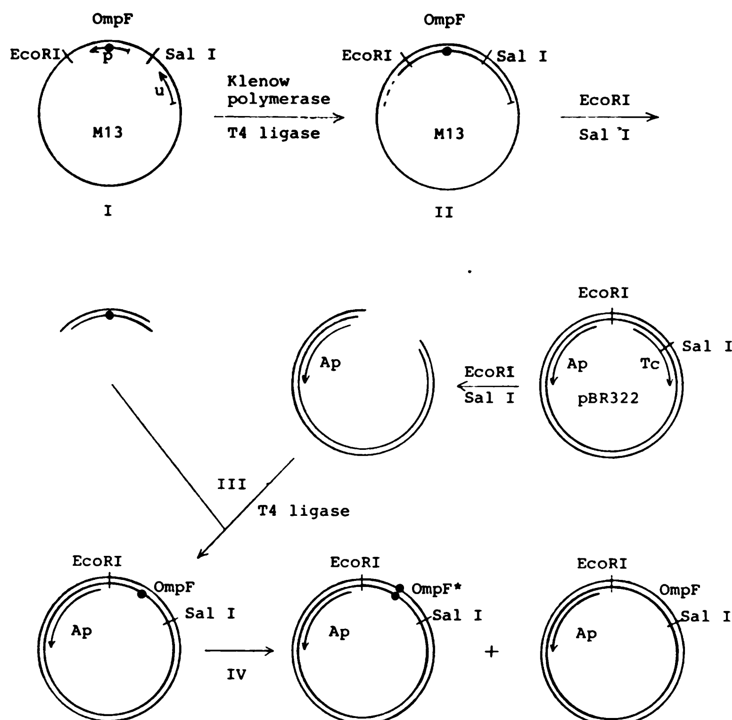


Fig. 2. Principle of the modified procedure. I: 5'-$^{32}$P-labeled 17-mer mismatch primer (p) and 15-mer universal dideoxy sequencing primer (u) annealed to circular single stranded M13 DNA carrying the ompF signal peptide gene insert. II: Extension by Klenow polymerase followed by ligation. III: Excision by EcoRI and SalI restriction endonucleases and ligation to EcoRI-SalI cut pBR322. IV: Transformation of E. coli.

was prepared by chemical synthesis. Primer induced mutation should result in an increase in the number of charged amino acids from 2 to 3 by a substituion of a Met(ATG) for a Lys (AAG) (see fig. 1). Simultaneously a new MboII site should be generated.

The scheme for generating the mutant is shown in fig. 2.

Preliminary pulse-chase experiments (1) demonstrated that the two primers initiated the extension reaction only at the correct position, although at low temperature (0° C) additional priming sites were observed (data not shown).

The 17-mer mismatch primer was $^{32}$P-labeled in the 5'-position, mixed with unlabeled 15-mer "universal" dideoxy sequencing primer and single stranded M13 with the ompF insert. The extension and

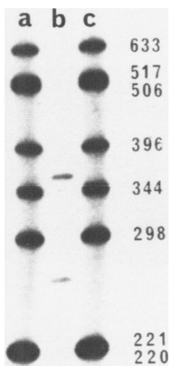


Fig. 3. Denaturing PAGE patterns, lane b, of the extension and ligation mixture (fig. 2) after digestion with EcoRI and SalI. The upper band corresponds to a correct extension and ligation product (361 bases). The lower band corresponds to unligated fragment (268 bases). Lane a and c are $^{32}$P-labeled DNA size markers of pBR322 cut with HinfI and EcoRI. Sizes of the markers in bases are shown to the right of the autoradiogram.

ligation reaction was carried out at room temperature. After digestion of the resulting mixture with EcoRI and SalI restriction endonucleases a sample was analyzed by polyacrylamide gel electrophoresis (fig. 3).

Two bands were obtained, one of 361 bases corresponding to a correct extension and ligation reaction and the other corresponding to unligated fragment (268 bases). A ligation reaction was performed on the unfractionated mixture in order to join the EcoR1-SalI ompF fragment to the EcoR1-SalI cut pBR322 fragment previously added as a carrier.

Upon transformation of competent E. coli cells $6.7 \cdot 10^5$ Ap resistant, Tc sensitive colonies were obtained per pmole of ompF DNA.

