

1. Close out technical report for Grant #DE-FG02-01ER63141

Genomic Plasticity in *Ralstonia eutropha* and *Ralstonia pickettii*: Evidence for Rapid Genomic Change and Adaptation

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2. There are no patents pending or protected data reported on herein.

3. Executive summary

The proposed foci of our investigations were on *Ralstonia eutropha* and *Ralstonia pickettii*. We have 18 derived lineages of the former as well as their progenitor and eleven isolates of the latter. Our goal was to measure the level of plasticity in these strains and attempt to derive a mechanistic understanding of how genomic plasticity formed. Extensive attempts to reproducibly induce conformational changes in the genome of *R. eutropha* were unsuccessful. We thought that we had a reasonable lead on this inasmuch as we had shown that the ancestral strain along with many of the derivative lineages exhibited “temperature induced mutation and mortality akin to *R. metallodurans*. However we were unable to get subtractive hybridization working to the degree that it revealed differences between the lineages. During this time the *R. pickettii* analysis was proving quite fruitful and so we concentrated our efforts on our analyses of *R. pickettii*. These strains were isolated from a copper-contaminated lake sediment and were resistant to copper at 800 µg/ml (CuSO₄).

Our results in the investigation of *R. pickettii* permitted a view into the adaptation of a beta-proteobacteria to an extreme environment. Our work revealed that within the same ecosystem two genomovars with structurally different genomes and genome sizes were present and apparently filling similar if not identical niches. The genomovars were detected with REP & BOX-PCR, pulse field gel electrophoresis, and DNA:DNA hybridizations. Moreover there were different metal resistance patterns associated with the different genomovars, one showing resistance to Zn and Cd while the other had resistance to Ni. Five of the isolates had a high-copy number extrachromosomal element that was identified as the replicative form of a filamentous phage. Mature virions were isolated from culture broth using PEG precipitation and CsCl density centrifugation. The DNA associated with the filamentous particles was single stranded and had a sequence identical to the intracellular replicative form. Using PCR targeting phage-specific genes we showed that seven out of the eleven isolates carried the phage. The seven isolates positive for phage were formed one of the genomovar groups, hence the presence of the phage may have generated the divergent lineage. One representative of each of these genomovars was sequenced by JGI. While the genomes have not been closed completely, the results so far are provocative. Strain 12J, which carries the phage, revealed four integrated copies of the phage genome, each copy at a different level of divergence from the active replicative form. A comparative analysis of the common genes found in these integrated phage copies revealed that the gene complements were incongruent with one another within a phage copy, suggesting that the copies had become sites of recombination or that the cell had recruited genes for different functions. One of the integrated copies had the exact sequence as the replicative form and we assume this to be the most recent integration event. Evidence for a recent integration was revealed in a repeated sequence element found on each terminus of the phage genome. Finally, these isolates are of interest in bioremediation and reclamation of metals from waste streams. We

have determined that each *Ralstonia* cell is capable of binding 6×10^7 Cu (II) ions and that 90% of the binding occurs within 12 hours of exposure to Cu(II).

Our studies have revealed a species with a uniquely fluid genome. This *Ralstonia* population has been under extreme selective pressure over the past hundred years as it responded to the accumulation of copper in the lake sediment as a result of unregulated mining practices. In addition to the selective pressure of copper, the population was repeatedly infected with a filamentous phage that may have contributed to the divergence of the genomovars. This dynamic population could help reveal the selective forces and their consequences on genome structure in a proteobacterium that has potential for remediation.

4. Comparison of original goals with accomplishments.

Our original goal and specific aims were stated in the original proposal as:

“The overall goal of our proposed studies with *R. eutropha* TFD41 and *R. pickettii* is to better understand the dynamics of the genome in the natural environment.

Our specific aims are:

- 1. Identify patterns of genomic change among 18 replicate lines of *R. eutropha* TFD41. This will be achieved by determining the differences in the genomic structure between the ancestral *R. eutropha* TFD41 and the 18 derived genotypes.***
- 2. Identify the sequence surrounding the deletions/rearrangements in the evolved populations to identify controlling elements.***
- 3. Identify the differences in genomic structure between the two sub-populations of *R. pickettii*.***
- 4. Determine if genomic rearrangement elicited by environmental stress accelerates the rate of evolution.”***

Our first year of work focused on both *R. eutropha* & *R. pickettii*. We repeatedly tried to delineate the differences in the 18 strains of *R. eutropha* using subtractive hybridization but were unsuccessful. Moreover we were unable to induce genomic rearrangements using a temperature-nutritional regiment described previously for a *R. eutropha* strain. For these reasons we abandoned our work on *R. eutropha*. The work on *R. pickettii* proved quite successful and we completed our specific aims as delineated in the original proposal and extended our understanding of this population beyond our original design.

