Microbial diversity of *mer* genes in bacteria isolated from mercury contaminated environments

Chris Asakiewicz*, Jeffra Schaefer, and Tamar Barkay

Department of Biochemistry and Microbiology,
Rutgers, The State University of New Jersey,
New Brunswick, New Jersey, 08901-1406

* Rutgers Undergraduate Research Fellow

Keywords: hydrothermal vents; mercury resistance; mercuric reductase; microorganisms

Abstract

Isolated mercury resistant bacteria obtained from mercury-contaminated soils of the Carson River, NV, were analyzed for the diversity of their mercuric reductase gene, *merA*, by restriction fragment length polymorphisms (RFLP). It was hypothesized that the diversity would be high because the host bacteria had evolved in presence of high mercury concentrations. Following improvement of the RFLP protocol, the restriction enzymes NaeI and NciI were used in the analysis. Using NciI and NaeI, 5 and 4 distinct *merA* patterns were identified, respectively. None of these patterns was similar to previously described RFLP from enteric bacteria reflecting the difference between soil and gastrointestinal tract environments. Since *merA* loci were grouped differently by the NciI and NaeI RFLPs, loci with different DNA sequences were most likely grouped together. The improved RFLP procedure is now available for the study of *merA* diversity in various mercury-contaminated environments.

Introduction

The Carson River Mercury Site is a 50-mile length of the Carson River beginning at Carson City and ending downstream in the Lahontan Valley (EPA, 2002). Contamination of the soil at this site occurred in the late 1800's, during the gold and silver mining era in this region. Ore that was mined was transported to mills along the river and mixed with mercury during processing (EPA, 2002). Mining in the region has discharged around 7500 tons of mercury. Today, some of this mercury is still present in the flood plain of the Carson River (EPA, 2002). Mercury can exist in the forms Hg^0 (elemental mercury), Hg^{II} (mercuric ion), and CH_3Hg^I (methyl mercury). Some microorganisms in the soils of the Carson River have developed resistance to the different forms of mercury, resistance that is based on the functions encoded by the mercury resistance (*mer*) operon (Barkay et al., 2000). In the *mer*-based system MerP transfers Hg^{II} from the periplasm of the cell to MerT, an inner membrane spanning proten that transports Hg^{II} into the cell. In the cytoplasm the product of *merA*, the enzyme mercuric reductase, takes Hg^{II} and reduces it to gaseous Hg^0, which readily diffuses across cell membranes. Therefore, resistance is mediated by the elimination of Hg from the cell's local environment. The *mer* operon is

specific for Hg^{II} although the system can handle methyl mercury with the help of the gene, *merB*, which encodes for the organomercurial lyase. (Figure 1)

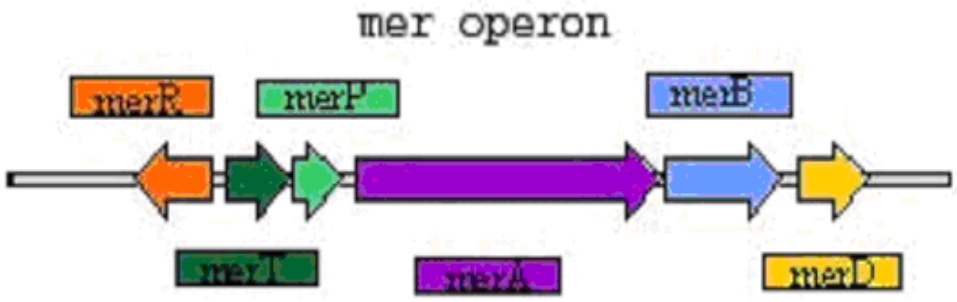

Figure 1. The *mer* operon including the regulators (*merR* and *merD*), transporters (*merP* and *merT*), mercuric reductase (*merA*) and organomercurial lyase (*merB*).

