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Multiple-Mutation Reaction: a Method for Simultaneous Introduction of Multiple Mutations into the *glpK* Gene of *Mycoplasma pneumoniae*

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In *Mycoplasma pneumoniae*, the UGA opal codon specifies tryptophan rather than a translation stop site. This often makes it difficult to express *Mycoplasma* proteins in *E. coli* isolates. In this work, we developed a strategy for the one-step introduction of several mutations. This method, the multiple-mutation reaction, is used to simultaneously replace nine opal codons in the *M. pneumoniae glpK* gene.

Mycoplasma pneumoniae is a pathogen that lives on mucosal surfaces and causes diseases such as mild pneumonia, tracheobronchitis, and complications affecting the central nervous system, the skin, and mucosal surfaces (9, 14, 26). This bacterium possesses one of the smallest genomes of any free-living organism known so far. This reduced genome makes *Mycoplasma* spp. interesting from two points of view: (i) the analysis of these bacteria may help to identify the minimal set of genes that is required for independent life (7), and (ii) *M. pneumoniae* and its close relative *M. genitalium* are well suited for the development of the methods of the postgenomic era (10, 27). Another interesting aspect of the small genome is the observation that several enzymes of *Mycoplasma* spp. are "moonlighting"; i.e., they have multiple unrelated functions (11). This was discovered for glycolytic kinases, which are also active as nucleoside diphosphate kinases in *M. pneumoniae* and other *Mycoplasma* spp. (18).

However, the analysis of proteins from *Mycoplasma* spp. is hampered by a peculiarity of the genetic code of these bacteria: they use the UGA opal codon to incorporate tryptophan rather than as a stop codon as in the universal genetic code (8, 21). Thus, if cloned into *Escherichia coli* or other hosts, the genes from *M. pneumoniae* may contain many stop codons that prevent heterologous expression. Several strategies have been developed to solve this problem. For example, some *M. pneumoniae* genes, such as *ptsH* or *hprK*, do not possess UGA codons and thus require no special care (24). Expression of mollicute genes in *Spiroplasma* spp. that read the UGA as a tryptophan codon was reported, but these bacteria are difficult to handle (23). *E. coli* suppressor strains expressing an opal suppressor tRNA were developed, but they fail if multiple opal codons are present (22). *M. pneumoniae* genes containing few UGA codons have been expressed in *Bacillus subtilis* with low efficiency (12). In cases with only a few opal codons, these were changed by site-directed mutagenesis to allow expression in *E. coli* (13, 17). The *M. pneumoniae* P1 adhesin gene contains 21 opal codons, and a large-scale purification of the protein, though highly desired, has so far not been possible. In this case, protein fragments were expressed and purified (3). Finally, *Mycoplasma* genes could be synthesized in vitro from oligonucleotides; this strategy is, however, quite expensive. In this work, we present a PCR-based method that allows the simultaneous introduction of several mutations in a single step. Using this strategy, 9 of the 10 opal codons of the *glpK* gene from *M. pneumoniae* were modified, leading to expression of glycerol kinase in *E. coli*.

Outline of the multiple-mutation reaction (MMR) strategy. Several methods for PCR-based site-directed mutagenesis have been developed. Among these, the combined chain reaction method (1, 2) proved to be very rapid and reliable. The principle of this method is the use of mutagenic primers that hybridize more strongly to the template than the external primers. The mutagenic primers are phosphorylated at their 5′ ends, and these are ligated to the 3′ OH groups of the extended upstream primers by the action of a thermostable DNA ligase. Moreover, the DNA polymerase employed must not exhibit 5′ → 3′ exonuclease activity, to prevent the degradation of the extended primers. In our view, *Pfu* and *Pwo* polymerases are both well suited (15, 19). The original protocol describes the introduction of two mutations simultaneously. In a previous study, we used a combined chain reaction to mutagenize four distant bases in a DNA fragment in a one-step reaction (our unpublished results).