Plasmid Analysis

Plasmid DNA was prepared from 24 individual colonies and analyzed by cleavage with MboII followed by polyacrylamide gel electrophoresis. Two types of cleavage pattern were observed. 10 out of the 24 plasmids (42%) gave rise to a band corresponding to the 169 bp fragment expected for MboII cleavage of mutated plasmid. Under the same conditions only partial MboII cutting of pBR322 was observed due to $N^6$-adenine methylation of the MboII-Sau3A (MboI) sites GAAG(m)ATC present in four positions in the pBR322 molecule (20).

It is interesting to notice that the presence of two GATC sites (fig. 1A, Sau 3A sites) in the M13 template between the

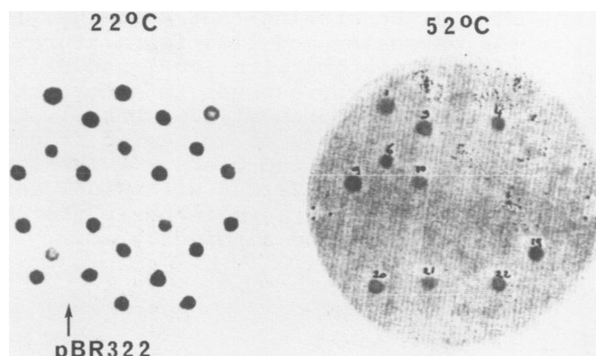


Fig. 4. Dot blot hybridization of 24 immobilized plasmids to $^{32}$P-labeled mutagenic primer. 10 mutants were identified after washing at 52° C as described under materials and methods. No hybridization to pBR322 was observed.

two primers was not a serious problem with regard to the methylation instructed mismatch repair system of the host organism (21, 22). The M13 template was isolated from a dam$^{+}$ host (JM103) (23) i.e. resulting in fully N$^{6}$-methylated GATC sites, while the complementary GATC sites in the in vitro primer extended strand are unmethylated. However, the large fragment of pBR322 into which the double stranded restriction fragment containing the mismatch was ligated contains 19 dam methylated GATC sites in each strand (24).

Dot blot hybridization

As site specific mutagenesis only in certain cases will result in creation or destruction of a restriction site, a more generally applicable method was investigated, the so-called dot blot hybridization (1). The method is based on the difference in thermal stability of a perfectly matched hybrid and a hybrid with a mismatch (5). Plasmids were immobilized to filter paper and then hybridized to $^{32}$P-labeled 17-mer. The filter was then washed at increasing temperatures (10° C intervals) and autoradiographed after each wash. As seen in fig. 4 identification of the plasmids carrying mutated ompF insert was possible after washing at 52° C. The results of the dot blot hybridization agrees with the results of the MboII cleavage analysis.

Retransformation

The "mutant" colonies obtained after the first transformation

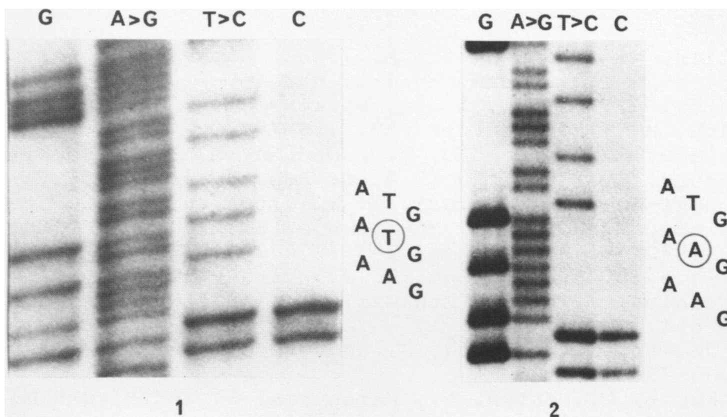


Fig. 5. DNA sequence determination of wild type (1) and mutant pBR322-ompF (2). The T to A transversion is encircled.

contain plasmids which are the progeny of a heteroduplex. Within a single cell a 1:1 mixture of plasmids derived from the mutated and the wild type strand, respectively, would be expected. Due to the low efficiency of the transformation process only one plasmid is normally taken up by the competent host cell. Therefore pure mutants could be obtained by retransformation of $CaCl_2$ treated LaC1 cells with plasmid from three of the "mutant" colonies identified as described above. From each plate four ampicillin resistant clones were isolated and plasmid DNA was prepared. As expected 50% or 6 out of 12 of the plasmid preparations were shown by MboII cleavage analysis and by dot blot hybridization to be mutated.

Sequence analysis

One of the mutated plasmids were selected for sequencing as described in the experimental part. The sequence was found to be identical to that of the wild type ompF region except for the T to A change at the desired position (fig. 5).

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