When the Carson River site was contaminated, it is likely that the microbial populations in the soil and water were affected by the toxicity of mercury. The *mer* operon was likely induced and certain populations gained resistance while others remained sensitive. Over the years, *mer* genes were transferred to sensitive strains and thus, additional populations gained resistance and the *mer* system increased in diversity (Barkay et al., 1993). The microorganisms that exist at the site today are acclimated to the mercury toxicity. To study the diversity of the *mer* system in microorganisms of the Carson River, polymerase chain reaction (PCR) primers were designed to amplify *mer* genes. Restriction digests were performed on the PCR product in order to easily classify *merA* amplicons with different genetic sequences into groups, based on their restriction digest patterns. This method had been previously performed on primate fecal flora at the University of Georgia (Liebert et al, 1997). That study's goal was to determine how evolution of the genes occurred. A similar approach was applied here with the Carson River bacterial isolates that were resistant to Hg^{II}. The goal of this study was to examine the diversity of *merA* PCR products from mercury resistant isolates from three mercury-contaminated sites. The three sites were the Meadowlands, NJ, the Carson River, NV, and the Experimental Lakes Area (ELA) in Ontario, Canada. Each site contains a different level of mercury contamination from different sources. High levels of mercury contamination occurred in the Meadowlands water and Carson River soil samples. In the ELA water samples mercury contamination is low. Here we report preliminary results for mercury resistant bacteria from Carson River soils.

Methods: *Amplification of the merA gene*.

The first of step was a PCR using *merA*-specific primers with DNA isolated from mercury resistant environmental isolates (Yagi, 2002). This gene is essential in the reduction to detoxify Hg^{II}. Degenerate primers have been designed to target highly conserved regions of *merA* from Gram-negative bacteria. Amplifications were carried out in reactions (final volume 50 µL), containing 1X PCR buffer provided by Fisher ©, 1.5 mM $MgCl_2$, 0.2 mM dNTP's, 0.4 mM primer concentration, 5 ml of isolated DNA and 0.025 U/ml of Taq Polymerase. Samples were then subjected to a (1 cycle) denaturation for 3 min at 94°C, (40 cycles) denaturation for 10 sec at 94°C, annealing step for 30 sec at 54°C, followed by an extension for 1.5 min at 72°C, (1 cycle) then a final extension for 5 min at 72°C, (1 cycle).

Visualization of *merA*.

Once the gene was amplified, the PCR product was loaded and run in a standard 1.0% agarose gel. By using a 6X gel- loading buffer, for visualization of how far the product has run, we were able to stop the gel at the proper time in order to visualize the band that contains the gene. The correct PCR product is a 1.2 Kb fragment determined from the region flanked by the primers. Gel Doc © (BioRad) was used to calculate the migration value R_f as well as the size in base pairs (bp) of each band detected in the gel.

Gel Purification.

Bands of the 1.2 kb amplicons were cut out from the gel and purified using the Clontech ™ Gel Purification Kit.

Reamplification and Purification.

A second PCR reaction was then performed on the purified samples. Amplification was carried out as described above for the first round of *merA* PCR amplification, and the product was separated and purified as above.

Restriction Fragment Length Polymorphisms.

The next step used restriction enzymes, NaeI and NciI, to digest the amplicons. Diversity of the products was ascertained in by RFLP. Samples were digested in total reaction volumes of 20.5 µL, containing 7.3 µL of deionized water, 2 µL of 10X Buffer, 0.2 µL acetylated BSA, 10 µL of DNA and 0.5 µL of restriction enzyme. Incubation of these samples was carried out overnight. The procedure has been optimized so that two additions of the enzyme were needed for full digestion; therefore after one hour another 0.5 µL of the enzyme was added.

Visualization of the Digests and Data Treatment.

At the end of the digestion, digested samples were separated on a 3% agarose gel for visualization of RFLP patterns. Software provided with the Gel Doc © system was used to calculate the R_f values as well as the size of each fragment that resulted from the digestion of *merA* amplicons. By comparison of the digests, similarities between the patterns were grouped into families (Figure 2).