For the introduction of up to nine mutations in a single experiment, we developed the MMR. This method requires the efficient binding of all the mutagenic primers to the target DNA. To ensure that extension of a PCR product is not possible beyond the next (i.e., more downstream) mutation site without ligation to the corresponding mutagenic primer, special care needs to be taken in primer design. This reaction is based on an accurate calculation of melting temperatures. For this purpose, the formula T_m (melting temperature in °C) = 81.5 + 16.6(log[Me^+]) + 0.41 × %G+C − (500/oligonucleotide length) − 0.61 × % formamide was used (16). Only bases that match between primer and template were used for the calculation. One consideration was made when designing the mutagenic primers: ligation was facilitated by placing a G or C at the 5′ end of the oligonucleotide to favor close duplex formation between the primer and the target DNA. The external primers were selected to have melting temperatures

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TABLE 1. Oligonucleotides used in this study

Oligonucleotide	Sequence (5′–3′)[a]	Mutation	T_m (°C)
CH7	AAAAGTCGACATGGATCTAAAACAACAATACATTCTTG	None	59
CH8	TATAAAGCTTGTCTTAGTCTAAGCTAGCCCATTTTAG	A1512G	63
CH9	AAAAGTCGACATGGATCTAAAACAAC	None	57
CH10	TATAAAGCTTGTCTTAGTCTAAGCTAG	None	59
CH11	P-GATCCCTTAGAAATTTGGTCAGTCCAATTAG	A165G	64
CH12	P-CCATTGTGTTATGGAACAAAGAAAATGGTTTG	A273G	63
CH13	P-CACTAAGATTGCTTGGATCTTGGAAAATGTTC	A438G	64
CH14	P-CCTGGTTAATTTGGAAACTAACGGGTG	A522G	64
CH15	P-CCATGACATGGTCACAAGAGTTAGGC	A606G	65
CH16	P-TACCGAGTCATTGGTCTACTAGTGC	A705G	65
CH17	P-CCTTAAAGTGGTTAAGGGATAGTCTTAAGG	A966G	65
CH18	P-GCAGTTAATTATTGGAAGGACACTAAACAAC	A1386G	63
CH19	P-GAAATCAAAGCGTTGGAACGAAGCTG	A1482G	65

[a] The "P" at the 5′ end of oligonucleotide sequences indicates phosphorylation.

considerably lower (about 4°C) than those of the mutagenic primers. The MMR was performed with 2.5 units of *Pfu* DNA polymerase (MBI Fermentas, Lithuania) and 15 units of Ampligase (Epicentre, Madison, WI) in MMR buffer (20 mM Tris-HCl [pH 8.5], 3 mM $MgCl_2$, 50 mM KCl, 0.4 mg/ml bovine serum albumin, and 0.5 mM NAD^+) in a total volume of 50 μl. Conditions for MMR included denaturation at 95°C for 30 s, primer annealing at 57°C for 30 s, and elongation at 65°C for 6 min, for 35 cycles. Initially, the DNA fragment (100 ng) was denatured for 5 min at 95°C. Ten picomoles of each primer was used. The sequences and the arrangements of the oligonucleotides used in this study are shown in Table 1 and Fig. 1, respectively.

