used to create a vector which was eventually resuspended in 50 µl sterile distilled water. The insert was created from restriction digest of either plasmid DNA or PCR-generated DNA. The fragment was isolated from a preparative agarose or 7.5 % polyacrylamide gel, purified as described in Section 2.7.7, and resuspended in 20 µl of sterile distilled water. The insert was not dephosphorylated. Therefore, the ligation reaction consisted of dephosphorylated vector DNA and an excess of phosphorylated insert DNA. This prevented the religation of vector without insert DNA. A typical ligation reaction contained 2 µl dephosphorylated vector DNA, 5 µl insert DNA, 4 µl 5 × T4 DNA ligase buffer and 1 unit of T4 DNA ligase (in a volume made up to 20 µl with sterile water). The reaction was left overnight at room temperature. 10 µl of the ligation mixture was used for the transformation of competent *E. coli*.

2.11 PCR

2.11.1 Routine PCR

The Polymerase Chain Reaction (PCR) is an amplification procedure which can produce large amounts of DNA from a small amount of DNA template (reviewed by Arnheim and Erlich, 1992). Two oligodeoxynucleotide primers (usually ≥20 bases), which can anneal specifically to sequences upstream and downstream from the double-stranded target DNA, are synthesised. The primers are designed such that, when they anneal to the template, their 3' ends face each other. The PCR is composed of a series of cycles and each cycle contains three steps. The first step is the denaturation, during which the template DNA is heated to 94 °C, for about 1 minute, to render it single-stranded. In the second step the reaction mixture is cooled, allowing the primers to anneal specifically to the single-stranded DNA. The temperature used for the annealing step is a few degrees below the lowest melting temperature (T_m) of the two primers. Finally, in the third step the reaction, the sample is heated to 72 °C, enabling the thermostable DNA polymerase to synthesise complementary DNA sequences starting from the 3' end of the primers. The time of this step is calculated from the extension rate of *Taq* polymerase (1 kb/min). This three step cycle is repeated 30-50 times, causing the concentration of the amplified DNA fragment to increase exponentially. Heat-resistant DNA polymerase, from bacteria such as *Thermus aquaticus* (*Taq*), is employed in PCR. This allows the annealing and extension steps to be carried out at elevated temperatures and just one sample of enzyme to be used during the entire cycling process (Saiki *et al.*, 1988). Thus, PCR reactions are easily automated using thermocyclers.

A typical PCR reaction was set up in a 0.2 ml thin walled PCR tube with the following components: 10 µl 10 × Expand™ buffer, 4 µl mix of dNTPs (5 µM each), 10 µl each primer (10 µM), and 10 µl template DNA (~2 fmol target DNA, usually 5 µl of 1/500 dilution of a plasmid miniprep) in a final volume of 100 µl. The components were mixed and briefly centrifuged in a microcentrifuge. 1 unit of Expand™ DNA polymerase was then added with gentle mixing.
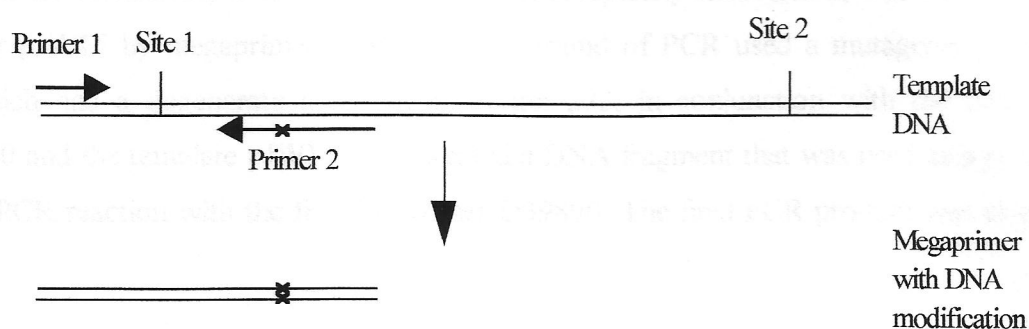
For error-prone PCR mutagenesis of gene sized DNA sequences, *Taq* DNA polymerase and buffers were used (Bioline).

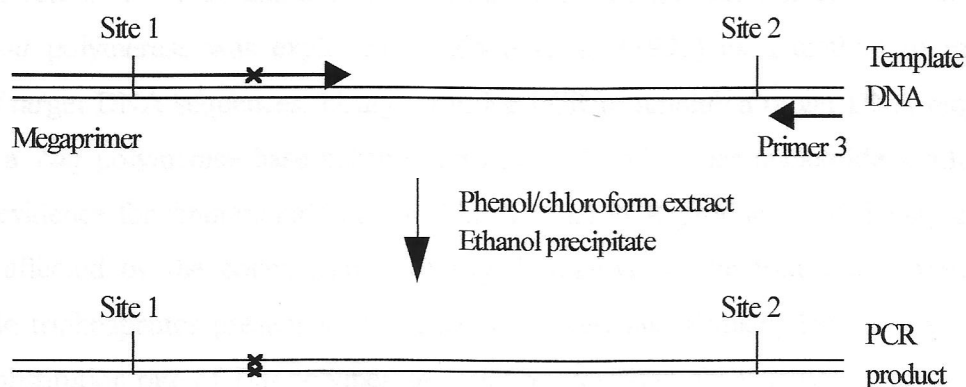## 2.11.2 Modification of sequences by megaprimer PCR