5. Summary of Project Activities.

Eleven strains of *Ralstonia* were isolates from copper contaminated lake sediment. The strains were isolated based on their resistance to high levels of copper (up to 1200 $\mu\text{g/ml}$) and the apparent sequestration of copper in colonies. Our first paper on these isolates (REF) reported on i.) the phylogeny of the isolates using 16S rRNA comparative sequence analysis ii.) the resistance to Zn, Co, & Ni in the 11 isolates, and iii.) SEM and EDR of the isolates. Results from these analyses indicated that the 11 isolates displayed two different metal resistance patterns and that growth in the presence of copper produced greater amounts of extracellular material. Subsequent analyses conducted via support from Grant # DE-FG02-01ER63141 was as follows.

- 1. *R. pickettii* strains were further examined using TEM and high resolution TEM with EDS. These techniques revealed that when the strains were grown under high concentrations of copper, the cells responded by sequestering copper in the outer envelope (Fig. 1). The location of the copper was verified with EDS. The cell volume increased by a factor of 2-3 fold as a consequence of this sequestration.***

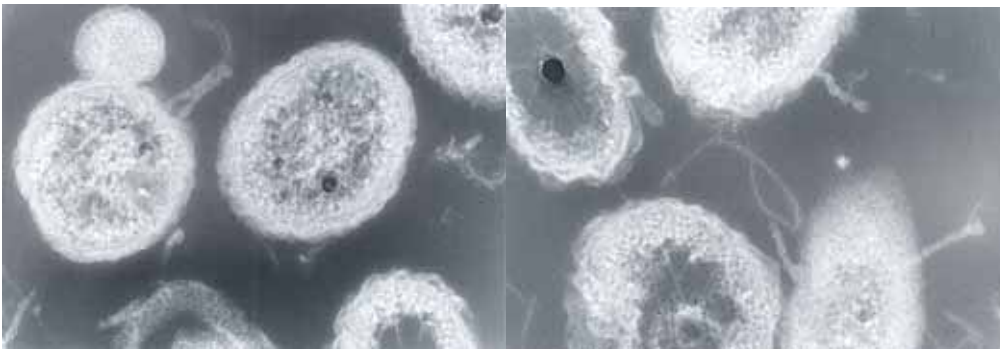


Fig. 1. Two TEM micrographs showing increase in the thickness of the cell envelope after growth in the presence of Cu. Note filamentous phage being extruded from the cell.

2. In addition to the location of copper, the TEM analysis revealed the presence of filamentous structures that were later identified as phage.
3. The differences in metal resistances among the isolates led us to examine plasmid distributions and genome structure across the eleven isolates. We detected three extrachromosomal elements in the eleven strains with a diverse distribution pattern. Four of the eleven had a 44 kb element. The remaining seven had a 57 kb plasmid and five of the seven had a 7.3 kb high copy number plasmid (Fig. 2).

