Cloning and Characterization of a mer Operon From Natural Aquatic Bacteria

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ABSTRACT

Mercury is a highly toxic element and has caused numerous cases of human toxicity throughout history. Methylmercury is the most toxic form of mercury and bioaccumulates in fish consumed by humans. Mercury resistant bacteria exist in nature that have the ability to detoxify methylmercury by a two-enzyme system encoded on the mer operon. Currently only mer operons cloned from clinical isolates are available which may not be relevant to molecular microbial ecology studies performed in aquatic environments. The purpose of this study is to clone a mer operon from natural aquatic bacteria. Ten strains of bacteria resistant to at least 2 ppm methylmercury were isolated from aquatic habitats. Genomic libraries were created in Escherichia coli from four of these strains. One clone was obtained from strain LO13 that conferred mercury resistance. The clone has a 10.5 kb fragment that presumably contains a mer operon. A restriction map was created and the fragment was sub-cloned and sequenced. The DNA sequences were compared to known sequences on the NCBI database using a "blastx" protein search. Once characterized this mer operon will be more relevant for use in bioremediation and gene expression studies in aquatic environments.

INTRODUCTION

Mercury is a highly toxic element and has caused numerous cases of human toxicity throughout history. Mercury is found in many forms in the aquatic environment and undergoes a complex biogeochemical cycle (Fig 1). The elemental form of mercury (Hg0) is very volatile and is readily released into the atmosphere. An ionic form (Hg(II)) exists which maybe methylated to methylmercury (CH3Hg+) by bacteria (Winfrey and Rudd, 1990). Methylmercury is the most toxic form of mercury and is bioaccumulated in fish and other aquatic animals. Human consumption of fish with elevated levels of

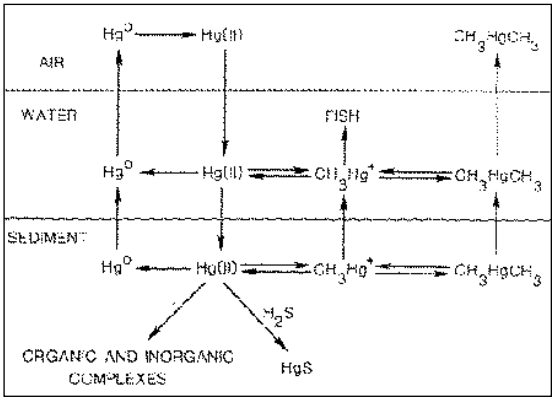

Fig. 1. The biogeochemcial cycling of mercury in freshwater lakes.

methylmercury can cause serious health concerns.

Mercury resistant bacteria have the ability to convert the methylmercury to the more volatile Hg^0, thereby detoxifying methylmercury and removing mercury from the aquatic environment. This is carried out by a two-enzyme system that is encoded for within the *mer* operon (Silver and Mirsa, 1988; Fig 2). The *mer* B gene encodes for organomercury lyase, which converts CH_3Hg^+ to Hg(II). Mercuric reductase, encoded for by the *mer* A gene then converts Hg(II) to Hg^0 which is released into the environment. The *mer* R gene product is a regulatory protein that both represses and activates the operon (Hongri et al, 1996). The *mer* P and *mer* T genes encode proteins involved in transport of mercury into the cell.

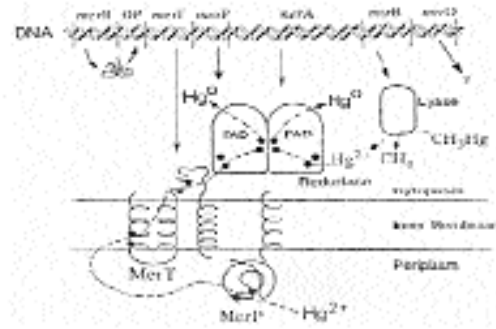

Fig 2. Model of the mercury resistance genes (mer operon) from plasmid pDU1358 found in a clinical isolate of Serratia marcescens.

Currently *mer* operons are used in molecular microbial ecology and bioremediation studies. However, only *mer* operons cloned from clinical isolates are readily available. These *mer* operons may not be relevant for use in gene expression and bioremediation studies performed in aquatic environments. The purpose of this study is to clone and characterize *mer* operons from natural aquatic bacteria.

Materials and Methods

Bacterial Isolations. Ten strains of mercury resistant bacteria were isolated by Brain Marks from natural aquatic habitats on plates containing at least 2 ppm phenyl mercuric acetate (PMA) (Table 1). The identity of strain LO13 was determined by biochemical reactions (APITM test strip) and 16s ribosomal RNA sequencing.

DNA Isolation. Bacterial strains L013, L014, L016, and MB5 were streaked out on R2A agar containing 4 ppm PMA and incubated at 25ºC for 24 hrs. A tube containing 5 ml of R2A broth plus 2 ppm PMA was inoculated from the isolated colonies. This culture was transferred to a 100 ml flask containing R2A +2 ppm PMA. DNA was then isolated according to the procedure described in Winfrey et al, 1997. The concentration of the DNA was estimated using spectrophotometry (Winfrey et al, 1997).

