# STRAIN-LEVEL GENOMIC AND PHYSIOLOGICAL VARIATION IN FOUR *MICROBACTERIUM SPP*. CHROMATE REDUCERS

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This is dedicated to my parents John and Michele and my brothers JP and Matthew. Your constant encouragement and support provided me every opportunity I have been given.

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ABSTRACT

STRAIN-LEVEL GENOMIC AND PHYSIOLOGICAL VARIATION IN FOUR *MICROBACTERIUM SPP*. CHROMATE REDUCERS

by Michael W. Henson

Hexavalent chromium [Cr(VI)], a soluble carcinogen, has caused widespread contamination of soil and water in many industrial nations. Bacteria have been shown to play an active role in the reduction of Cr(VI) to Cr(III), which is insoluble and less toxic, however, the biological mechanisms governing this reaction appear to vary greatly across species. Here, we utilized genome sequencing analysis and physiological characterization of four Cr(VI) reducing *Microbacterium* isolates (Cr-K1W, Cr-K20, Cr-K29, and Cr-K32) to elucidate key genes involved in chromate reduction. While their 16S gene sequences were nearly identical, growth and chromium reduction analyses revealed physiological differences among the strains. Specifically, Cr-K29 and Cr-K32 had reduced 2 mM of Cr(VI) within 48 hours, while it took longer (approximately 120 hours) for Cr-K1W and Cr-K20 to reduced 0.5 and 1.2 mM of Cr(VI), respectively. Moreover, there appears to be a positive correlation between reduction and resistance to chromate. While all the genomes share a large number of core proteins (2,810 proteins), the two fastest chromate reducers (Cr-K29 and Cr-K32) shared an additional 610 proteins. All four isolates contain putative genes related to chromate reduction (i.e. *chrR* and *yieF*) but their sequences had low homology when compared to other chromate reducing bacteria. We predict both genes may be involved in chromate reduction with one driving reduction and the other providing low levels of resistance. This study illustrates the lack of conservation of chromate reductases and the need for greater research into the mechanisms governing chromate cycling in the environment.

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# CHAPTER I

## Overview

Heavy metals [e.g. Pb (lead), Mg (Mercury), Cd (Cadmium), Cr (Chromium)] are naturally occurring elements originating from the lithosphere. With the advent of the industrial age, the anthropogenic inputs (e.g. mining, smelting, fossil fuel consumption) of heavy metals have outweighed natural inputs, such as eruptions, weathering, and forest fires (Callender 2003; Duruibe, Ogwuegbu et al. 2007). When in excess in the biosphere, heavy metals occur as environmental contaminants and present health issues for both ecosystems and organisms (Boyd 2010). Unlike persistent organic pollutants, heavy metals cannot be completely degraded or destroyed, which makes them a major public and political concern (Cheng, Holman et al. 2012). Having been exposed to toxic heavy metals for billions of years, microbes have developed diverse mechanisms that allow them to survive and resist these toxic elements (Silver and Phung 2005; Díaz-Pérez, Cervantes et al. 2007). Concomitantly, microbes' ability to mineralize and transform these elements has garnered increasing attention for its economic and environmentally friendly aspects when compared to chemical and physical environmental remediation alternatives (He, Li et al. 2010). While numerous different microbial species have been indicated as important in the transformation of these toxic heavy metals (e.g. Cr), the mechanisms and pathways employed by these microbes have yet to be resolved (Duruibe, Ogwuegbu et al. 2007; Giller, Witter et al. 2009; Henne, Nakatsu et al. 2009; Boyd 2010; Cheng, Holman et al. 2012; Viti, Marchi et al. 2013).

Accurate modeling of complex environmental systems requires measuring not only the taxonomic diversity of microorganisms, but also the diverse metabolic pathways utilized by these organisms (Ottesen, Marin et al. 2011; Shi, Tyson et al. 2011; Stewart, Ulloa et al. 2012).

The genetic pathways known to be involved in heavy metal transformations are only partially understood and remain enigmatic. Recently, high throughput sequencing (e.g. whole genome sequences, metagenomics, metatranscriptomics) has provided the opportunity to reconstruct whole genomes of environmental organisms, allowing for the assessment of potential metabolic roles (Tyson, Chapman et al. 2004; Venter, Remington et al. 2004; Baker, Sheik et al. 2013). When combined with physiological studies, these techniques allow for a more detailed approach to connect genetic potential to function within the environment.