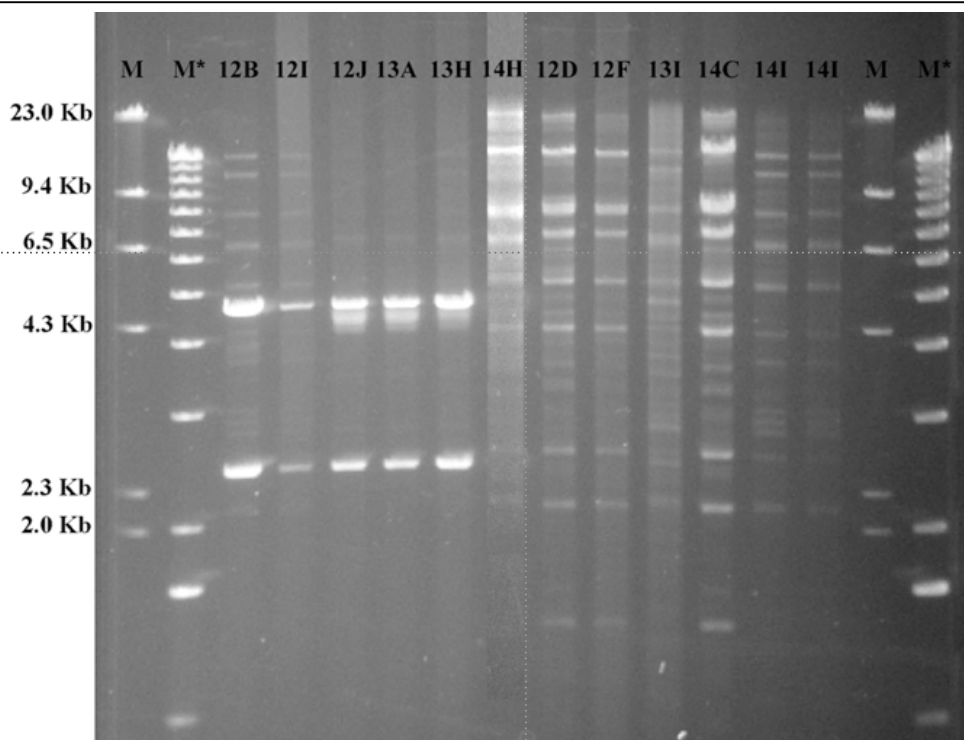
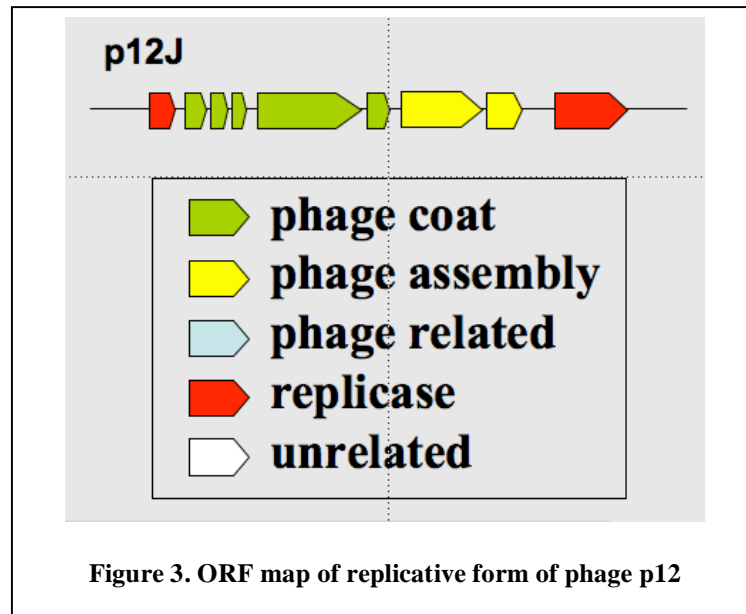
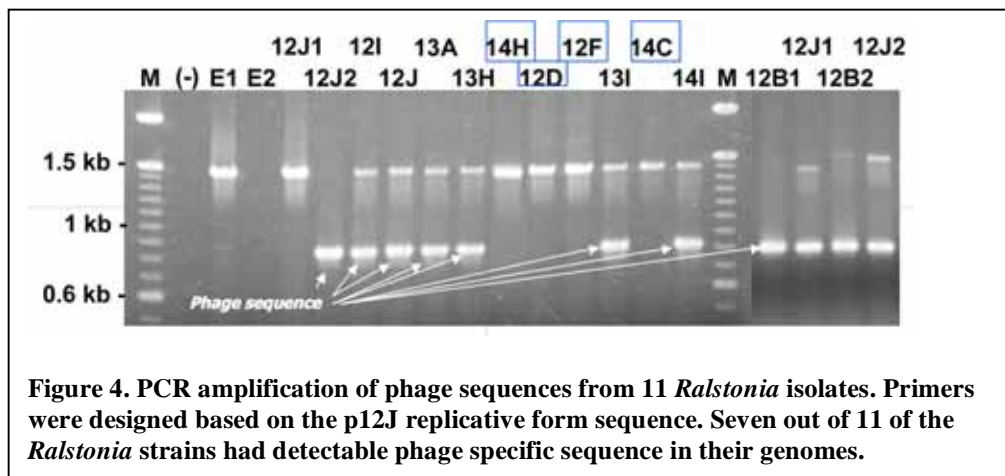


Figure 2. RFLP analysis of plasmids isolated from *Ralstonia* strains.

- The 7.3 kb extrachromosomal element was sequenced and proved to be a filamentous phage. Nine ORFs were identified (Fig. 3).