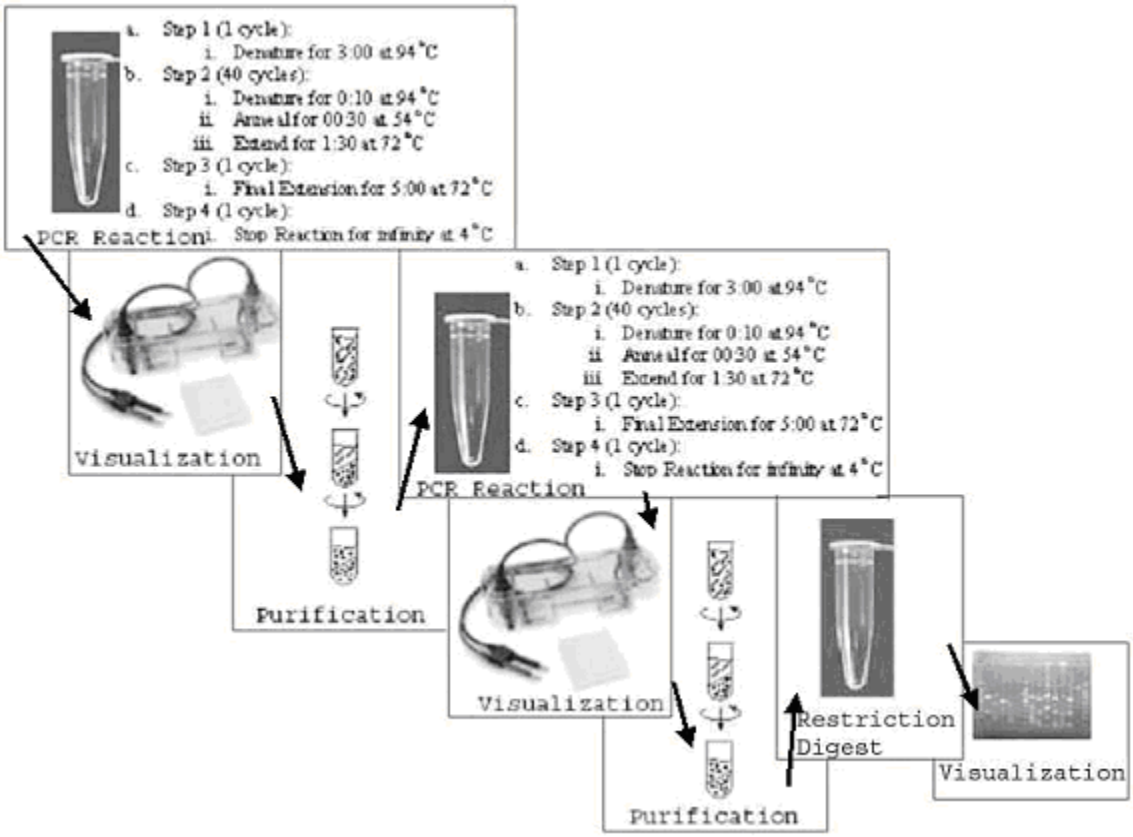

Figure 2. Restriction Fragment Length Polymorphisms (RFLP) protocol. Steps include an initial PCR reaction followed by a purification, reamplification, and second purification. The purified product is then cut with the appropriate restriction enzyme for visualization and data treatment.

Results

Since environmental samples were being tested, the degenerate primers only picked up sequences in 38% of the isolated samples. As a result of this low yield, few samples were characterized by restriction patterns. A total of 15 samples were successfully PCR-amplified and digested to give restriction patterns for diversity analysis. NaeI consistently gave restriction fragments that totaled more than the expected 1.2 Kb. These patterns were therefore determined to be the result of incomplete digestion (Figure 3). Incomplete digestion is evident in Figure 3 in lanes 7 and 13 in comparison with lane 14, which contains an undigested PCR fragment. Samples displaying similar restriction patterns were grouped together. The gel (Figure 3), lanes 9, 10 and 11 contain similar patterns. Lastly, some samples had such frequent cutting that no restriction patterns were displayed, as in the case of the sample in lane 5.

