

4.1. INTRODUCTION:

It is now quite an established fact that elevated levels of mercury, whether by natural processes or by anthropogenic input, exerts selection pressure on microbial communities. Those communities that can be adapted are going to be the ones that will live through in environments contaminated by this toxic heavy metal. Many species of bacteria have developed several ways to cope up with mercury toxicity. A detailed and all encompassing review by Osborn et al. (1997) lists uptake of mercuric ions (Hg^{2+}) despite low cellular permeability, demethylation of methyl mercury followed by conversion to mercuric sulfide compounds, sequestration of methyl mercury by continuous production of hydrogen sulfide, methylation of mercuric mercury to the volatile methylmercury, and the enzymatic reduction of Hg^{2+} to Hg^0 as the major bacterial mechanisms to deal with Hg toxicity.

Resistance against mercury compounds, mediated by the microbial *mer* operon was discovered in the early 1970's (Summers & Lewis, 1973). Since then, several "archetypical" *mer* operons (Brown et al., 1991; Liebert et al., 1999) have been studied in depth with respect to structure, function and regulation of the individual gene products (Ji & Silver, 1995; Nies, 1999; Barkay et al., 2003). Genes conferring resistances to mercury compounds are clustered in an operon in most known naturally occurring systems (Silver & Phung, 1996; Barkay et al., 2003). The *mer* resistance components can be subgrouped into three categories based on their functional roles: transporters of Hg(II) into the cells, converters (enzymatically) of toxic mercury compounds into a relatively nontoxic form [$\text{Hg}(0)$], and regulators of operon expression. The *mer* operons from both gram-positive and gram-negative bacteria have been cloned and sequenced. Genetic and biochemical studies have advanced our knowledge, leading to in-depth understanding of

resistance mechanism, gene evolution, and regulation of expression of the *mer* genes (Misra, 1992).

This energy consuming reaction is based on the ubiquitous *mer* operon. Most *mer* operons contain at least the mercury-resistance genes *merR*, *merD*, *merT*, *merP* and *merA* (Silver & Phung, 1996; Osborn et al., 1997). Expression of *mer* operon is regulated by the products of *merR* and *merD* and is inducible by Hg (II). The product of *merR* represses operon expression in the absence of inducer and activates transcription in the presence of inducer. The product of *merD* coregulates expression of operon (Misra, 1992; Silver & Phung, 1996). Products of periplasmic *merT* and inner membrane-*merP* take part in the transport of metal across the cell membrane. Products of *merC* and *merF*, both membrane proteins were found to act as mercury transport system (Kusano et al., 1990; Wilson et al., 2000). Bacteria that are resistant only to Hg (II) have a so-called “narrow spectrum resistance”, whereas others that are resistant to both Hg (II) and certain organomercurials are with broad-spectrum resistance. Resistance against organomercurials depends on the organomercurial lyase MerB that cleaves the carbon-mercury bond of the organomercurials, and the resultant product Hg (II) can be subsequently reduced by the mercuric reductase (Silver & Misra, 1988). In some cases, the *mer* operon contains other functional genes. The *merG* product provides phenylmercury resistance, presumably by reducing the in-cell permeability to phenylmercury (Kiyono & Pan-Hou, 1999).

As expression of these genes is induced with low levels of mercury salts, Hg(II) functions as an environmental sensor, switching on the synthesis of mercury resistance protein components. Both MerC and MerT contain two pairs of cysteine and periplasmic MerP contains one pair of cysteine. It is hypothesized (Misra, 1992) that these cysteine pairs (thiol groups) sequester Hg(II)

and transfer Hg (II) to the thiols of cytoplasmic mercuric reductase via the transmembrane protein MerT. Thus Hg(II) is never free to interact with cellular constituents, and only the elemental form of mercury [Hg(0)] is released in the cytoplasm. Mercuric reductase (product of *merA*) is the key enzyme in reduction and detoxification of Hg (II). It is a flavin adenine dinucleotide (FAD) -containing disulfide oxidoreductase that transfers electrons of NADPH or NADH to Hg (II). The resulting metallic mercury Hg (0) is harmless to bacteria and diffuses out of the cell (Miller et al., 1986; Misra, 1992; Silver & Phung, 1996; Hobman & Brown, 1997; Osborn et al., 1997). In aerobic environs, metallic mercury volatilizes and thus the cell detoxifies its surrounding microenvironment. This mechanism of bacterial resistance to ionic mercury is illustrated in figure 4.1. The induction of *mer* expression by Hg (II) binding to MerR occurs at nanomolar concentration of Hg (II) (Condee & Summers, 1992). Organomercurials induce *mer* operon expression after their cleavage by MerB to Hg (II). But, in some cases, operon expression could be induced by organomercurials themselves before cleavages (Nucifora et al., 1989; Kiyono et al., 1997, 2000). Without the induction by low concentrations of Hg (II) (Lund et al., 1986), the “mercury resistance” is not present and cells are toxified at otherwise non-toxic Hg (II) concentrations (Horn et al., 1994). Organomercurial lyases differ in their substrate specificity; some lyases cleave mercury-carbon bonds of only a small number of organomercurials, while many others can cleave many bonds (Nakamura et al., 1990).

Bacterial strains examined from many distinct environments, reveal that *mer* operons occur on plasmids (Brown et al., 1986; Griffin et al., 1987; Radstrom et al., 1994) as well as on chromosomes (Wang et al., 1987; Inoue et al., 1991) and, also, are often components of transposons (Misra et al., 1984; Kholodii et al., 1993). Further, sequence analyses revealed that *mer* operon is a genetic mosaic with a novel modular arrangement (Summers, 1986) of essential

genes and accessory genes. Although the presence of mercuric reductase is essential for enzymatic detoxification - and hence resistance to inorganic mercury, expression of *merA* gene (and *merR*), has been reported in a high proportion of gram-positive environmental strains sensitive to mercury, suggesting the presence of non-functional *mer* operons in which the mercury transport genes are either absent or non-functional (Bogdanova et al., 1992). Although the physical arrangement of the *mer* operons may vary, all contain the essential genes but surprisingly, only limited studies have attempted to characterize mercury resistance at the molecular level in marine bacterial isolates (Barkay et al., 1989; Rasmussen & Sorensen, 1998). Barkay et al. (1989) found that only 12% of culturable MRB from estuarine environments hybridized to *mer* (Tn21) probe suggesting that such MRB from the marine environment encode novel *mer* genes or other mechanism (s) that provide Hg resistance (Reyes et al., 1999). It has been proposed that *mer* is an ancient system, which evolved at a time when levels of available mercury kept rising in natural environments, possibly as a consequence of increased volcanic activity (Osborn et al., 1997).

In this thesis mercury resistant marine bacteria have been investigated for the *mer* genes from genomic DNA and an attempt was made to investigate the presence and involvement of plasmid mediated resistance by two indirect assays.

