CHAPTER 4

DISCUSSION

Pentachlorophenol (PCP) degradation by *Sphingomonas chlorophenolica* leads to the formation of tetrachloro-*p*-hydroquinone (TeCH) as the primary intermediate. PCP is oxidized to TeCH by PCP 4-monooxygenase (PcpB) in the presence of oxygen and NADPH (Orser et al., 1993). Usually, oxygenase system requires its reductase as a electron transfer protein for transferring electron from NAD(P)H to an active site of terminal oxygenase. Recently, amino acid sequence analysis of *pcpD* gene shows high similarity to those of oxygenase reductases. Therefore, *pcpD* should encode for PCP 4-monooxygenase reductase protein involved in transferring electrons from NADPH, through the redox centers of PcpD, to the FAD prosthetic group of PCP 4-monooxygenase (Lange et al., 1994). To investigate the functional of *pcpD* and PCP degradation in *S. chlorophenolica*, in this work, mutant strain of *S. chlorophenolica* containing mutated *pcpD* gene was constructed by gene replacement technique and studied the role of these mutants in PCP degradation. Lange et al. (1995) and Chanama and Crawford (1997) were successful to use gene replacement technique for disruption *pcpB* and *pcpA* gene in *S. chlorophenolica*, respectively.

Gene replacement is a gene knockout tool for genomic analysis. This technique is very useful for genomic study in microorganisms, plants and mammalian cells. One of the advantages of this technique is that it can be carried out in any wild type regardless of genetic background. Since it is not necessary to use a strain that is *recA, recBC sbcB* or *recD* deficient, thus useful for carrying out gene replacements in enteric bacteria other than *E. coli*. The use of a wild type genetic background also prevents

any problems that might occur when uncharacterized mutations or deletion constructs are introduced into strains that are *recA, recBC sbcB* or *recD* deficient (Hamilton et al., 1989). The only requirement for host strain is that it be recombination proficient. The requirements for the gene of interest are it must be dispensable and must have been cloned. The strategy for the gene replacement was shown in Fig 1.7.

The criteria for optimal transformation as described by Saunders and Saunders, 1988 are: first no endogenous plasmids should be present; second, the possibility of any existing restriction systems should be ruled out; third, a compatible replicating plasmid must be found; and fourth, a selectable marker must be known to work for the specific host. For *S. chlorophenolica,* it possesses at least one plasmid of approximately 100 kb in size. Second, *S. chlorophenolica* DNA is not sensitive to certain restriction enzyme, and the sensitivity is independent of GC content of the restriction enzyme recognition sequence. This indicates the presence of a DNA modification system whereby the DNA may be methylated at certain restriction enzyme recognition sequence, preventing their cleavage by restriction enzyme. These systems are generally regarded as protection system from foreign DNA. Finally, a plasmid capable of replicating in *S. chlorophenolica* has not been detected therefore no marker for test the transformation condition and transformation efficiency. Anyway Lange et al. (1995) and Chanama and Crawford (1997) succeeded in transformation of recombinant plasmid into *S. chlorophenolica* by electroporation.

The targeting vector was transformed into *S. chlorophenolica* and selected transformant in medium with kanamycin. Kanamycin resistance of transformant should be mediated by kan^r gene that inserted into genome because pUC 19 could not replicating in *S. chlorophenolica*. In this work transformation with targeting vector or pUC 19 gave no transformant cell. It is difficult to conclude that the electrotransformation in this work was successful or not because there were no marker

to test transformation efficiency. Anyhow electroporation condition and cells preparation for transformation in this work were performed according to Lange et al. (1995) and Chanama and Crawford (1997). Accordingly the targeting vector should be introduced into cell successfully.

Meletzus et al. (1990) reported about the effect of pulse length in the electrotransformation on the transformation efficiency, transformation frequency, and cell survival. Long time constant decreased the percentage of cell survival but increased transformation efficiency. In this work, electroporation settings of 2.5 kV and 600 Ohms resulted in a 11.5-13.5 ms pulse length, consequently short pulse length were applied to used in this work. A resistance 200 and 400 Ohms were applied and resulted in 4.0-9.5 ms pulse length. All three conditions (200, 400 and 600 Ohms) gave no transformant cell.