Cloning of *M. pneumoniae glpK* and expression of the protein in *E. coli*. An analysis of growth behavior and the in vivo protein phosphorylation pattern identified glycerol as a key carbon source associated with regulatory phenomena. This substrate triggered in vivo phosphorylation of the HPr protein of the phosphotransferase system by the metabolite-sensitive HPr kinase/phosphorylase (5, 24). We were therefore interested in studying glycerol metabolism and its regulation in more detail. As a first step, we intended to purify the glycerol kinase. This enzyme is known to be a key target of catabolite regulation in gram-positive bacteria (4, 25). However, the corresponding *glpK* gene contains 10 opal codons and was therefore a good subject for MMR in order to change these codons to tryptophan codons for *E. coli*. The *glpK* gene was amplified using the oligonucleotides CH7 and CH8 and chromosomal DNA of *M. pneumoniae* M129 (ATCC 29342) as a template. With CH8, the most C-terminal opal codon was replaced by a TGG codon. The amplicon was cloned between the SalI and HindIII sites of the expression vector pWH844 (20). The resulting plasmid, pGP253, was used as a template for MMR with CH9 and CH10 as external primers and CH11 through CH19 as mutagenesis primers. Five independent MMRs were carried out, and the MMR products were individually cloned as a SalI/HindIII fragment into pWH844. The inserts of one clone resulting from each MMR were sequenced. Out of the five candidates, three contained the nine desired mutations without any additional mutations. One plasmid contained seven out of nine mutations, and the fifth plasmid bore all nine mutations and one additional undesired 1-bp deletion in one of the primer regions. Plasmids bearing all nine desired mutations but no additional mutations were designated pGP254. pGP254 allows the expression of *M. pneumoniae* glycerol kinase fused to an N-terminal hexahistidine sequence under the control of an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible promoter. To test the success of the mutagenesis, we compared the protein contents of *E. coli* cultures carrying either pWH844, pGP253, or pGP254. A prominent band corresponding to an approximate molecular mass of 56 kDa is detectable in the strain bearing pGP254, while no such protein is expressed from pGP253 encoding the unmutated *glpK* gene (Fig. 2A). The glycerol kinase was purified to apparent homogeneity by Ni^{2+}-nitrilotriacetic acid chromatography as described previously (Fig. 2B) (15). Thus, MMR was successful in achieving

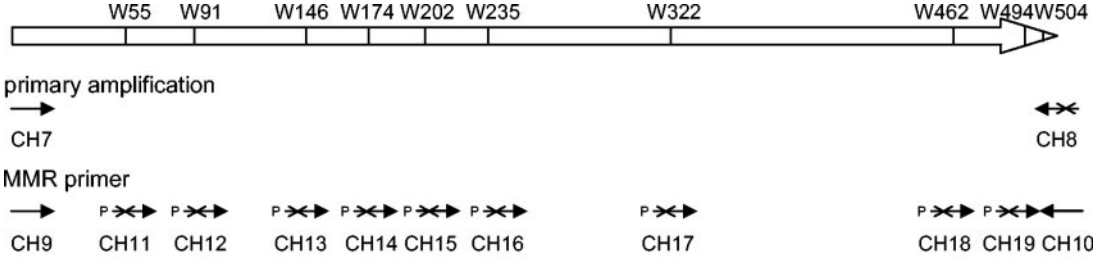

FIG. 1. Strategy for amplification and mutagenesis of the *M. pneumoniae glpK* gene (MPN050 [6]). The positions of the opal codons in the wild-type *glpK* gene (indicated by a W followed by the number corresponding to the amino acid) and the position and orientation of the external and mutagenic oligonucleotides are shown. The annealing site of each oligonucleotide is indicated by an arrow. Oligonucleotides bearing an A→G transition are depicted by crossed arrows.