4.2. MATERIALS AND METHODS:

All the thirteen isolates that were subjected to 16S rDNA sequencing were subjected to molecular analysis for detection of *merA* genes. Eleven of them viz. GO02 (*A. faecalis*), GP06 (*A. faecalis*), GP08 (*B.iodinium*), GP13 (*B.iodinium*), GP14 (*B. iodinium*), GP15 (*A.faecalis*), GP16 (*A.*

faecalis), GP17 (*A. faecalis*), CM10 (*Bacillus* sp.), CH07 (*P.aeruginosa*) and CH13 (*B.pumilus*) were BHRM (chapter 3) and were also capable of volatilizing mercury (chapter 5). As these strains were found to be promising potential candidates for detoxifying varieties of toxic chemicals, they were subjected to detailed molecular studies.

4.2.1. Detection of the *mer* operon:

Extraction of genomic DNA: as described in chapter 3.

Purification of DNA: as described in chapter 3.

PCR amplification of *mer* genes:

Purified DNA samples from all the MRB strains were stored at -20° C until taken up for PCR analyses. For all the PCR analyses, 20 μ l reaction mixture was used (Table 4.2.1) and the amplification carried out following a suitable program (Table 4.2.2) using a programmable DNA thermocycler (Eppendorf, Germany). The *merA* region was amplified using primer pairs synthesized by Invitrogen (Invitrogen GmbH, Karlsruhe, Germany) that were designed after aligning all available *mer* operon sequences from the EMBL database with ClustalW (Table 4.2.3). To amplify the complete region of *mer* several specific primers (Figure 4.2) were used and sequences of them are listed in Table (4.2.4). PCR mixture was prepared and appropriate primers were used prior to loading the samples into the thermocycler. At the end of the program, the microfuge tubes were taken out and a 2 μ l aliquot of PCR amplicon from each run was electrophoresed through TAE agarose (0.8%) gel for 45 minutes at 85 Volts using proper loading dye and marker. At the end of the electrophoresis, the gels were stained in ethidium bromide (EtBr) (1 μ g ml $^{-1}$) and then photographed using UV transilluminator (Herolab, Germany). Appropriate positive and negative controls were included in every PCR run. One of the *merA* products derived from the isolate *P. aeruginosa* (CH07) was extracted from the gel, cleaned and

sequenced by GATC-BIOTEC AG (Konstanz, Germany) with commonly used dideoxy chain termination method (Sanger et al., 1977) using an automated DNA sequencer (ABI PRISM model 3700).

4.2.2.1. Analysis of plasmid DNA using Nucleospin kit:

Cell preparation:

Seven bacterial isolates (GO02, GP06, GP14, GP15, GP16, GP17, CH07) which were also positive for *merA* were grown in Luria Bertani (LB) medium for 24 h and 1.5 ml of fresh culture was centrifuged in a 2 ml eppendorf tube at 11000g for 30 sec.

Cell lysis and clarification:

The supernatant was removed as much as possible. Two hundred fifty μ l buffer A1 was added and the pellet was resuspended till there was no clumps in the suspension. Two hundred fifty μ l buffer 2 was added and mixed gently by inverting the tubes for 6-8 times and were incubated at room temperature for 5 minutes. The lysate was centrifuged for 10 min at 11000g at room temperature.

Binding and eluting of DNA:

A Nucleospin Δ Plasmid column was placed in a 2 ml collecting tube; loaded the supernatant from the previous step onto the column and centrifuged for 1 min at 11000g. The filtrate was discarded and the column placed back into the collecting tube. Six hundred μ l buffer A4 (with ethanol added prior to use) was added into the column and centrifuged for 1 min at 11000g. The filtrate was discarded and the tubes were centrifuged once more at 11000g for 2 min to dry the

silica membrane. The tubes were kept on clean bench for additional 10 minutes to completely remove the residual ethanol. The column was placed on a 1.5 ml eppendorf tube and the silica membrane-bound DNA was eluted with 50 μ l sterile milliQ water and stored at -20° C.

Digestion of DNA:

Sample DNA (1 μ l) was taken into a sterile eppendorf tube and 4 μ l buffer 2 (New England Biolab) was added to it followed by addition of 4.5 μ l of milliQ water. Restriction enzyme Hind III (0.5 μ l) was added to this mixture (now 10 μ l) and was mixed well by tapping the tubes. The tubes were kept for incubation for 1 h at 37° C.

Gel electrophoresis:

Agarose gel (0.8%) was prepared in 1x TAE buffer (pH 8). The samples were diluted with milliQ water (1:5=sample: MQ water). The digested sample (3 μ l) was mixed with 2 μ l loading dye and was loaded into the well. The gel was immersed in running buffer (1x TAE) and was run at 90 V for nearly 2 h. The gel was stained in EtBr (1 μ g ml⁻¹) and was photographed using an UV transilluminator.

4.2.2.2. Plasmid Curing

Two different assays of plasmid curing (Figure 4.4) were performed on many strains to check whether Hg resistance was plasmid mediated. The resistant strains grown in two- strength (2S) SWNB (composition l⁻¹: peptone 10.0g, beef extract 6.0g, aged seawater 750 ml and deionized water 250 ml; final pH 7.5) with 10 ppm Hg were transferred to 2S SWNB (without Hg) and were allowed to grow for 24 h. After this, they were transferred to four-strength (4S) SWNB and allowed to grow for 12 h. A third transfer was done in to a fresh set of 4S SWNB tubes and

allowed to grow for six hours before inoculating into media containing different xenobiotics. Each time, a 50 µl inoculum was added to next set of experimental medium (i.e. either 2S or 4S). After every transfer, 15µl aliquots were spread plated on SWNA containing 0, 10 and 50 ppm Hg to note the frequency of loss if any, of mercury resistance. Acriflavine, a plasmid curing agent (Kim et al., 1990), was added to 2S SWNB at a concentration of 2.5 µg ml⁻¹ and changes in counts of colony forming units as well as their growth in the presence of different toxicants were monitored. This was based on the principle that if the number of colony forming units were reduced it meant that due to loss of plasmid-mediated resistance the no. of CFU became less. Else, there was no plasmid-mediated resistance.