Competent cells of *S. chlorophenolica* for transformation were resuspended in 10% glycerol and 30% polyethylene glycol (PEG) 8000. For PEG, there were many works reported about PEG-promote the transformation of plasmid or chromosomal DNA into cell (Akimemko et al., 1976; Levi-Meyrueis et al., 1980; Glumova and Prozorov, 1981; Gasparich et al., 1993; Okamoto et al., 1997). Akimemko et al. (1976) shown that when PEG concentrations were increased the relative transforming activity (RTA) at first (at PEG concentrations approximately 30-40 mg/ml) increased to the value of 200-250%, then began to decrease. At PEG concentrations approximately 100-125 mg/ml. These showed that the PEG-concentration in this work should be appropriate for transformation.

In some recipient cells other than *E. coli*, transformation efficiency were very low for plasmid DNA prepared from *E. coli* (Meletzus et al., 1990; Binotto et al., 1991; Klapath et al., 1996). This is possible due to an active restriction modification

system in recipient cells. Followed by Lange et al. (1995) *E. coli* HB 101 and JM 105 could be used as recombinant host in the successful transformation of plasmid into *S. chlorophenolica.* Therefore *E. coli* XL 1-BLUE which used as rebombinant host in this work should not give any problem for transformation efficiency.

Transformation frequency (transformant per survivor) increases linearly with DNA concentration in the range of 10 pg/ml to 7.5 μg/ml (Dower et al., 1988). Conley et al. (1984) reported the relationship of transformation frequency and concentration of DNA in transformation of pBR322 into *E. coli*. The saturating DNA concentration/transformation mixture was about 0.5-1 μg, whereas higher 1 μg of DNA did not increased the transformation frequency. On the other hand, Le-vi Meyrueis et al. (1980) reported about the transformation of chromosomal DNA into *Bacillus subtilis* protoplast in the presence of PEG that DNA concentrations higher than 1-2 μg/ml decreased the yield of transformant, without showing signs of general toxicity. This result corresponded to that of Simon and McEntee, 1989 which reported about the transformation of plasmid into *Saccharomyces cerevisiae* by electroporation. The frequency of transformation increased with the amount of plasmid DNA between 25 ng and 100 ng. At higher concentrations of DNA (1-1.5 μg) transformant yield decreased. This work used 5 μg of targeting vector in each transformation mixture. The use of high concentration of DNA was because the targeting vector could not replicated in *S. chlorophenolica*. Thus it had limited time to live in cell. Moreover, high concentrations of DNA might increase the chance to find the target site on genomic DNA.

Linear-DNA fragments have been used successfully by several laboratories to do effect gene replacement in the *E. coli* genome (Jasin and Shimmel, 1984; Winans et al., 1985; Arps and Winkler, 1987; Shevell et al., 1988; Russell et al., 1989). Oden et al. 1990 demonstrated that it was not necessary to linearize the plasmid DNA to

achieve the desired double crossover event on the cominant gene replacement in *E. coli* genome. Linearization of plasmid DNA at a unique site reduces the transformation frequency relative to circular molecule by 10^2-10^3 fold (Cohen et al., 1972). Gene replacement results from crossover with recircularized plasmid molecules. The estimated linerized plasmid DNA can recircularize in *E. coli* at about a 10^{-5}-10^{-6} frequency by recombinatory event. Therefore it is inefficiency for the gene replacement process by transformation with linear DNA.

Moreover, stage of the transforming DNA result in efficiency of double crossing over. Targeting frequency increased proportionally with copy number of the targeting was part of a linear amplicon, but remained unchanged when it was present on circles. Transformation with covalently closed circular plasmid DNA which storage at 4°C, decrease in efficiency of double crossover event. This may be due to a higher percentage of nick molecules, which may not be substrates for the system (Oden et al., 1990). But when transformed with linearized plasmid DNA which storage at 4°C, the event of crossover increased. This increase correlated with the appearance of linear dimer, trimer and higher oligomer forms.