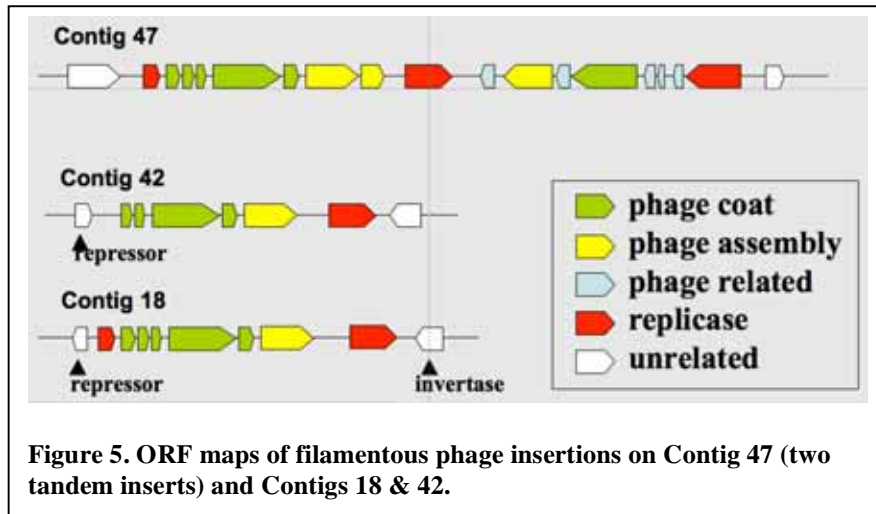


- The distribution of filamentous phage sequences was tested with PCR and hybridization. All seven strains with the 57 kb plasmid had detectable phage sequences while the remaining strains with the 44 kb element lacked phage sequences (Fig 4).

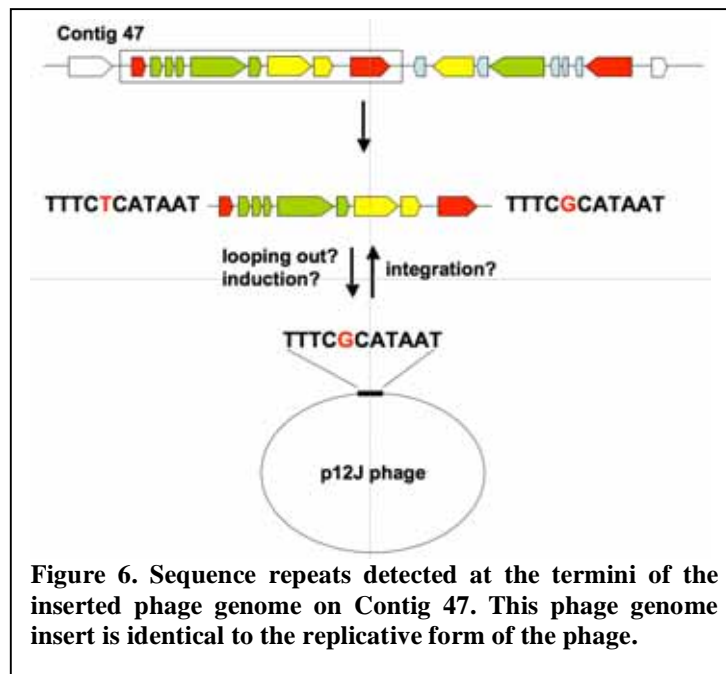


- We have prepared high molecular weight DNA from the two detected genomovars (see below) for sequencing at JGI. Draft sequences are now available and the genomes are in the finishing stage.
- JGI sequence from 12J revealed one exact copy of the replicative form of the phage integrated into the genome and three additional partial copies integrated at different sites. PCR targeting both genome and phage sequences revealed that 5 of the 6 strains similar

to 12J retained identical copies at the same locations. One of the isolates appeared to have a change in at least one of the copies (Fig. 5).