Megaprimer PCR was used to mutagenise genes in the absence of suitably placed restriction sites. Figure 2.1 outlines the technique, which is a two-step modification of the routine PCR described in Section 2.10 (adapted from a protocol by Perrin and Gilliland, 1990). In the first step two primers are used, one of these is mutagenic and the other overlaps a restriction site that will be utilised to clone the final PCR product into the vector DNA. The product of the first reaction is referred to as a megaprimer. In a second reaction the megaprimer is used in conjunction with a third primer, which overlaps a second restriction site, to generate a full-length product that is suitable for cloning.

2.11.3 Site-directed mutagenesis of *melR*

DNA fragments carrying *melR* cloned in pCM117-303, pVH-173, pJW15 or pLG314 were used as a template for mutagenesis. Mutagenic primers (Table 2.4) were used in conjunction with the flanking primer D29808, D27669, or D38105, depending on the template being used (see Table 2.5), to generate PCR products that were purified and used as a megaprimer in a second round of PCR with the appropriate flanking primer (D29809, D4600 or D38424). The resulting DNA fragments were restricted and cloned into pCM117-303, pVH-173, pJW15 or pLG314 as appropriate.

First PCR reaction

Primer 1   Site 1   Site 2

                                                                          Template
                                                                          DNA

Primer 2

                                                                          Megaprimer
                                                                          with DNA
                                                                          modification

Gel purify PCR product
Phenol/chloroform extract
Ethanol precipitate
Use as megaprimer in round 2

Second PCR reaction

Site 1   Site 2

                                                                          Template
                                                                          DNA
Megaprimer
                                                                          Primer 3

Phenol/chloroform extract
Ethanol precipitate

Site 1   Site 2

                                                                          PCR
                                                                          product

Cut with restriction enzymes 1 and 2,
purify and clone into appropriate vector

Figure 2.1 Schematic overview of megaprimer PCR (from Burr, T., 2000). The megaprimer is generated in a PCR reaction using a primer which flanks the upstream restriction site (primer 1) and a second primer carrying the desired modification (primer 2). This PCR product contains the desired modification and is used in the second PCR reaction in conjunction with a third primer that flanks a downstream restriction site (primer 3). The resulting full-length PCR product carries the desired modification as well as restriction sites for cloning.

2.11.4 Construction of a mutant library of codon 273 of *melR*

A library of *melR* derivatives, in which codon 273 was completely randomised, was constructed in the vector pJW15 by megaprimer PCR. The first round of PCR used a mutagenic primer, D38405, which had a degenerate sequence at codon 273, in conjunction with the flanking primer D4600 and the template pJW15. This created a DNA fragment that was used as a primer in a second PCR reaction with the flanking primer D39890. The final PCR product was cloned into pJW15.

2.11.5 Random mutagenesis of *rpoD*

Taq polymerase lacks a 3'→5' proof reading endonuclease activity. Tindall and Kunkel (1988) reported that, during a single round of DNA replication, *Taq* polymerase displayed a one-base frameshift error rate of 1/41,000 and a base substitution rate of 1/9000. The error-prone DNA synthesis of *Taq* polymerase was exploited by Zhou *et al.* (1991) as a method of random mutagenesis of target DNA sequences. Using a routine PCR to amplify a target DNA sequence they observed a *Taq* polymerase base substitution rate of 3.7×10^{-5} per nucleotide synthesised and found no evidence for "mutational hotspots" in the final PCR product. The fidelity of *Taq* polymerase is affected by the concentration of $MgCl_2$ relative to the total concentration of deoxynucleotide triphosphates present in the reaction (Eckert and Kunkel, 1990). They found that the base substitution rate of *Taq* polymerase could be increased, from 3×10^{-5} to 1.5×10^{-4} per nucleotide synthesised, by increasing the $MgCl_2$ concentration from 1 mM to 10 mM in the presence of 250 µM of each dNTP. Hence, by performing standard PCR and manipulating the error rate of *Taq*, DNA fragments can be generated containing random base pair substitutions. In this work, the method of Zhou and co-workers (1991) was used to prepare a library of random mutations in the 3' segment of the *rpoD* gene, which encodes the RNAP σ^{70} subunit. The PCR reaction contained the primers D38796 and D29200, and the plasmid pVRσ was used as a template. $MgCl_2$ was used at a concentration of between 2.5 and 3.5 mM and each dNTP was present at a concentration of 250 µM.

2.11.6 Construction of *melAB* promoter derivatives

The JK30 and JK31 fragments were generated by PCR, using primer D34119 for JK30 or D34120 for JK31, with the flanking primer D3407. pUC18 carrying the JK22 *melAB* promoter