General recombination proceeds by means of stand exchange between DNA molucules. In *E. coli* this process is mediated by the production of the *recA* gene (Radding et al., 1982). For *Bacillus subtilis*, Rec E product functionally homologous to that of the *E. coli recA* gene, was detected in cases where recombination in dependent on short region of homology. Homology requirements for recombination in *B. subtilis* were differ from *E. coli*. Phage lamda-plasmid recombination in *E. coli*, approximately 30-40 bp to be a critical value for homologous recombination in $recA^+$ cell (King and Richardson, 1986; Shen and Huand, 1986). In the other hand, *B. subtilis* was found that approximately 70 bp of homology is required for detectable homologous recombination by plasmid-chromosome cointegration. Homologous recombination was not detected when only 25 bp of homology between plasmid and chromosome were

provided (Khasanov et al., 1992). The different in minimal homology requirements for gene targeting in *E. coli* and *B. subtilis* could be accounted for, not only by the different in DNA substrates used, but also by different in the enzyme machinery between gram-negative *E. coli* and gram-possitive *B. subtilis*. As was suggested by Singer et al. (1982), the critical homology length is determined by site size requirements for DNA strand-transfer proteins. So the differences in minimal homology requirements for two system could deflect differences in the site size for RecA and RecE proteins. Whereas homologous recombination in protozoan parasite Leishmania were found that a decrease in the length of homologous sequence <1 kb on one arm of the vector linearly influences the targeting frequency. No homologous recombination was detected, when the flanking homologous regions were <180 bp (Papadopoulou and Dumas, 1997).

Insertional recombination was very specific in targeting homologous site while the recombination rate did depend strongly on the homologous size of the targeting insert in the donor plasmid (Lee et al., 1998). Russell et al., (1989) reported that the frequency of linear transformation decreased with decreasing lengths of homologous DNA franking. The frequency of transformation with 1900 bp and 600 bp homology was less than 20% and 2%, respectively, of frequency with 4300 bp homology. When homologous sequences was interrupted by nonhomologous sequences that approached the size of the total homology, the integration frequency was most closely correlated to the average size of the flanking homologies. If the nonhomologous sequence were less than the total homology present, the integration frequency resembled that of uninterrupted sequence. Total homology flanking sequence (3′ homology + 5′ homology) for insertion recombination in *E. coli* was 0.85 kb (0.3 kb + 0.55 kb), 1.45 kb (0.85 kb + 0.6 kb), 1.45 kb (0.6 kb + 0.85 kb), 2.1 kb (1.0 kb + 1.1 kb) and 2.1 kb (1.1 kb + 1.0 kb) gave the insertion frequency of 1.13×10^{-4} 2.8×10^{-4}, 2.79×10^{-4}, 16×10^{-4} and 20×10^{-4}, respectively (Hamilton et al., 1989). These reports support the

dependence of frequency of homologous recombination on homology length. From Haminlton et al. (1989), the total homology 850 bp gave the insertion frequency 1.13×10^{-4}. In this work had homology length approximately 800 bp therefore the mutant by insertion would be 1.13 of 10000 cells and concentration of competent cell was not less than 3×10^{10} cells/ml thus one reaction of electroporation should gave the mutant at least 1.8×10^{5}colonies. But Papadoulou and Dumas (1997) reported that different chromosomal locations were found to be targeted with significantly variable levels of efficiency. Different strains of the same species showed differences in gene targeting frequency. Therefore the expected amount of mutant in these work may be not true.

This work transformed the targeting vector with the same condition as Lange et al. (1995) and Chanama and Crawford (1997) 25 times, but obtained no transformant. The last transformantion experiment gave transformant on kanamycin (Kan) plates. This transformants were transferred to ampicillin (Amp)-Kan plates to test the event of crossover. For single crossover, transformant would resistant to Amp and Kan whereas a double crossover event would produce transformant which only resistant to Kan. Unfortunately, wild type strain of *S. chlorophenolica* was resistant to Amp. Thus Amp-Kan plate could not be used to test the crossover event in the transformants. Southern blot analysis was used to confirmed the structure of gene replacement and verified that a crossover event had occurred. Southern blot analysis between *Eco*RI-digestion genomic DNA and *pcp D* fragment probe gave one signal band. The approximate size of possitive band was 3 kb, this corresponding with the expected size of Lange and Orser (1994). All of the transformants were *S. chlorophenolica* because it had signal bands on X-ray film with *pcpD* fragment probe. All transformants were mutant strains of *S. chlorophenolica* resistant to Kan. Surprisingly, the size of signal band between genomic DNA from wild type and mutants were equal. Consequently, Kan^{r} gene was not inserted into *pcpD* gene. The expected function of *pcpD* gene product is reductase.