The research presented within aimed at detailing the mechanisms used by microorganisms for reducing and resisting hexavalent chromium [Cr(VI)]. Cr (VI) has caused widespread contamination of soil and water in the United States and other industrial nations (Barak, Ackerley et al. 2006; Brose and James 2010; Cheng, Holman et al. 2012). Because Cr (VI) compounds are highly toxic, mutagenic, and carcinogenic, chromium poses a threat to any organism that interact with it. This causes an additional concern as the toxicity of chromium to soil microorganisms can inhibit growth of natural communities and limit bioremediation of other pollutants (Kourtev, Nakatsu et al. 2009). Specifically, this project utilized both high throughput sequencing and physiological data collection to describe four *Microbacterium* spp. collected from a Cr-rich Department of Transportation site in Seymour, Indiana. Overall, we found considerable strain-level genomic and physiological variation between the four chromate reduction environmental isolates. Though a highly homologous chromate reductase was not identified, the genomes did contain putative genes that relate to chromate tolerance that can be investigated for the potential role in chromate reduction. Of the four isolates, only two provide strong evidence of capabilities of having high reduction and resistance; while one isolate showed little to no reduction or resistance. This study illustrates the need for caution when deriving

function from taxonomy. Further, the lack of known chromate genes present in the genomes identifies a knowledge gap in the understanding of chromium cycling within the environment. Future studies of these isolates should investigate the potential for multiple or a non-specific chromate reductase(s) involved in the reduction and resistance of Cr(VI).

This work (Chapter II) is currently in review for publication in the journal *Applied and Environmental Microbiology*.

# CHAPTER II

## Introduction

Chromium (Cr) has become a major environmental pollutant due to its wide use in metal and mining industries (Barak et al., 2006; Brose and James, 2010; Cheng et al., 2012). Specifically, hexavalent chromium has caused widespread contamination of soil and water in the United States and other industrial nations (He et al., 2010; Cheng et al., 2012; Beller et al., 2013). This is of particular concern because the toxicity of pollutants (e.g. chromium) to soil microorganisms could lead to the inhibition of growth of natural communities and limit bioremediation of other pollutants (Bååth, 1989; Said and Lewis, 1991; Nakatsu et al., 2005; Gough et al., 2008; Kourtev et al., 2009; Gough and Stahl, 2011). Within the environment, chromium mainly persists in two forms: Cr(III) and Cr(VI) (Bartlett, 1991). Cr(VI) is highly toxic, soluble, and can be easily transported across cell membranes (Ackerley et al., 2004b; Cheng et al., 2012). Conversely, Cr(III) is sparingly soluble, thermodynamically stable, and found in oxide, hydroxide, and sulfate minerals or complexed by organic matter and soil minerals (Oze et al., 2004; Barak et al., 2006; Oze et al., 2007; Brose and James, 2010; Viti et al., 2013). Because Cr(VI) compounds are highly toxic, mutagenic, and carcinogenic, the ability to differentiate the oxidation states of chromium compounds is essential (Suzuki et al., 1992). While the natural oxidation of Cr(III) is only mediated by a select number of abiotic substrates (e.g. manganese oxides and hydrogen peroxide), Cr(VI) reduction can be driven by various bacterial and abiotic factors (e.g. iron(II) and hydrogen sulfides) (Oze et al., 2004; Brose and James, 2010; Viti et al., 2013).

A limited number of bacterial species have developed unique mechanisms to overcome the toxicity of Cr(VI) in the environment (Silver and Phung, 2005; Diaz-Perez et al., 2007).