4.3. RESULTS:

4.3.1. Mer operon:

The PCR analyses for the presence of different genes of the *mer* operon from the thirteen MRB isolates were interestingly different. DNA samples from nine isolates: GO02, GP06, GP08, GP14, GP15, GP16, GP17, CH07 and GO01 were positive for *merA* (plates 4.1a & b). None of these isolates possessed *merC*, *merF* or *merP*, which are responsible for the transport system functioning in the *mer* operon. Four isolates (GO02, GP14, GP16, CH07) were positive for *merB* (plates 4.2a & b). *MerD* was present in three isolates (GO02, GP14 and GP16; plate 4.3). All of these 11 isolates were seen to volatilize mercury (chapter 5). *MerR* gene was found in four isolates (GO02, GP14, GP16 and CH07; plate 4.4). A maximum of four genes viz. *merA*, *merB*, *merD*, *merR* of the *mer* cluster were present in three isolates viz. GO02, GP14 and GP16 (Figure 4.3). In CH07 *Pseudomonas aeruginosa* three genes (*merA*, *merB* and *merR*) were detected. CH13 (*Bacillus* sp.) and CM10 (*Alcaligenes faecalis*) lacked all the genes looked for from this set

of marine isolates. However, they volatilised mercury from the medium. PCR results for different *mer* genes are shown in the Table 4.3.1.

4.3.2. Analysis of plasmid DNA:

No plasmid was detected following the extraction and digestion of the product with Hind III. There was no indication of loss of Hg resistance after the plasmid curing experiment in any of the three strains tested (Table 4.3.2). Also, resistance to antibiotics and the capability to grow in SWNB containing DDT and several other toxicants was not lost in any of these strains following curing by growing the MRB either in double/quadruple strength SWNB or in medium with acriflavine.

4.4. DISCUSSION:

Mercury-resistance genes *merR*, *merT*, *merP*, *merA* and *merD* (Silver & Phung 1996; Osborn et al., 1997) on several operons operate probably in unison for protecting the cell from this heavy metal toxicity. As the gene products of *merR* (repressor) regulate expression of the operon and *merD* (inducer) is inducible by Hg (II), the *merR* product represses operon expression in the absence of inducer and, activates transcription in the presence of inducer (i.e., Hg [II]). Also as Misra (1992) and Silver & Phung, (1996) describe, this repressor coregulates the operon expression. In essence, the binding of Hg (II) to MerR occurs at nanomolar concentration of Hg (II) thus activating *mer* expression (Condee & Summers, 1992). According to Liebert et al. (1997), the *mer* loci are based on the presence and order of essential genes, *merR*, *merT*, *merP*, and *merA* or the accessory genes (*merC*, *merF*, *merB*, *merD* and *merG*). A number of features of original *mer* model have been confirmed by studying resistance and/or Hg²⁺ uptake in genetically

manipulated strains, but conflicting data have been reported on the role of the *merC* product. Deletion of the genes *merT*, *merC*, and/or *merP* of the Tn21-encoded system showed that *merT* and *merP* were essential for full resistance (Lund & Brown, 1987; Hamlett et al., 1992; Morby et al., 1995), but that *merC* could be deleted without any effect on resistance, at least as measured (Hamlett et al., 1992). However, in *Thiobacillus ferrooxidans* there is no gene corresponding to *merT*, but rather one that is similar to *merC* (55% homology at the amino acid level). Cloning of *merC* from *T. ferrooxidans* and its expression in *Escherichia coli* indicated that *merC* was involved in the uptake of mercuric ions (Kusano et al., 1990). The Tn21-encoded *mer* operon is complicated by the existence of both *merC* and *merT*. Hamlett et al. (1992) demonstrated by constructing deletion mutants lacking both *merT* and *merP* that the roles of different proteins in Tn21 system were necessary for resistance to 50 μM Hg^{2+} . Deletion of *merT* had greater impact than deletion of *merP*. Results have shown that deletions or mutations in *merT* and /or *merP* decreased operon induction, and in order to exhibit the optimum response in whole cells, the MerR- Hg^{2+} sensor required an intact mercury transport system (Lund & Brown, 1987; Hamlett et al., 1992). It was observed that in the absence of a specific mercury transport system, the *mer* operon could be induced at a high Hg^{2+} concentration almost as low as that required for induction of the operon with intact operon (Selifonova & Barkay, 1994). So, mercuric ions can be effectively transported to the MerR receptor by mechanism(s) other than the *mer* transport system.

Results from the detailed analyses during this study suggest that there are similarities of sequences of *merA* (using specific primers for *merA*) with *merC* (70%) PCR products. When compared with the existing sequences further suggest that *merC* is probably a divergent version of *merA*. In a recent study (Felske A. D. M. personal communication) a *Pseudomonas*

aeruginosa Bro12 isolated from chloralkali waste bore low similarity to already known mercuric reductase (around 70% nucleotide identity) probably due to variation in *mer* genes in same species of bacteria, which are present in less polluted soils than in mercury-impacted soils. Bruce et al. (1995) also found non-hybridization of clones towards the Tn501 *merRTΔP* region, which further suggest divergence of the *mer* sequence in the environment. Although the presence of the *merT*, could not be checked in this study, mercuric mercury is believed to cross the cell membrane as an uncharged complex with chloride (Barkay et al., 1997), hydroxide (Barkay et al., 1997; Morel et al., 1998) and/or sulfide (Benoit et al., 1999). It is quite likely that either passive transport or *merT* mediated transport into the cell occur in these marine MRB.

Plasmid curing experiments using two different techniques (using acriflavin and nutrient enrichment) did not yield any HgCl₂ sensitive derivatives. Moreover, as no plasmid could be detected after the extraction, in all the tested strains of bacteria (CH07, CH03, CH12, GO02, GP06, GP14, GP15, GP16 and GP17) the mercury resistant genes are chromosomally encoded. Trevors (1986) also found similar results using ethidium bromide as a curing agent. Olson et al. (1999) suggested that the absence of plasmid could be attributed to the facts that 1) large plasmids (100 megadaltons) will not go through 0.8% agarose gel; 2) DNA bands localized in the same region of the chromosomal band will not be observed; and 3) breakage of large plasmids during extraction will often be interpreted as loss of plasmids.

A number of studies have utilized DNA probes to confirm the presence of *mer* genes encoded on plasmids and host genomes (Barkay et al., 1989; Rani & Mahadevan, 1994; Rasmussen & Sorensen, 1998). Previous studies in terrestrial and freshwater environments have shown that as many as 100% culturable mercury resistant environmental isolates contain genes that have

homology to either *merTn501* (Bruce et al., 1995) or *merTn21* (Barkay et al., 1989). However, there are conflicting findings on the frequency of these prototypic genes occurring in culturable mercury resistant isolates obtained from marine water and sediment samples (Reyes et al., 1999). In a recent study, 29 out of 33 mercury resistant marine sediment bacteria were reported to contain *mer(Tn501)* genes (Rasmussen & Sorensen, 1998). In another study, quite contrastingly it was found that only 13 out of 105 mercury resistant marine bacteria isolates contained *mer(Tn21)*-like genes (Barkay et al., 1989). Reyes et al. (1999) found only 4% of the mercury resistant isolates from the most heavily impacted estuarine site to exhibit homology to the *merA* and *merRTΔP* DNA probes. Plasmid curing experiment done in this study ruled out these concerns, as none of the strains became sensitive to Hg following both the curing assays. Thus it can be hypothesized that the mercury-resistance in the strains studied was chromosomally borne rather being plasmid-mediated.