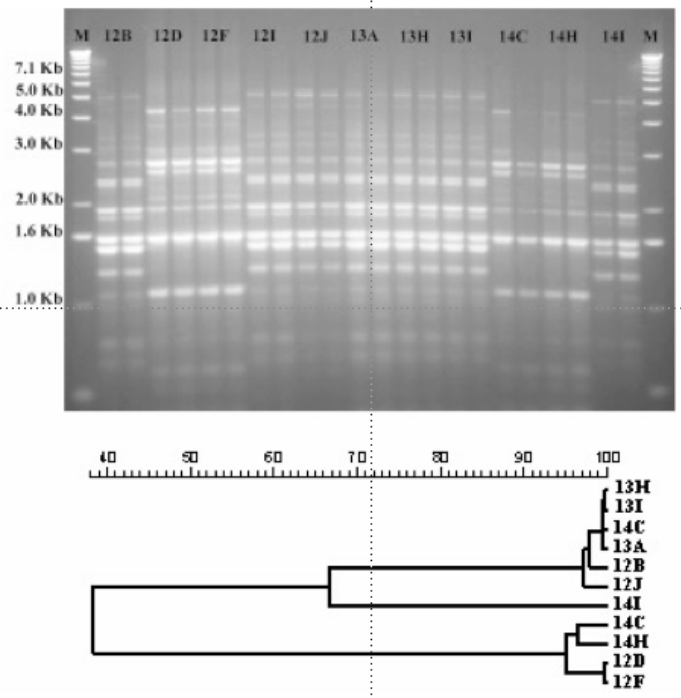
8. JGI sequence of 12D revealed two highly degenerate copies of the phage in the genome at different locations from 12D.
9. Sequence repeats were detected at the termini of the phage genome inserted into Contig 47 suggesting a possible mechanistic route for insertion (Fig. 6).



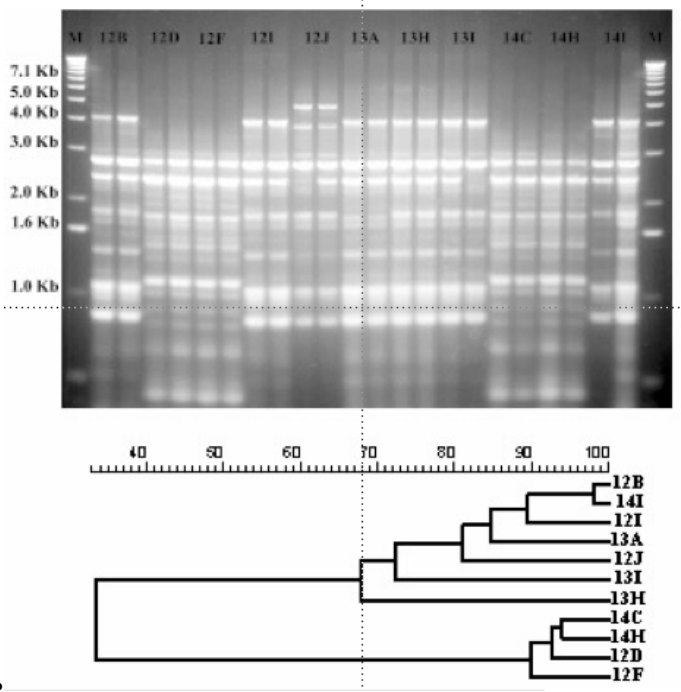
10. Growth rates of phage-bearing and phage-free strains were compared and as is common with strains carrying filamentous phage, the growth rate was diminished. However, because of the substantial differences between the genomes of phage-free and phage

bearing strains, we cannot conclude that the phage is causing the diminution of growth rate.

11. The eleven strains were analyzed with REP & BOX PCR. Both primer sets revealed that the eleven strains were composed of two groups with different genome structures. The genomic groups defined by REP & BOX matched the groups defined by metal resistance patterns and phage distribution (Fig. 7).



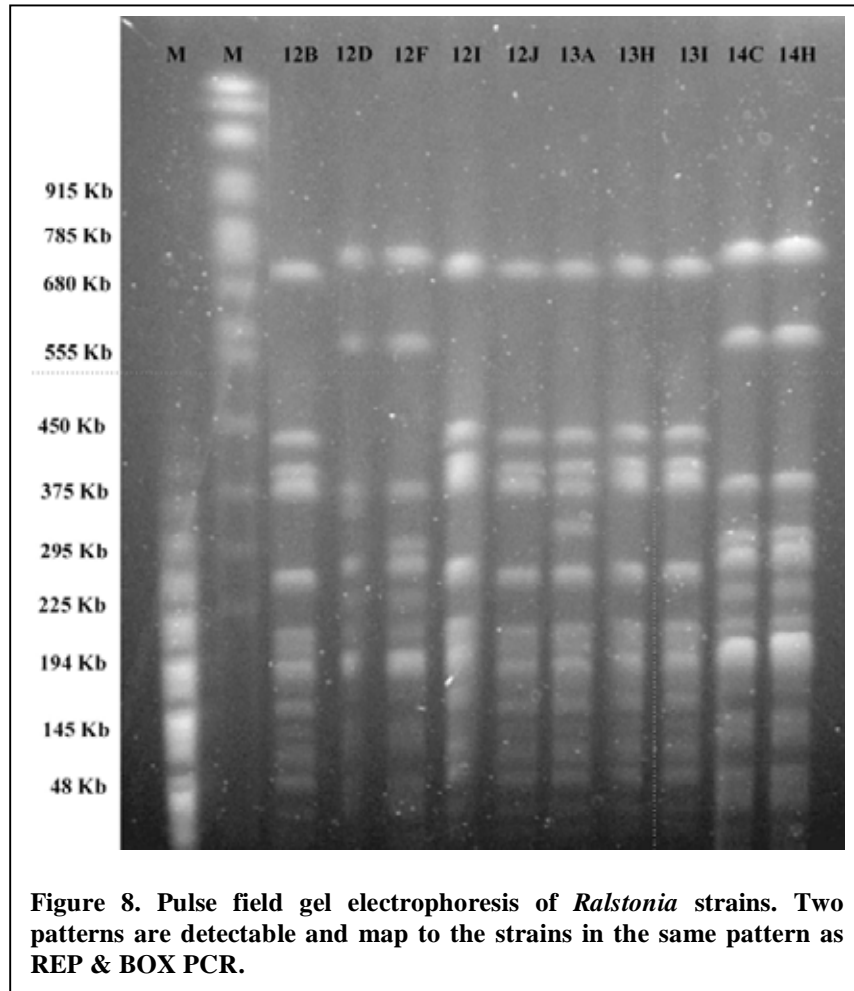
A.