While certain bacteria can use Cr(VI) as a terminal electron acceptor under anaerobic conditions (Sheik et al., 2012), other bacteria possess soluble enzymes that can facilitate aerobic reduction. For example, lipoyl dehydrogenase and cytochrome c oxidase reduce hexavalent chromium by one-electron transfer (Barak et al., 2006; Eswaramoorthy et al., 2012), leading to the formation of the highly reactive intermediate Cr(V) (Ackerley et al., 2004b; Barak et al., 2006; Cheng et al., 2012) Cr(V) can then transfer one electron to $O_2$, which regenerates Cr(VI) and produces reactive oxygen species (ROS) (Eswaramoorthy et al., 2012). Shuffling between Cr(V) and Cr(VI) within the cell generates large quantities of ROS and exhausts reducing power (Barak et al., 2006). This resulting oxidative stress has been considered the primary mechanism of Cr(VI) toxicity (Ackerley et al., 2004b; Eswaramoorthy et al., 2012). While several other genes have been identified as influential and potentially important in chromium resistance and reduction (e.g., *chr*A (transporter), *chr*R (reductase), and *yieF* (reductase) (Ackerley et al., 2004a; Gonzalez et al., 2005; Cheng et al., 2012; Eswaramoorthy et al., 2012; Viti et al., 2013)), their occurrence and genetic conservation in environmental chromium reducing isolates are poorly understood (Henne et al., 2009a; Henne et al., 2009b).

ChrA, part of the CHR superfamily, is a chromium transport protein that has been linked to Cr(VI) resistance. The mechanism of this efflux pump has been determined by examining two model organisms, *Cupravidus metallidurans* and *Pseudomonas aeruginosa* (Cervantes and Ohtake, 1988; Cervantes et al., 1990; Henne et al., 2009a). In an *in silico* study, Ramirez-Diaz et al. (Ramirez-Diaz et al., 2008) identified 135 ChrA orthologs that were dominated by Proteobacteria representatives. These ChrA orthologs have variable amounts of sequence similarity, questioning the broad functional role these orthologs may have in these organisms (Henne et al., 2009a). In addition, studies investigating the connection between ChrA and

resistance to Cr(VI) found varying ranges of resistance (Henne et al., 2009a), further illustrating the debate of whether the mere presence of ChrA confers resistance.

Aerobic chromate reduction is commonly thought to take place with soluble NADH/NADPH dependent chromate reductases. Of the chromate reductases, two have been well studied: ChrR in *Pseudomonas putida* (Park et al., 2000) and YieF in *Escherichia coli* (Barak et al., 2006). ChrR uses one and two electron transfers to first reduce Cr(VI) to Cr(V), the reactive intermediate, and then generate Cr(III). While the reactive intermediate can be re-oxidized into Cr(VI) (in the presence of oxygen), it was. YieF, a sequence homolog of ChrR, utilizes a four electron transfer to reduce Cr(VI) to Cr(III). The reductive mechanism of YieF also produces ROS similar to ChrR, however the YieF enzyme uses quinone reductase activity to help protect cells against ROS production (Ackerley et al., 2004a; Gonzalez et al., 2005; Cheung and Gu, 2007). Transcriptomic and proteomic studies detailing the regulation of these proteins in various other bacteria have been inconclusive on their direct role in Cr(VI) reduction (Barak et al., 2006; Brown et al., 2006; Thompson et al., 2007; Mugerfeld et al., 2009; Thompson et al., 2010; Beller et al., 2013; Viti et al., 2013).

While numerous bacteria have been shown to reduce Cr(VI) compounds, a globally conserved molecular mechanism has yet to be resolved (Kourtev et al., 2006; Ramirez-Diaz et al., 2008; Patra et al., 2010; Sheik et al., 2012; Beller et al., 2013; Viti et al., 2013). The objective of this study was to further our understanding of key Cr(VI) reduction mechanisms by examining four chromium reducers isolated from a Cr-rich environment using genomic and biochemical analyses. Overall, we found considerable strain-level genomic and physiological variation between these environmental isolates. While a highly homologous chromate reductase was not identified, the genomes contained putative genes that might relate to chromate tolerance.

## Materials and Methods

*Sample Collection and Isolation*

Soil was collected in Seymour, IN from a Department of Transportation site known to have contamination from chromium, lead, and organic solvents (Nakatsu et al., 2005; Kourtev et al., 2006). Bacterial strains were isolated from the contaminated soil as described in Kourtev *et al*. (Kourtev et al., 2009). Briefly, isolates were enriched on 50% trypic soy agar (TSA) and 0.25 mM Cr(VI). Isolates were selected based on their various abilities to resist and reduce Cr(VI). Isolates were stored in glycerol stocks at -80 ° C.