Nine of the 11 MRB isolates that volatilized Hg²⁺ to Hg⁰ (as seen from the Hg volatilization experiment) were positive for *merA* PCR. Interestingly, two of the 11 isolates that did not hybridize to the well-characterized *mer* genes were able to volatilize mercury and this indicates that non-*mer* mediated volatilization exists in the marine system. Excepting for CH07, all other strains lacked phenylmercury acetate (PMA) resistance [in sweeter nutrient broth with 10 ppm PMA concentration] thus allowing to suggest that the *merG* is probably not expressed by most marine MRB. In addition, the three other *merB* positive strains viz (GO02, GP14 and GP16), were unable to resist PMA unlike the CH07. Thus, it is likely that both *merB* and *merG* are required for PMA resistance in the marine MRB. Baldi et al., (1993) reported such non-*mer* mediated volatilization like the one found in this study. Findings from this study as well as some previous ones (Barkay et al., 1989; Reyes et al., 1999) provide evidence that an important portion

of the culturable mercury resistant bacteria from the marine environments might be lacking genes homologous to well characterized *mer* genes. And, there is a strong possibility of existence of non *mer*-mediated mercury volatilization system in marine microbes.

Table 4.2.1: Composition of PCR reaction mix for detecting *mer* genes

Composition	Volume
Water	6.4 μ l
10X Buffer (LSP)	2 μ l
MgCl ₂ (25 mM)	2 μ l
Q-solution	4 μ l
DNTP	2 μ l
Primer (F)	0.5 μ l
Primer (R)	0.5 μ l
DMSO	0.4 μ l
Taq polymerase	0.2 μ l
DNA	2 μ l
Total	20 μ l

Table 4.2.2: Thermocycler programme followed for PCR for the *mer* genes

Program:	Time	Temperature
For 30 cycles	Min: sec	°C
Pre denaturation	5:00	95
Denaturation	0:15	94
Annealing	0:30	Different
Elongation	1:00	68
Post elongation	4:00	68

Table 4.2.3: Various sets of primers used for detecting *mer* genes through PCR

<i>mer</i> gene	Primers	Annealing Temp.
Mer A	A1 (F) & A5 (R)	50.7°C & 58.1°C
Mer B	B1 (F) & B2 (R)	55°C
Mer C	P1 (F) & A10 (R)	63°C ~ 64.6°C
Mer R	R3 (F) & R1 (R) or T3 (R)	56°C
Mer D	D6 (F) & E1 (R)	56°C
Mer P	P3 (F) & A10 (R)	57.6°C
Total Mer	R3 (F) & E1 (R)	57.6°C

Table 4.2.4. Primer sequences used for PCR for *mer* genes

<i>mer</i> primer s	Directio n	Nucleotide sequence (5'-3')	Annealing Temp. (°C)
A1	F	ACC ATC GGC GGC ACC TGC GT	68
A5	R	ACC ATC GTC AGG TAG GGG ACC AA	70
B1	F	TCG CCC CAT ATA TTT TAG AAC	58
B2	R	TTT CTT TGC ATC TGT CCC GAC	60
P1	F	GGC TAT CCG TCC AGC GTC AA	64
A10	R	GAT CAT GAT CTT GGA CGG CAC ACA	74
R3	F	ATC AGC GGG CAG GAA ACG TT	62
R1	R	TTG GAG AAT GTG ACT ATG GC	55
T3	R	TTG NGG TTC AGA CAT RGG CT	60
D6	F	TGT TCA ATG ARC GCC TAC AC	59
E1	R	ATC GGT TTG TGC GTC TCG GA	NA
P3	F	AAG GCS WTK WKC RAG GTC GA	63

Table 4.3.1. Summary of results on detection of different *mer* genes, presence of plasmids in marine mercury-resistant bacteria and their ability to volatilize mercury

MRB	<i>merA</i>	<i>merB</i>	<i>merR</i>	<i>merD</i>	<i>merP</i>	Volatilization	Plasmid assay
GO02	+	+	+	+	-	+	-
GP06	+	-	-	NT	-	+	-
GP08 ^a	+	-	+	-	-	+	NT
GP13	-	-	-	NT	-	+	NT
GP14	+	+	+	+	-	+	-
GP15	+	-	-	NT	-	+	-
GP16	+	+	+	+	-	+	-
GP17	+	-	-	NT	-	+	-
CH07	+	+	NT	-	-	+	-
CH13	-	-	NT	NT	-	+	NT
CM10 ^a	-	-	NT	NT	-	+	NT

+, positive; -, negative; NT, not tested; ^a tested for both *merC* and *merF*, but they were absent

Table 4.3.2. Average plate counts (number ml⁻¹) of mercury resistant bacteria from plasmid curing assays

	Growth medium								
	SWNA with no Hg			SWNA with 10 ppm Hg			SWNA with 50 ppm Hg		
Isolates >>	CH 03*	CH 07	CH 12	CH 03	CH 07	CH 12	CH 03	CH 07	CH 12
Assay									
2S SWNB without mercury									
I transfer	3360 ±84.8	10496 ±362.1	4224 ±169.7	3360 ±56.6	6144 ±116	3840 ±178.2	3256 ±79.2	12096 ±517.6	3024 ±178.2
4S SWNB without mercury									
II transfer	7680 ±362	8320 ±905.1	16896 ±794.8	6656 ±461	12544 ±4344	11904 ±373.4	3584 ±370.5	4440 ±1742	8960 ±602.5
III transfer (0 h)	6720 ±518	6080 ±1358	3840 ±181	4160 ±226	5760 ±3620	2560 ±181	4352 ±461	2512 ±939	6912 ±362
III transfer (6 h)	4608 ±45.3	7936 ±3982	10368 ±594	2560 ±48.1	7760 ±792	5952 ±175.4	3552 ±121.6	6912 ±4242	2944 ±181
2S SWNB with acriflavine, without mercury									
I transfer	5760 ±232	15872 ±509	3520 ±181	5568 ±238	4992 ±175.4	3456 ±362	5632 ±913.6	8192 ±213.5	2880 ±79.2
II transfer	8640 ±453	14720 ±1991	10112 ±526.1	7680 ±175	10176 ±2987	6528 ±645	5760 ±489	1324 ±164	2260 ±221
III transfer (0 h) ^a	3040 ±56.6	4144 ±792	5248 ±461	4096 ±50.9	4440 ±1867	3840 ±215	2880 ±325.3	2664 ±758	4800 ±354
III transfer (6 h)	4128 ±122	7680 ±1991	7680 ±181	5632 ±192	6432 ±1747	5120 ±175.4	3456 ±36.77	2733 ±1204	3072 ±178.2

*isolates, CH03 and CH12 belong to Enterobacteriaceae and CH07 is a *Pseudomonas*. ^aPlating was done immediately following the III transfer (0 h) and after six hours of incubation. Please refer Figure 4.4 for details.