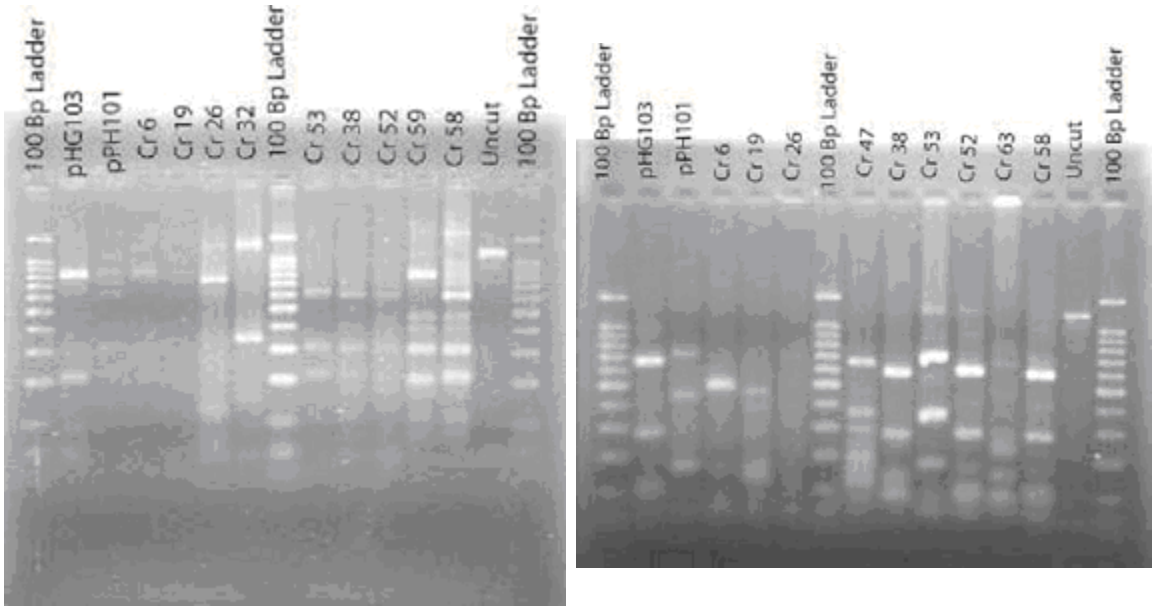

Figure 3.
Left- Sample restriction digest with NaeI. Samples loaded include: *lane 1* (100 Bp ladder), *lane 2* (pHG103), *lane 3* (pPH101), *lane 4* (Cr6), *lane 5* (Cr 19), *lane 6* (Cr 26), *lane 7* (Cr 32), *lane 8* (100 bp ladder), *lane 9* (Cr53), *lane 10* (Cr38), *lane 11* (Cr52), *lane 12* (Cr 59), *lane 13* (Cr58), *lane 14* (Uncut sample) and *lane 15* (100 bp ladder).
Right- Sample restriction digest with NciI. Samples loaded include: *lane 1* (100 bp ladder), *lane 2* (pHG103), *lane 3* (pPH101), *lane 4* (Cr6), *lane 5* (Cr 19), *lane 6* (Cr 26), *lane 7* (100 bp ladder), *lane 8* (Cr47), *lane 9* (Cr38), *lane 10* (Cr53), *lane 11* (Cr52), *lane 12* (Cr63), *lane 13* (Cr58), *lane 14* (Uncut sample) and *lane 15* (100 bp ladder).

To try to remedy the problems of incomplete digestion with NaeI, NciI, the enzyme used by Liebert *et al.* (1997) was used for further digestions. The digests displayed had little to no incomplete cuts. Some isolates did display bands totaling over the expected 1.2 kb, but this problem could be solved by totaling only the bands with the highest intensity. This is seen in the samples digested in lanes 11 and 13 of Figure 3 Next we compare the results obtained with NaeI and NciI. Some of the groups were conserved while others were regrouped. NaeI displayed unique restriction patterns leading to 5 families. Four of these families were complete, meaning they displayed restriction fragments that totaled to 1.2 Kb. The fifth group displayed restriction patterns indicative of incomplete digestions. Two categories were identified in the restriction patterns seen in this unknown group. If the bands totaled to less than 1.2 Kb, they were given the designation: bands lost. Incomplete digests were assigned to restriction patterns that totaled to a number greater than 1.2 Kb (Figure 4). NciI led to 6 distinct restriction patterns. Five of the families had totals around 1.2 Kb. The sixth family still had problems of incomplete digestion, but in comparison to NaeI, most of the unknown had patterns adding up to < 1.2 Kb. Overall, NciI led to a higher probability of obtaining too few bands than too many (Figure 4).