Cloning Techniques. The chromosomal DNA from these strains was partially digested with the restriction endonuclease *Sau* 3A I at 37ºC. Aliquots were removed from the digestion at various time points. The DNA fragments were visualized by agarose gel electrophoresis on a 0.8% gel stained with ethidium bromide (Fig 4). Restriction fragments between 5kb and 10kb were purified by a Gene Clean™ procedure (Gene Clean™; Fig 3). The plasmid pGEM –3Zf(+) was digested with *Bam*H I at 37ºC for four hours and ligated to the compatible ends of the *Sau* 3A I fragments. The ligation was allowed to proceed for eight hours at 16ºC.

Preparation of competent cells. *Escherichia coli* DH5? was streaked on TSA plates and incubated at 37ºC for 24 hours. Isolated colonies were inoculated into 5ml of LB at 37ºC

with shaking (300rpm) overnight. Four milliliters of culture was transferred to a 1000 ml flask containing LB to make a 2% inoculum and placed on a shaker at 37ºC. The Flask was allowed to incubate until the absorbance read 0.58. Cells were treated with cold $CaCl_2$ according to the procedure described by Winfrey e. al, 1997 and frozen in a dry ice/ethanol bath.

Transformations. The recombinant DNA plasmids were then transformed into competent E. coli strain DH5? by the procedure described in Winfrey et al, 1997. The transformation mixture was plated on plates containing 5ppm Hg(II) plus 75µg/ml ampicillin and incubated at 37ºC for 24hrs.

Restriction Mapping of pMJP3. The recombinant plasmid containing the *mer* clone (pMJP3) was isolated by alkaline lysis mini-prep procedure (Winfrey et. al., 1997). Single, double, and triple digests with selected restriction endonucleases were performed on pMJP3 to make a restriction map (Fig. 5).

Subcloning of pMJP3. pMJP3 was cut with *Sal* I and *Bgl* II restriction endonucleases in separate digests at 37ºC for 1 hour to generate smaller fragments of the cloned DNA (Fig. 5). Each fragment was ligated to pGEM –3Zf(+) and transformed into *E. coli* DH5? to generate two subclones. Subclones from the *Bgl* II and *Sal* I fragments were designated pMJP4 and pMJP5 respectively (Fig. 5).

Sequencing Techniques. The recombinant plasmid DNA from pMJP3, pMJP4, and pMJP5 was isolated using a Qiagen™ mini-prep kit (Qiagen). Polymerase Chain Reaction (PCR) based sequencing reactions were completed using a BIG dye™ kit and reaction products sent to UW-Madison Biotech Center for sequence analysis (BIG dye). DNA sequences were compared to known sequences on the NCBI database using a “blastx” protein search.

RESULTS

Table 1. Characteristics of mercury resistant bacteria

Strain	Resistance ppm PMA[a]	Gram reaction	Oxidase reaction	Morphology
MB1	2	-	-	single rods
MB2	3	-	-	short rods
MB3	2	-	-	oval rods
MB4	3	-	-	single rods
MB5	4	-/+	+	oval rods
L020	3	-	+	rods
L014	3-4	-/+	+	short rods
L013	4	-	+	single rods
YB3	2	-	-	rods
PA07b	5	-	-	short rods
L016	4	-	-	oval rods

[a] phenyl mercuric acetate

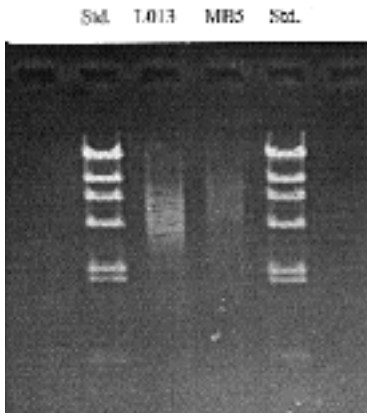

FIG. 3. An agarose gel of DNA from the 4-10 kb range of Sau 3A1 partial digests of DNA from strains LO13 and MB5. Standards are a Hind III

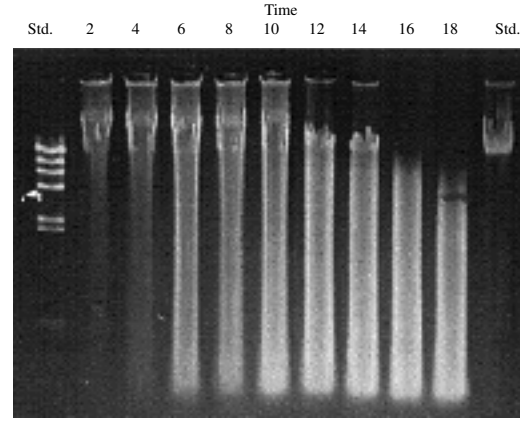

FIG. 4. An agarose gel of chromosomal DNA strain L013 partially digested using the restriction endonuclease Sau 3A I. Time refers to the amount of time the DNA was allowed to digest. Standards are a