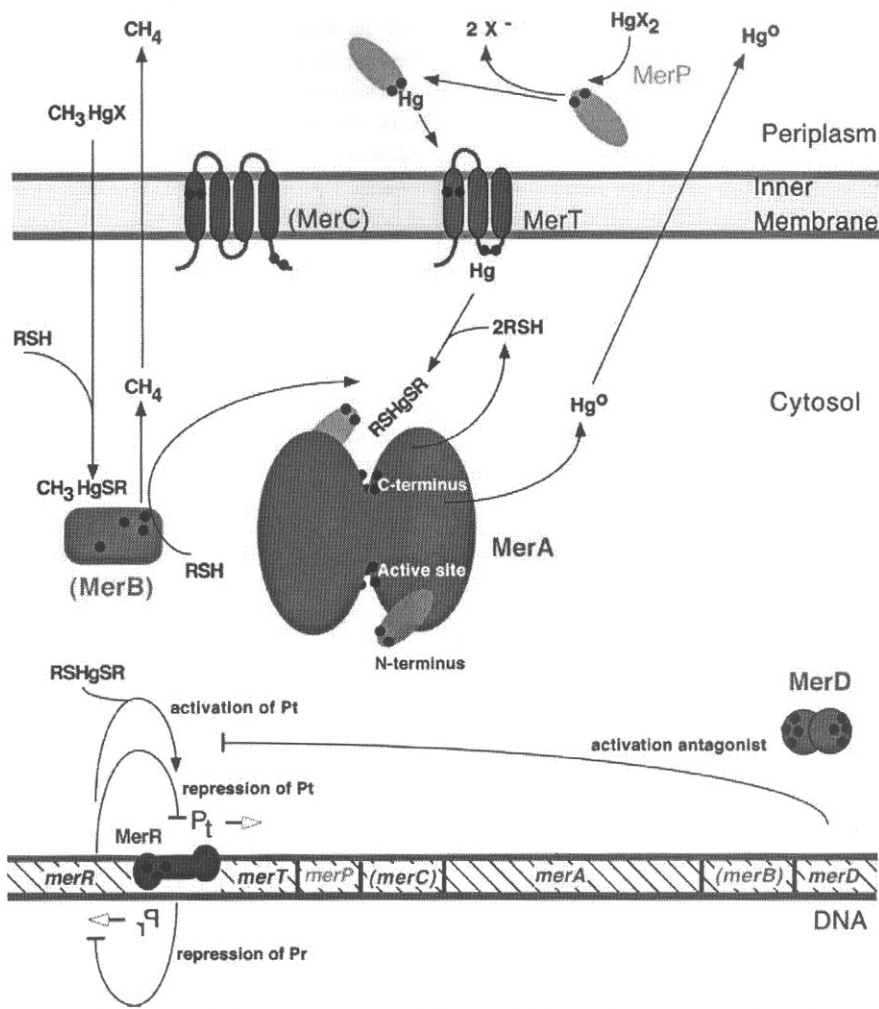


Figure: 4.1. Molecular mechanism of mercury resistance (Figure adapted from Barkay et al., 2003)

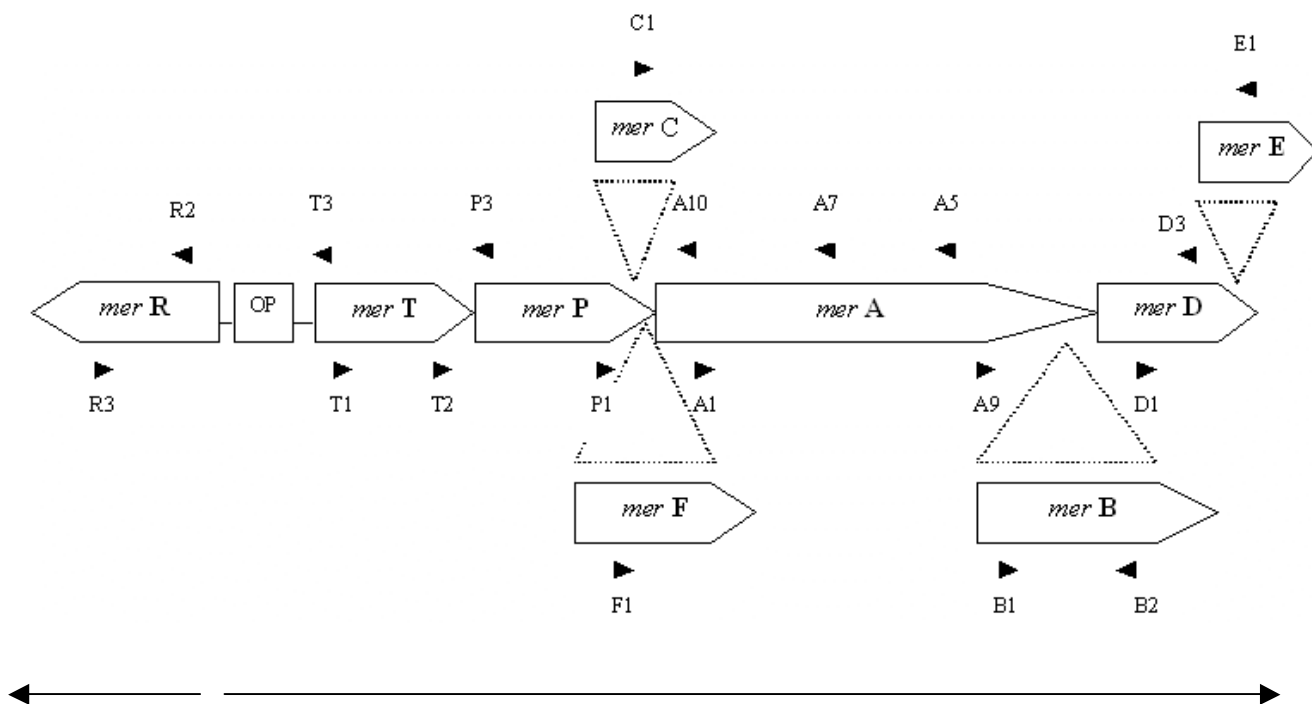


Figure 4.2. Schematic representation of the complete *mer* operon of gram negative bacteria. The *merR* gene is transcribed in opposite direction from the other *mer* genes as indicated by the large arrows below the figure. The arrowheads indicate the location and direction of the *mer* primers (after Liebert et al., 1997).