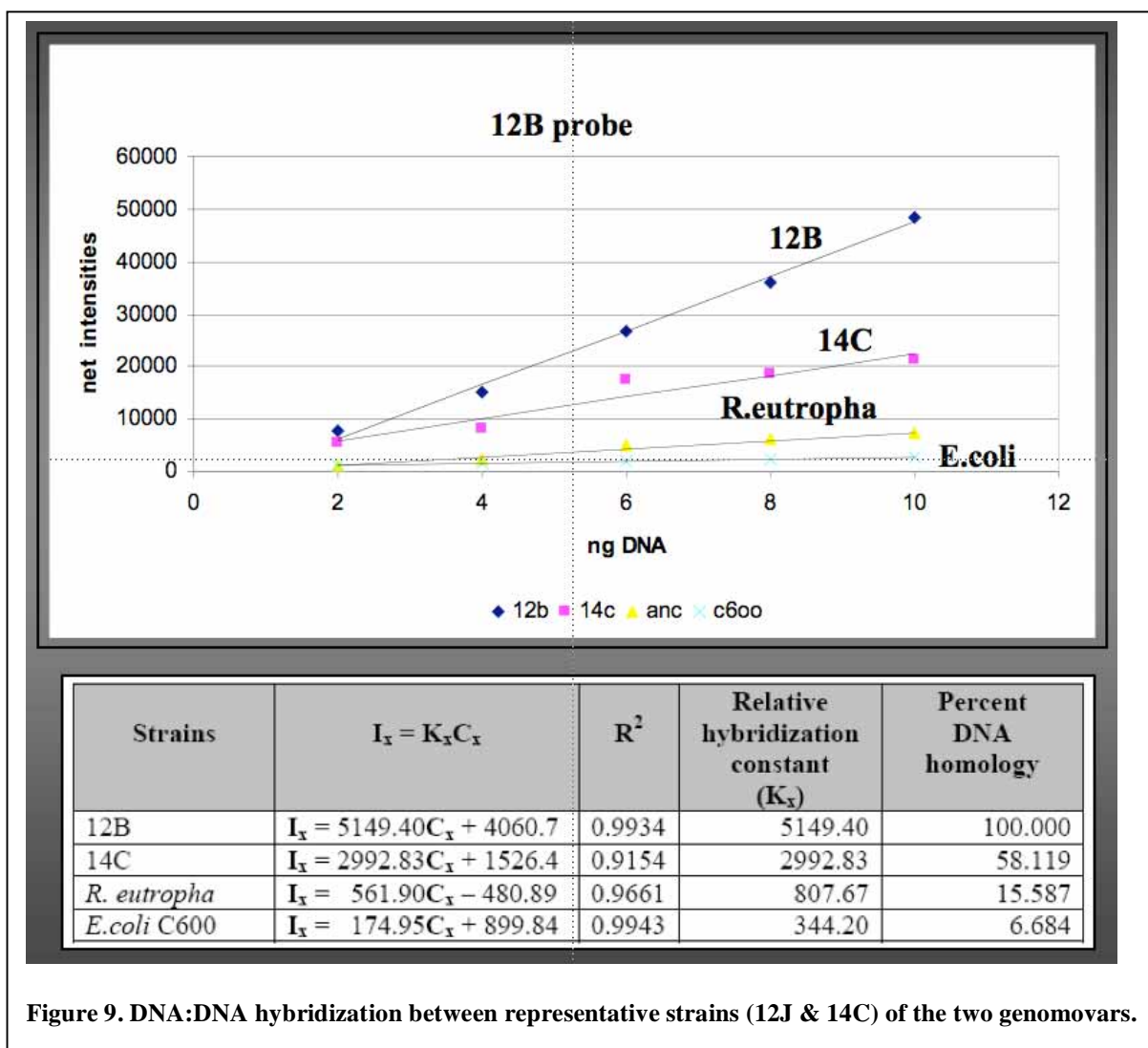
B.