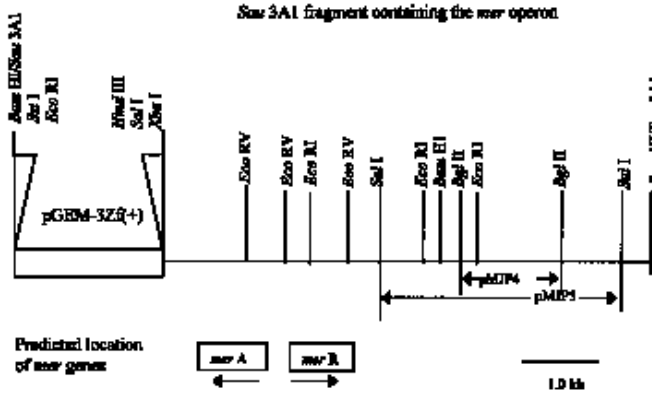

FIG. 5. Restriction map of pMJP3. The recombinant plasmid contains a 10.6 kb fragment (from a Sau3A 1 partial digest) that confers mercury resistance to E. coli DH5a. Subclones pMJP4 and pMJP5 refer to the individual fragments ligated to pGEM 3Zf(+). The predicted location of mer A and mer R genes based on DNA sequence of pMJP3 and pMJP5.

RESULTS AND DISCUSSION

Mercury resistant bacteria were isolated and characterized by resistance to phenyl mercuric acetate (PMA), Gram reaction, morphology, and oxidase reaction (Table 1). Although the identity of these strains is unknown, they are all Gram negative rods. The identity of strain LO13 was determined to be Pseudomonas putida by biochemical tests and 16S ribosomal RNA sequence comparison.

The recombinant plasmid pMJP3 containing the *mer* operon has a 10.6 kb insert (Fig. 5). The restriction map generated allowed us to subclone portions of this insert making pMJP4 and pMJP5 (Fig. 5). Recombinant plasmid pMJP4 contains a *Bgl* II fragment that is approxi-

mately 1.3 kb long, while pMJP5 contains a *Sal* I fragment that is approximately 5.2 kb long. The subclones were cut with restriction endonucleases to orientate the direction of the clones compared to the original pMJP3. This allows determination of the exact locations and directions of DNA sequences obtained.

DNA sequence from terminal ends of the three clones was obtained in order to locate the *mer* operon within pMJP3 (Fig. 5). The sequences were compared to sequences on the NCBI database using "blastx", a protein alignment search. Segments of the *mer* A gene encoding for mercuric reductase and the *mer* R gene encoding a regulatory protein were found. A transposase gene characteristic of other *mer* operons was identified adjacent to the *mer* A gene. An ATP synthase gene and a gene involved in cell wall synthesis, glucosamine-1-phosphate acetyltransferase, were also identified. By comparing the location of these sequences to known *mer* operons, the location and length of the *mer* A and *mer* R genes were predicted.

According to this prediction there is enough room between the *mer* R regulatory gene and the *mer* A gene for a promoter/operator region. However, there is insufficient room for the *mer* B, *mer* P, *mer* T, or *mer* D genes present in most other *mer* operons indicating that this organism has a novel organization of the *mer* genes. The *mer* B gene product an organomercury lyase converts CH_3Hg^+ to Hg(II), which can then be converted to Hg^0 by mercuric reductase. Strain LO13 conferred resistance to organomercurials indicting it had a *mer* B gene. Since the clone from strain LO13, pMJP3, was selected for on plates containing Hg(II), it is possible that the *mer* B gene may not be present in the cloned DNA.

Further sequencing will allow us to determine if other *mer* genes are present in pMJP3. However, it is clear that strain LO13 has a novel organization of *mer* genes. Ultimately, this *mer* clone will be more relevant than current *mer* genes (obtained from clinical isolates) for bioremediation and gene expression studies performed in natural habitats.

FUTURE WORK

Clone pMJP3 will be plated on media containing phenyl mercuric acetate to confirm whether or not it contains the mer B gene. We will continue sequencing from the located mer R and mer A genes. New sequence will be compared to known mer genes to locate other mer genes. This will allow us to generate a complete genetic map of the mer clone. Since this clone was derived from a native aquatic bacterium, it will provide a valuable set of mercury resistance genes for use in developing mercury bioremediation systems, monitoring mer gene expression in natural environments, and other molecular microbial ecology studies.

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REFERENCES

BIG dye. 2000. Manual for DNA sequencing using BIG dye kit.

Hongri, Y., L. Chu, T. Misra. 1996. Intracellular inducer Hg^{2+} concentration is rate determining for the expression of the mercury-resistance operon in cells. 1996. J. Bacteriol. **178:**2712-2713.

Qiagen. 2001. Qiagen mini-prep kit manual.

Silver, S., T. K. Misra. 1988. Plasmid-mediated heavey metal resistances. Annu. Rev. Microbiol. **42:**717-743.

Winfrey, M.R., M.A. Rott, and A.T. Wortman. 1997. Unraveling DNA: molecluar biology for the laboratory. Prentice-Hall Inc., New Jersey.

Winfrey, M.R., and J.W.M. Rudd. 1990. Environmental factors affecting the formation of methylmercury in low pH lakes. Environ. Toxicol. Chem. **9:**853-869.