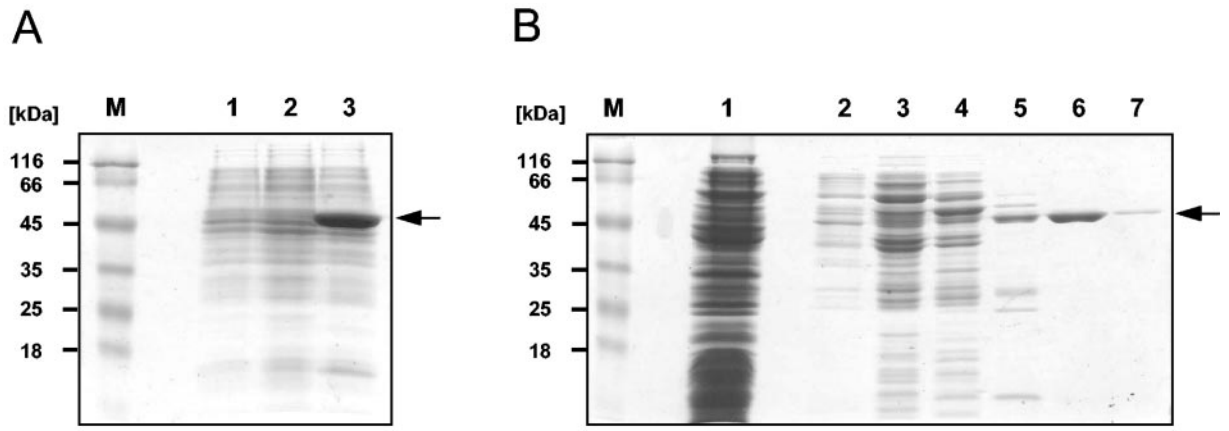

FIG. 2. Overproduction and purification of *M. pneumoniae* GlpK. (A) Sodium dodecyl sulfate (SDS)- polyacrylamide gel electrophoresis for the detection of His_6-tagged GlpK in crude extracts of *E. coli* DH5α bearing either the empty expression vector pWH844 (lane 1); the expression vector including the wild-type *glpK* allele, pGP253 (lane 2); or the vector including the mutated *glpK* allele, pGP254 (lane 3). Cells were grown to an optical density at 600 nm of 0.8, and expression from the IPTG-inducible promoter was induced by addition of 1 mM IPTG (final concentration). After 2 h, cells were harvested and disrupted by sonication. The insoluble fraction was pelleted in a centrifugation step and solubilized using 6 M urea, and sample aliquots were separated on an SDS–12% polyacrylamide gel. (B) SDS-polyacrylamide gel electrophoresis to monitor the purification of His_6-tagged GlpK. Crude extract of the GlpK expression strain (*E. coli* DH5α bearing the plasmid pGP254) that had been grown in the presence of 1 mM IPTG was passed over a Ni^{2+}-nitrilotriacetic acid superflow column (5-ml bed volume; QIAGEN) and washed extensively with a buffer containing 10 mM Tris-HCl (pH 7.4) and 200 mM NaCl, followed by elution with an imidazole gradient (from 10 to 500 mM imidazole). Aliquots of the individual fractions were separated on SDS–12% polyacrylamide gels. A prestained protein molecular mass marker (Fermentas) served as a standard (lane M). Lane 1, flowthrough; lane 2, 10 mM imidazole; lane 3, 20 mM imidazole; lane 4, 50 mM imidazole; lane 5, 100 mM imidazole; lane 6, 200 mM imidazole; lane 7, 500 mM imidazole.

efficient overproduction of *M. pneumoniae* glycerol kinase for biochemical studies.

This study demonstrates that MMR can be used for the rapid and highly efficient introduction of multiple mutations into a gene. Out of five individual clones, four had the desired mutations. Of these four, only one candidate contained an extra mutation, which was most probably due to an impure oligonucleotide mix. Indeed, other experiments indicated that the quality of the oligonucleotides is the limiting factor for MMR. Obviously, this method is useful not only for the expression of *Mycoplasma* species genes, but also to change codon usage patterns or for any other purpose that requires the introduction of many mutations or combinations of mutations at the same time. What is the maximum number of mutations that can be introduced by MMR in a single step? Our results suggest that the target of nine mutations is still far from a theoretical limit, and we are confident that this method can be made even more effective by taking care of the quality of the oligonucleotides (see above) and by using mutagenic primers that alternate between the two strands of the DNA. With this method at hand, even the expression of a functional P1 adhesin gene in *E. coli*, which has so far been beyond imagination (3), now seems feasible.

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