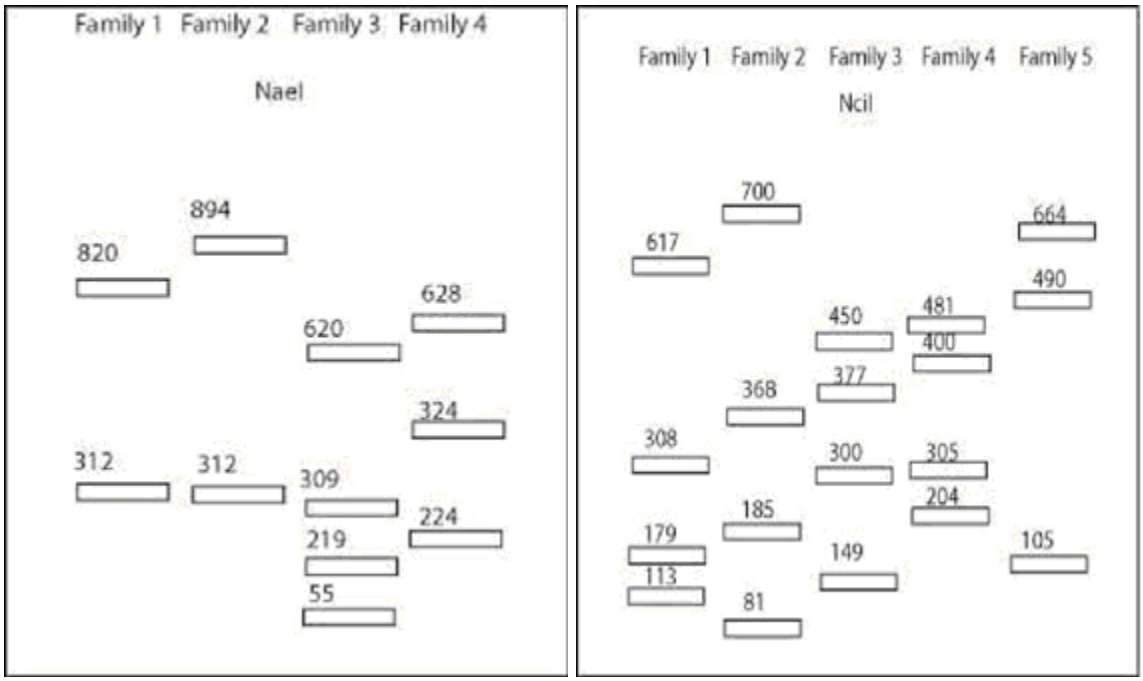

Figure 4.
Left- Restriction patterns displayed by the NaeI enzyme. Isolates included in the designated families are: *Family 1* (Cr38, Cr48, Cr52, Cr 53, and Cr58), *Family 2* (Cr43), *Family 3*, (Cr8 and Cr5) and *Family 4* (Cr69).
Right- Restriction patterns displayed by the NciI enzyme. Isolates included in the designated families are: *Family 1* (Cr38, Cr52 and Cr 58), *Family 2* (Cr53), *Family 3*, (Cr66), *Family 6* (Cr67) and *Family 5* (Cr43).

Discussion

When RFLP patterns were compared to *merA* RFLP patterns using NciI (Liebert et al., 1997), there was no similarity between data sets. The dissimilarities probably reflect sampling differences. We looked at the characteristics displayed by isolates of soil microbes while C. Liebert et al. looked at isolates from fecal flora of primates. Further, C. Liebert's primers do not contain degeneracy, while the primers used in this study contain many degeneracies (Liebert *et al.*, 1997). Degeneracy was introduced into the primers in order to detect a larger number of environmental sequences. In the case of Liebert's primers, additional degeneracy was not needed because the primers were designed to detect only enteric organisms with low diversity of *merA* (Liebert *et al.*, 1997). Highly degenerate primers may be able to detect more diverse sequences in a larger genetic pool, but a loss in specificity can be a significant problem with increasing degeneracy. Increasing the stringency by using C. Liebert's primers should have a lower probability of binding outside the specified gene (Liebert *et al.*, 1997). However, using these primers did not remedy the problem of amplification of secondary products, and so bands needed to be purified prior to digestion.