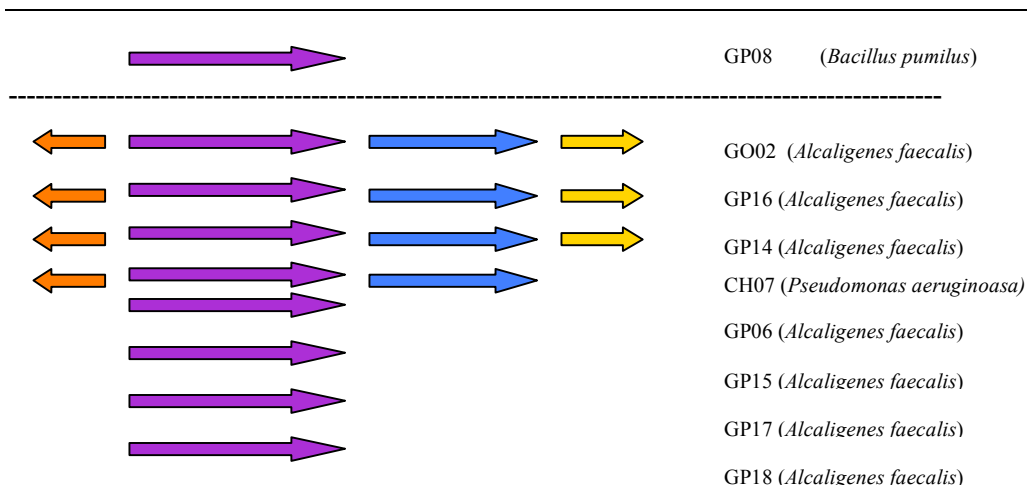
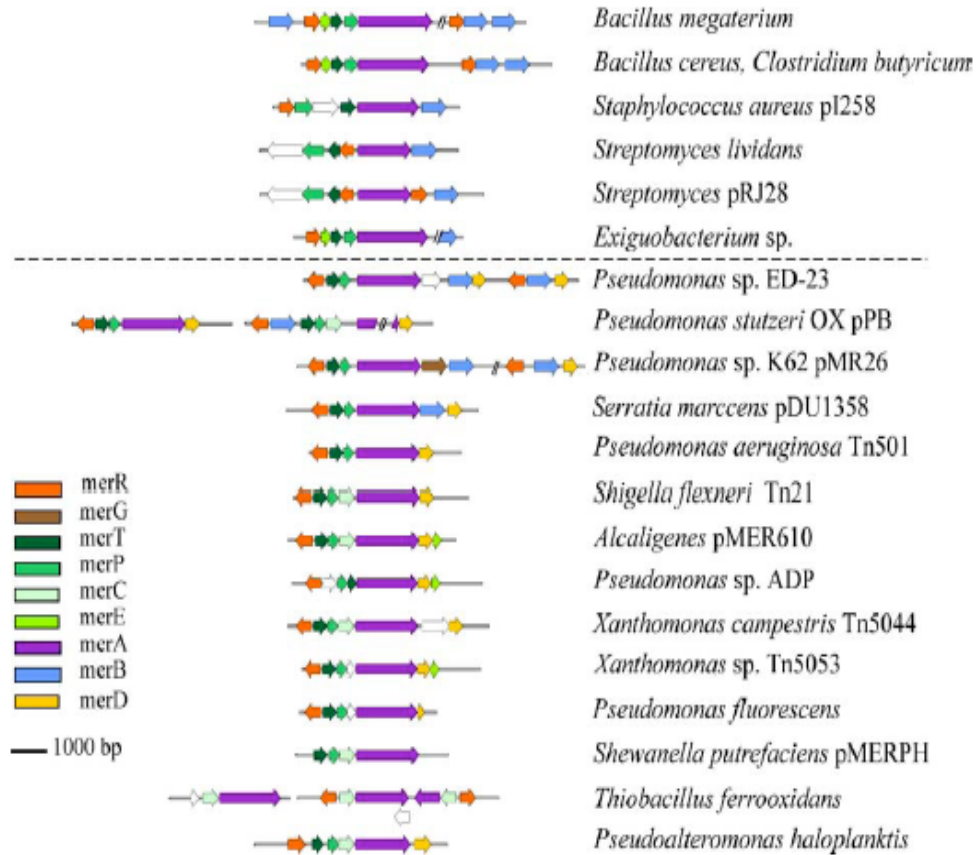


Figure 4.3. Diversity of *mer* operons. Sequenced *mer* operons from gram-positive (above dotted line) and gram-negative (below line) bacteria. Arrows indicate the direction of translation of each gene product. Colorless arrows indicate ORFs with unknown functions. The upper panel is adopted from Barkay et al, 2003. The lower panel shows the presence of different *mer* genes in marine MRB examined during this study. It is however to be noted that only 6 different *mer* genes were checked during this study.