Figure 7. REP (A.) and BOX (B.) PCR profiles of *Ralstonia* isolates. Dendrograms represent the similarities between profiles. In each case, two genome structures are detected.

12. The eleven strains were analyzed with PFGE and two genomic digestion patterns were detected. The distribution of strains in the two genomic groups defined by PFGE matched the groups defined by REP & BOX PCR, phage distribution and metal resistance. PFGE indicated that one genomovar had a chromosome size of 3.0 MB while the second was 3.5 MB (Fig. 8).



13. DNA:DNA hybridizations were performed on two representative strains from each genomovar type using a membrane-based detection system and employing digoxigenin labeled probe. Only 58% of the genomic DNA in strain 14C was homologous to strain 12B. When probed in the other direction, 88% of the genomic DNA in strain 12B was homologous to strain 14C. This non-reciprocal homology might be due to the differences in size of the genomes (Fig. 9).



14. Ribotyping was performed on two representative strains of the genomovars. Distinct differences were detected indicating that some of the ribosomal operons of the two genomovars were in different genomic settings.
15. Biolog™ analysis of the strains were performed to determine if there was any phenotypic differences beyond the metal resistance patterns that could be detected in the genomovars. We used Biolog™ plates targeting carbon, nitrogen and phosphorus source to screen the isolates. No significant differences were detected with Biolog™.

16. Other strains of *Ralstonia* are known to be genomically unstable when incubated in liquid culture for extended periods or when subjected to high concentrations of nutrients (rich broth, eg. 2xYT) and relatively high temperatures (36-37°C). To test for this phenotype in our strains we maintained two cultures continuously for 80-100 generations and monitored changes in genome structure with REP and BOX PCR. No changes in genome structure were detected over 100 generations and after growth at 36°C on rich media.
17. We used an indirect method to compare the two genomovars. Based on the published genome sequence of *R. solanareum*, we constructed a microarray with 6,000 oligomers targeting *R. solanacearum* along with 400 oligomers targeting membrane proteins of *Burkholderia* LB400. The results allowed us to identify groups of genes that were common and unique to the two genomovars. The analysis was not sufficiently fine so as to allow us to map gene contigs or infer gene clusters that were present or absent in the two genomovars, but it did provide data on some of the genetic differences between the two genomovars.
18. We have investigated the ability of the strains to sequester copper. We find that strains in exponential phase and stationary phase have both a slow and fast binding of Cu⁺⁺. Detectable binding can be discerned in as little as 10 minutes. Approximately 50% of binding occurred 2.8 hours after exposure to copper sulfate and the binding was 90% saturated within 12 hours. We are in the process of determining what cellular component binds the copper.