The RFLP protocol was optimized in order to create a versatile method that can be used to easily characterize *merA* genes from diverse environmental isolates. Throughout the optimization many problems had to be remedied. Originally, NaeI did not cut to completion. Initially, an overnight digest was used to remedy this problem. When an overnight digests did not cleanup the restriction patterns another enzyme was used. Incomplete cutting may have been a result of enzymatic STAR activity. STAR activity is when the enzyme cuts at a 4 bp sequence instead of the 6 bp sequences and can be the result of deviation from the optimized conditions. Since the enzyme continuously displayed these results, NciI was used in further purified samples. Some incomplete restriction patterns were assumed to be the result of secondary bands that were being purified from the gel. The solution to this problem is a second PCR run. Since the primers are optimized with the 1.2 Kb fragment they should dilute out the other bands. Another problem was that gel purification decreased the yield so significantly that the bands were unable to be amplified. If the band was not in a significant quantity, restriction patterns could not be seen. When the fragment was in low quantity and small, the ethidium bromide staining would not resolve a band. To fix this problem the gel purification and the second PCR were optimized. Therefore we started to elute the DNA band from gel slices for 2-3 min at 50oC, which increased yields by 10%. Elution was followed by a second PCR amplification. Lastly, the percentage of agarose in gels increased to 3% since originally all of the samples were run on 1% gels. Since many of the restriction digest fragments were very small there was no resolution when 1% gels were used.

The optimized protocol described here may be used to study diversity of *merA* from many environments. At this point we have used it to identify 5 groups based on the observed RFLP. Restriction fragment length polymorphism, however, does not mean sequence identity as can be seen in the differences between the NaeI and NciI cuts. Since the enzymes cut at different sequences, different groupings were determined. For example, with NaeI Cr53 is grouped with isolates Cr38, Cr48 and Cr 58 (Figure 3). With the NciI

enzyme, Cr53 displays a pattern different that all the other isolates. If only NaeI had been used to characterize these strains Cr53 would have been grouped with the others. Note that sharing a restriction pattern for *merA* does not mean that the host bacteria are the same. Restriction patterns can only be used to characterize similarities between genes. Since restriction enzymes cut at specific sites there is no information provided about outlying sequences. Only sequencing can provide this information. Ultimately, the most representative of each RFLP group will be sequenced.

Conclusion

Optimization of the PCR-RFLP protocol included changing to nondegerate primers to allow for greater specificity during amplification, and changing restriction enzymes to NciI to increase the degree of digestion. Following these improvements, the *merA* gene from 15 different environmental isolates was classified into 5 different families distinct from previously sequenced *merA*s, suggesting a large degree of genetic diversity in the Hg resistance genes from the highly Hg contaminated environment of Carson River, NV. Sequencing of representative members within each group, will help us to look at the actual codes that cause the samples to be different. These small genetic differences can lead to a variety of restriction patterns.

Once the procedure is fully optimized, the project will continue on samples isolated from other sites. These sites include a highly mercury-contaminated creek in the Meadowlands, NJ and the low-Hg lakes of ELA in Canada. It will be interesting to see whether the genetic diversity of *merA* is directly related to the degree of Hg contamination.

Acknowledgments

Special thanks to Jane Yagi, Jonna Coombs, and Rachel Kaletsky.

References

Barkay, T. (2000). Mercury cycle, Encyclopedia of Microbiology (Vol. 3, 2nd ed, pp 171-181), Academic Press.

Barkay T., Liebert C., and Gillman M. (1993). Conjugal gene transfer to aquatic bacteria detected by the generation of a new phenotype. Applied and Environmental Microbiology, 59 (3), 807-814.

EPA: Environmental Protection Agency. (2002). Carson River Mercury Site. (EPA Publication No. NVD980813646). Washington, DC: U.S. Government Printing Office

Liebert C., Wireman J., Smith T., and Summers A. O. (1997). Phylogeny of mercury resistance (*mer*) operons of gram-negative bacteria isolated from the fecal flora of primates. Applied and Environmental Microbiology, 5, 1066-1076.

Yagi J. (2002). Log of daily accounts. (Available at Cook College: Rutgers University, New Brunswick, NJ, 08901)