Actually, similar oxidoreductase genes are in genome. It should have many genes in the genome of *S. chlorophenolica* which code for reductases. Therefore our targeting vector carrying kan^r gene might recombine at the other sites, which have high homologous sequence with *pcpD* and lead the mutants resistance to Kan. This was confirmed by Southern blot analysis with kan^r gene probe. Unfortunately, there was no signal band on X-ray film. Even though kan^r gene was inserted or replace into other site, it should be detected because there was no *Eco*RI site in kan^r gene. All mutants were resistant to Kan without insertion of kan^r gene.

Drug resistance is a state of insensitively or of decreased sensitivity to drug that ordinary cause growth inhibition or cell death. Kanamycin belongs to the group of aminoglycosides antibiotic. The term aminoglycoside refers to any carbohydrate containing amino function, which is joined via glycosidic linkage to an aglycone moiety. Kan^r of *S. chlorophenolica*, gram-negative bacteria, could be explained as Kan^r of gram-negative bacteria. There are three know mechanisms by which gram-negative bacteria develop resistance to aminoglyciside (Kucers et al., 1997).

a. Ribosomal mutation: This causes an alteration at the site of aminoglycoside attachment to the ribosome thereby interfering with the drug-ability to bind to the ribosome. Gram-negative bacteria readily develop high-level resistance to streptomycin by this mechanism, because this drug appears it bind to a single site on the 30s subunit of the ribosome. Other aminoglycosides, such as kanamycin and gentamicin, appear to bind to multiple sites on both ribosomal subunits, and high-level ribosomal resistance to these drugs is rare and cannot be selected in vitro by a single mutation step. Low level gentamycin-resistance due to changes in ribosomal protein has been reported, and stepwise high-level resistance may develop if an organism is exposed in vitro to progressively higher concentrations of drug. These mutants are avirulent and revert rapidly to sensitivity when grown in the absence of gentamycin (Foster, 1983; Moellering, 1983)

b. Decreased cell permeability: Uptake of aminogylcosides in sensitive strain of gram-negative bacteria occurs in three stages. These antibiotics initially associate with the cell wall and then pass passively through outer membrane pores. The next phase is energy-dependent and involves binding of the antibiotics to membrane structures and respiratory quinones to form complexes for their transport into cell. The third phase is also energy-dependent and involves membrane-bound aminoglycosides becoming bound to ribosome. Uptake of aminoglycoside is dependent on energy derived from aerobic metabolism. All anaerobic bacteria are aminoglycoside resistant because intracellular transport to these agents is makedly impaired in the absent of oxygen. Aminoglycosides which are modified by acetylation etc. appear to be unable to react with ribosomes and inhibit protein synthesis, and they only exhibit the first two phases of transport into the bacterial cell (Foster, 1983; Taber et al., 1987).

Gram-negative bacilli do not possess inactivating enzymes or ribosomal mutations, yet they are resistant to these drugs. In some of these resistance is due to a transport defect. These mutants may have changes in cytochromes, respiratory quinones or a reduction of the synthesis of components of the electron transport chain (Bryan and Kwan, 1981). This type of resistance leads to generalized cross-resistance to all aminoglycoside, although the level of resistance may be low (Davies, 1983). It probably occurs by a chromosomal mutation affecting the gene for aminoglycoside transport (Hardy et al., 1980). In other bacterial strain resistance may due to a permeability barrier at the cell wall; in some strains a low level aminoglycoside-resistance is associated with a change in the structure of the lipopolysaccharide in their cell walls (Bryan et al., 1984).

c. Aminoglycoside modifying enzyme: High-level resistance most commonly occurs due to the acquisition of plasmid, which code for the production of enzyme, modifying by either acetylation, adenylylation or phosphorylation (Shannon et al., 1978; Shaw et al., 1993). At the molecular level, resistance to aminoglycosides is determined by a group of relatively small genes, which may be located on the

chromosome, plasmids, transposons and possibly bacteriophages (Davies, 1983; Lupcki, 1987; Shaw et al., 1991; Coleman et al., 1994).