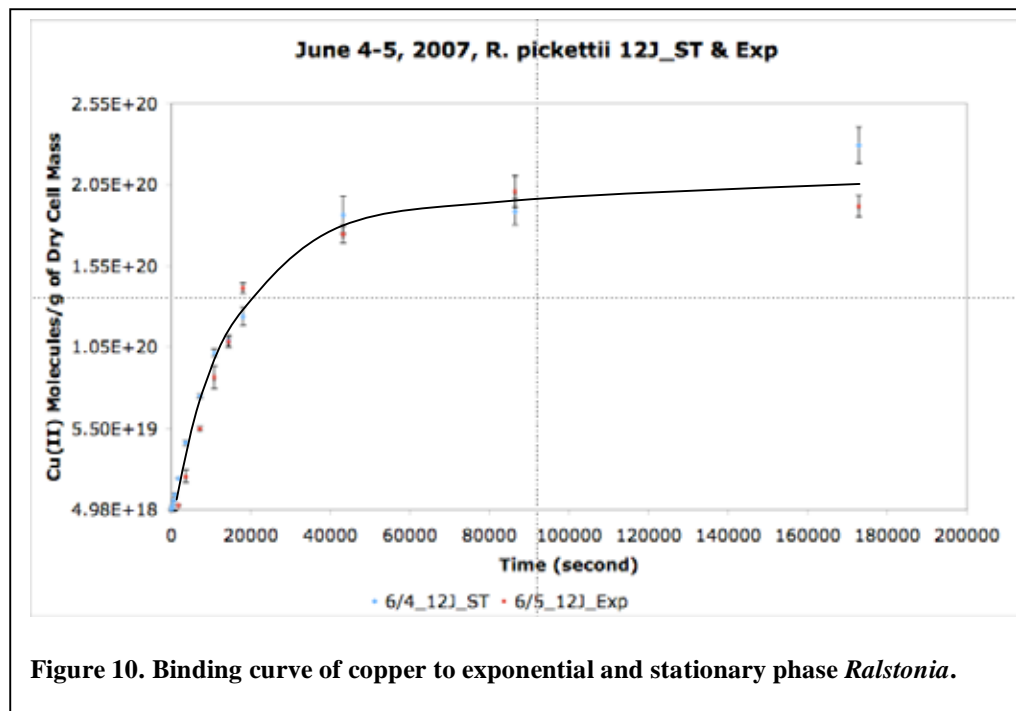


Figure 10. Binding curve of copper to exponential and stationary phase *Ralstonia*.

19. X-ray absorbance spectroscopy indicated that the bound copper was Cu(II) and liganded to oxygens and nitrogens.

20. A paper describing the filamentous phage has been written and submitted. A paper describing the genomic variations of the strains is in preparation. A paper that will compare the two sequenced genomes is in preparation but awaits the finished genomes for final analysis.

Summary. 18 lineages of *Ralstonia eutropha* were established and maintained for 1000 generations under either a structured environment or an environment driven by mass action, i.e. agar surface or liquid media respectively. These lineages were further analyzed and showed significant phenotypic radiation in many traits. Unpublished work indicated that the ancestor displayed “temperature induced mutation and mortality” (TIMM) similar to a *Ralstonia* strain described previously. The 18 lineages were found to have evolved various levels of TIMM. The levels of survival for the 18 strains at 37°C on rich media was as follows: L1, 1.5×10^{-4} ; L2, 1.3×10^{-5} ; L3, 1.3×10^{-5} ; L4, 2.8×10^{-6} ; L5, 1.2×10^{-4} ; L6, 5.3×10^{-7} ; L7, 1.3×10^{-5} ; L8, 1×10^{-8} ; L9, 9.2×10^{-6} ; L10, 2.9×10^{-8} ; L11, 4.4×10^{-5} ; L12, 1×10^{-8} ; L13, 1; L14 1.3×10^{-5} ; L15, 1.5×10^{-5} ; L16, 1×10^{-8} ; L17, 4.1×10^{-5} ; L18, 1.8×10^{-3} . Moreover PFGE of the 18 derivative lineages showed evidence of large deletions. Some deletions were detected in all strains and some deletions were lineage specific. Repeated attempts at isolating regions that were deleted using subtractive hybridization and differential PCR amplification were unsuccessful. Additional experimentation directed at inducing genomic rearrangements with TIMM were unsuccessful, hence most of our subsequent work was performed on the *Ralstonia pickettii* isolates described above.

Publications:

- Konstantinidis, K. T., N. Isaacs, J. Fett, S. Simpson, D. T. Long, and T. L. Marsh.** 2003. Microbial diversity and resistance to copper in metal-contaminated lake sediment. *Microb Ecol* 45:191-202.
- Kim, S-H, D. Engelmoer, M. Anderson, N. Cosgrove-Isaacs, T.L. Marsh.** A filamentous phage detected in copper resistant *Ralstonia pickettii*. Submitted
- Cosgrove-Isaacs, N, S-H Kim, P. Namphranchan, T.L. Marsh.** Genomic plasticity in *Ralstonia pickettii*. In preparation.
- Engelmoer, D., S-H Kim, N. Cosgrove-Isaac, T.L. Marsh.** The distribution of a filamentous phage in *Ralstonia* isolated from copper contaminated lake sediment. In preparation
- Marsh, T.L., S-H Kim, F. Yang, D. Engelmoer.** Comparison of *Ralstonia* genomes. In preparation.
- Yang, F, S. Kelly, K. Kemner, T.L. Marsh.** Sequestration of high concentrations of copper by *Ralstonia pickettii*. In preparation.