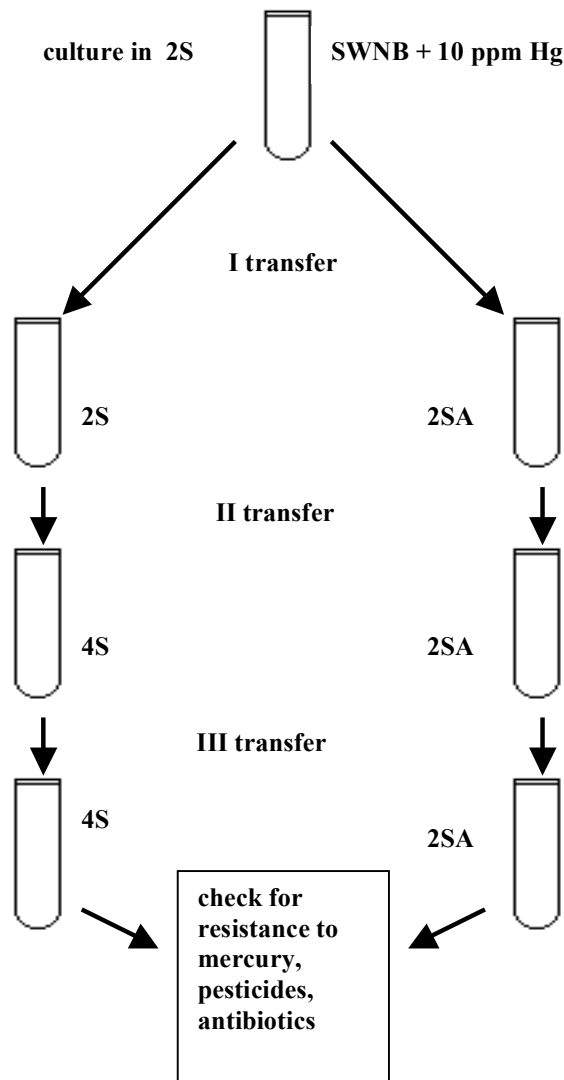
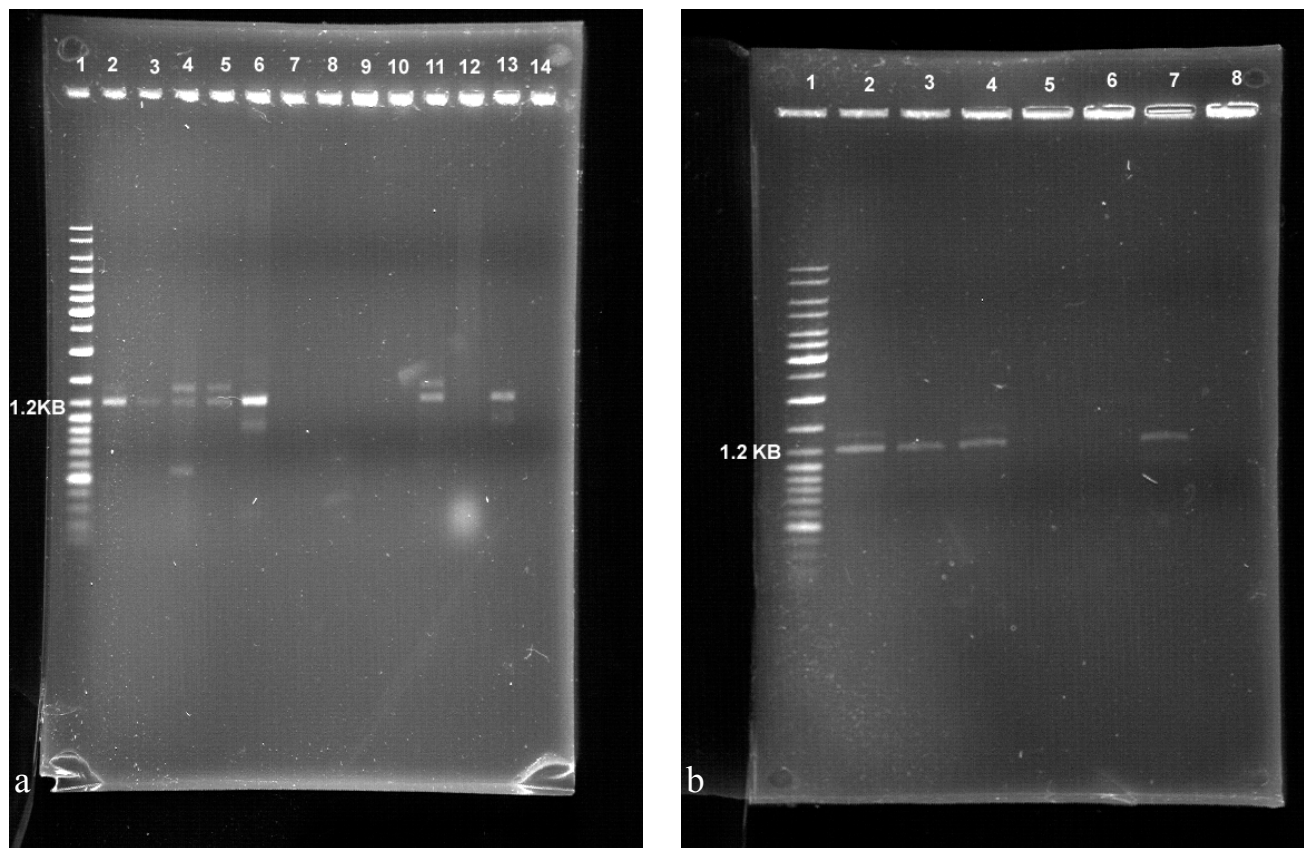


Figure.4.4. Experimental protocol for plasmid curing assays. Cultures of CH03, CH07 and CH12 were grown initially in two-strength seawater nutrient broth (2S SWNB) containing 10 ppm Hg. The first transfer into 2S and 2SA (A denotes acriflavine) was done after 24 h growth. Mercury was not added to any culture tube beginning from I transfer. While the growth was allowed for 24 h after I transfer, it was allowed only for 12 h after II transfer and for 6 h after the III transfer. At each transfer stage, plate counts were determined by spreading 15 μ l of culture on to SWNA with 0, 10 and 50 ppm Hg. After incubating for 6 h, cultures from III transfer sets were inoculated into appropriate media to check for resistance to mercury, pesticides and antibiotics.



Plates 4.1. Gel electrophoresis of PCR products showing *merA*

- a.** Lanes from left to right: lane 1, marker (DNA ladder mix, MBI Fermentas), lanes 2-13, GP14, GP15, GP16, GP17, CH07, CH13, CM10, 11C, S3, GO01, -ve control, Tin2 (+ve control), PCR mix (-ve control)
- b.** Lanes from left to right: lane 1, marker (DNA ladder mix, MBI Fermentas), lanes 2-8, GO02, GP06, GP08, 3C (contaminant), GP13, Tin2 (+ve control), PCR mix (-ve control)

Nine isolates viz. GO02, GP06, GP08, GP14, GP15, GP16, GP17, CH07 and CH01 were positive