Kanamycin resistance of bacteria is most commonly due to plasmid-mediated production of a series of aminoglycoside-modifying enzyme. In this work, kan^r gene from targeting vector did not inserted into genome of mutant strains and the targeting vector does not have origin of replication in *S. chlorophenolica* thus it could not be replicated in this bacteria. This was confirmed by plasmid extraction in mutant strains. Mutants did not have the targeting vector therefore resistance to Kan was not plasmid mediated. High-level ribosomal resistance to kanamycin is rare and cannot be selected in vitro by a single mutation step therefore resistance to Kan by mutant strains in this work should not be mediated by the mechanism of ribosomal mutation.

In the further study should be tested the mutants that how they were resistant to Kan. Mutant strains in this work were resistant to Kan might be mediated by the mechanism of decreased cell permeability. For this mechanism should be confirmed by testing for Kan uptake in mutant cells. For example, culturing the mutants in medium containing Kan, detection of amount of Kan inside. The amount of Kan in mutant cells is compared with that of wild type. If the amount of Kan in mutant cells is lesser, resistance to Kan by mutants may be mediated by the mechanism of decreased cell permeability. Sometime, mutants might have chromosomal mutation affecting the gene responsible for aminoglycoside transport system. Restriction fragment length polymorphism (RFLP) or random amplified polymorphic DNA (RAPD) should be used to test DNA of the mutants and wild type. If RFLP or RAPD patterns profiles of DNA of mutants are different from the wild type, mutant strains possible have mutated DNA. Further studying mutated DNA will let us to understand the mechanism of resistance to Kan.

Inability to detect the mutant strain containing mutated *pcpD* gene which mediated by homologous recombination might come from:

a. Promoter of kan^r gene can not work in *S. chlorophenolica*. Kanamycin resistance gene was used as selectable marker for transformant. Kanamycin resistance gene which use in this work (npt I) coordinates from transposon Tn903 in the sequence 1083-2038, which code for Kan phosphotransferase. Origin of replication starts at 1133-1977. The DNA sequence shows an ATG at position 1162 that is preceded by the Shine-Dalgarno sequence A-A-G-G at 1152. Upstream from this lies a region with the characteristic of a transcriptional promoter: T-A-T-C-A-T-C (1106-1112) for the Pribnow box and T-G-T-T-A-C-A-T-T-G at the -35 region (Grindley and Joyce, 1980). Lange et al. (1995) could used kan^r gene from Tn 5 as a selectable marker gene in *S. chlorophenolica*. Both Tn 903 and Tn 5 are from *E. coli*, therefore kan^r genes from both transposons have *E. coli* promoter. From nucleotide sequence analysis, promoter of kan^r gene from Tn 903 is differ from promoter of kan^r gene from Tn 5. With different *E. coli* promoter, kan^r gene from Tn 903 may not work in *S. chlorophenolica.*

b. Unsuccessful transformation of targeting vector into *S. chlorophenolica.* Because there are many parameters affecting the transformation and there are no marker available to test transformation efficiency. Lange et al. (1995) reported that the previous attempt at transforming and mutagenized *S. chlorophenolica* were unsuccessful therefore this strain may be difficult to transform.

c. Homologous DNA length in the targeting vector might not be adequate for homologous recombination. Lange et al. (1995) reported that the homology length for disrupted *pcpB* gene in *S. chlorophenolica* was 2.3 kb whereas in this work it was about 0.8 kb on total homology length.

Therefore, in the further study one should use the kan^r gene from Tn 5 (npt II) to interrupt the ORF of *pcpD* and increase the homologous DNA length in the targeting vector because recombination rate did depend strongly on the homologous size, high homology length increases the recombination rate.