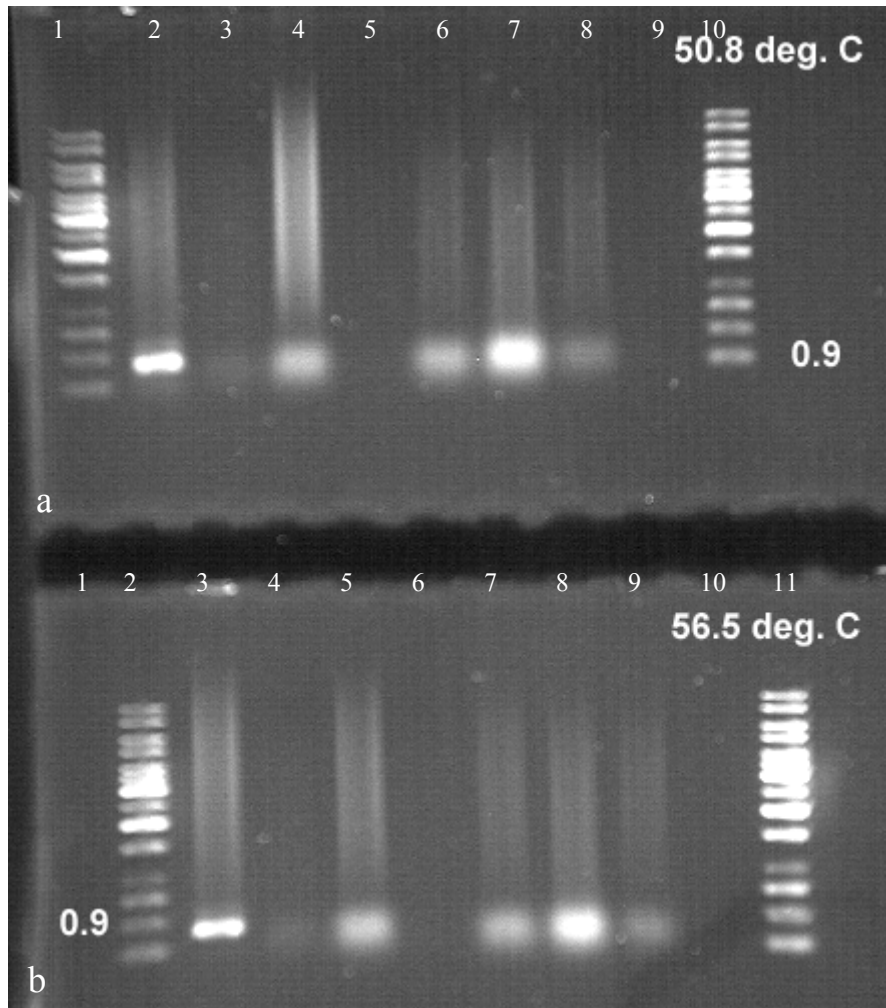


Plate 4.2. Gel electrophoresis of PCR products showing *merB*

a. Lanes from left to right: lane 1, marker (DNA ladder mix, MBI Fermentas); lanes 2-10, KT2442::*mer* 73 (+ve control), Hyp1, GO02, GP08, GP14, GP16, CH07, -ve control, marker ((DNA ladder mix, MBI Fermentas)

b. Lanes from left to right: lane 1, blank; lanes 2-11, marker ((DNA ladder mix, MBI Fermentas), KT2442::*mer* 73 (+ve control), Hyp1, GO02, GP08, GP14, GP16, CH07, -ve control, marker ((DNA ladder mix, MBI Fermentas)

Four isolates viz. GO02, GP14, GP16 and CH07 were positive

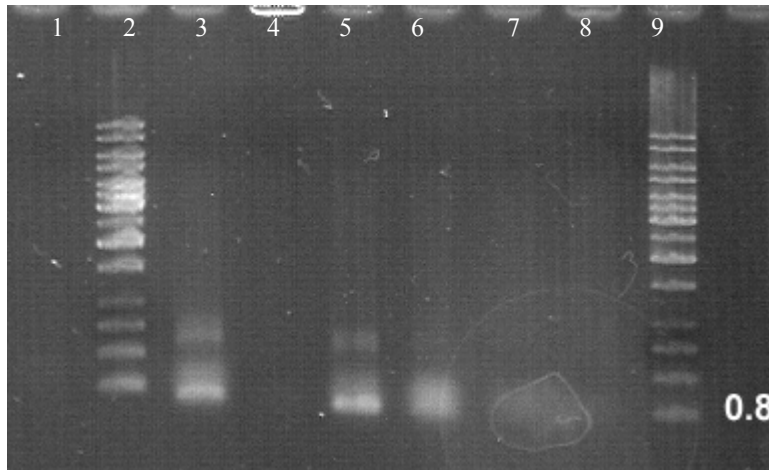


Plate 4.3. Gel electrophoresis of PCR products showing *mer D & E*

Lanes (from left to right): 1-9, -ve control, marker, GO02, GP08, GP14, GP16, CH07, KT2442::mer 73, marker (DNA ladder mix, MBI Fermentas).

Three isolates viz. GO02, GP14 and GP16 were positive

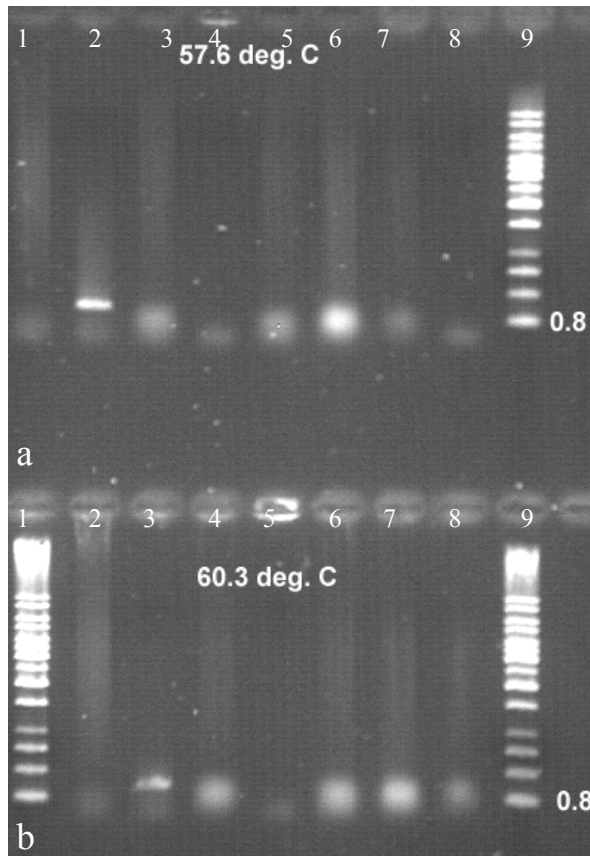


Plate 4.4. Gel electrophoresis of PCR products showing *merR*

a. Lanes from left to right: lane 1, KT 2442::*mer73*; lanes 2-9, Hyp1, GO02, GP08, GP14, GP16, CH07, -ve control, marker (DNA ladder mix, MBI Fermentas)

b. Lanes from left to right: lane 1, marker (DNA ladder mix, MBI Fermentas); lanes 2-9, KT 2442::*mer73*, Hyp1, GO02, GP08, GP14, GP16, CH07, marker (DNA ladder mix, MBI Fermentas)

Four isolates viz. GO02, GP14, GP16 and CH07 were positive

APPENDIX:

Buffers for isolation of genomic DNA:

Lysis buffer:

50 mM EDTA
50 mM Tris/HCl
0.5% Tween 20
0.5% Triton X-100
Lysozyme

Equilibration buffer:

750 mM NaCl
50 mM MOPS
15 % ethanol
0.15% triton X-100
pH 7.0 (room temp.)

Washing buffer:

1.0 M NaCl
50 mM MOPS
15% ethanol
pH 7.0 (room temp.)

Elution buffer:

1.25 M NaCl
50 mM Tris/HCl
15 % ethanol
pH 8.5 (room temp)

Buffers for PCR Purification:

Binding buffer:

7 M Guanidine-HCl in 200 mM MES buffer pH5.6 or
5.3 M Guanidine-HCl in 150 mM KAc buffer pH4.8

TE buffer:

10mM Tris-HCl, pH 7.5
1mM EDTA

Buffers for preparation of Plasmid DNA (alkaline Lysis)

SolutionI

50 mM Glucose
25mM TRis-cl (pH 8.0)
10mM EDTA (pH 8.0)

Solution II (Freshly Prepared)

0.2 N NaOH

1% SDS

Solution III

5 M potassium acetate 60ml

Glacial acetic acid 11.5 ml

H₂O 28.5ml

These buffers used in different analyses listed in chapters 3 and 4 of this thesis.