*DNA Extraction*

Bacterial isolates used in this study (Cr-K1W, Cr-K20, Cr-K29, and Cr-K32) were grown in 250 ml of 50% trypic soy broth (TSB) amended with 2 mM potassium chromate ($K_2Cr_4$). Following inoculation, isolates were incubated at 30°C, 225 rpm for 24-72 hours. Cells were harvested by centrifuging the cultures in 250 ml Nalgene bottles at 8000 x g for 18 minutes. The supernatant was removed and the pellet was washed with autoclaved nanopure water following the same procedure. Cell pellets were extracted using the FastDNA® Spin Kit (MP Biomedical, Santa Ana, CA) with one modification. Before extraction, cell pellets were resuspended in autoclaved nanopure water and 200 µl were transferred to the Lysis Matrix tube provided by the kit. DNA samples were recovered in 100 µl of DES solution and stored at -20 ° C.

*16S rRNA gene sequencing and analysis*

Bacterial 16S rRNA genes were amplified from the DNA extracts using the primer set 8F and 787R (Lane, 1991) as previously described by Ryu *et al.* (Ryu et al., 2013). The PCR products were sequenced in both directions in the Children's Hospital DNA Core Facility (Cincinnati, OH) using an Applied Biosystems Prism 3730XL DNA analyzer. The raw gene

sequences were processed using the Sequencher software (Gene Codes, Ann Arbor, MI). Sequences were submitted to Greengenes for alignment using the Nearest Alignment Space Termination algorithm. The clone libraries were compared using the Naive Bayesian rRNA Classifier(v 2.0) of the Ribosomal Database Project (RDP) with a 95% confidence threshold (Cole et al., 2009). For 16S rRNA gene sequences, DNA homology searches in GenBank were undertaken with the National Center for Biotechnology Information (NCBI) BLASTn (http://www.ncbi.nlm.nih.gov/BLAST/) program (Altschul et al., 1997).

*Chromate Resistance and Reduction Experiments*

All growth and reduction experiments were conducted in 50% TSB with 2 mM $K_2Cr_4$ at 30°C with shaking at 225 rpm. Cultures, inoculated from -80 ° C stocks, were grown overnight and then used to subculture fresh medium to an initial $O.D._{600}$ of 0.004. Cultures were prepared in triplicates for each growth and reduction experiment. The $O.D._{600}$ and Cr(VI) reduction were measured at 6, 12 and then every 24 hours for 5 days. The Cr(VI) reduction assay was performed following the modified protocol of Urone *et al.* (Urone, 1955). Briefly, one mL of each culture was centrifuged at 7000 rpm for seven minutes to remove all unwanted solids. Ten µL of the supernatant were added to 966 µL of deionized water in a one mL cuvette. The pH was adjusted by adding 3.40 µL of sulfuric acid. Finally, 20 µL of 1,5-Diphenylcarbazide, a colorimetric reagent, was added to the sample and allowed to set for 10 minutes for full color development. Readings were taken on a Carry UV-Vis spectrophotometer (Agilent technologies, Santa Clara, CA) at 540 nm. Negative controls of 50% TSB and 2 mM $K_2Cr_4$ were used to correct the reduction curves since TSB can reduce small amounts of Cr (VI) abiotically. Growth was measured from cultures on the UV-Vis spectrophotometer at 600 nm.

Minimum inhibitory concentration (MIC) determinations were performed in 5 ml of 50% TSB to which chromate ($K_2Cr_4$) was added to a final concentrations of 5, 10, 20, 40, 60, 80, and 100 mM. Cultures were prepared in triplicates for each MIC experiment and the $O.D._{600}$ was taken at 0 and 96 hours.

*Sequencing, De Novo Assembly, and Analysis*

Whole genome shotgun sequencing was performed using the Illumina MiSeq platform with 100 bp paired end reads at Cincinnati Children's Hospital Medical Center's Genetic Variation and Gene Discovery Core facility. Raw Illumina genomic reads were trimmed of their adapter sequences using the default setting of the program Trimmomatic (version 0.27) (Lohse et al., 2012). Trimmed reads were checked for quality using FastQC (version 0.10.2) (*http://www.bioinformatics.babraham.ac.uk/projects/fastqc/*) and then trimmed for quality using the fastx_trimmer (-Q33 -l 70) and fastx_quality_filter (-Q33 -q 30 -p 50) functions of the FastX toolkit program (version 0.13.2) (*http://hannonlab.cshl.edu/fastx_toolkit/*). To determine the appropriate range of Kmer length for assembly, the program KmerGenie (version 1.5856) was used (Chikhi and Medvedev, 2013). Cleaned pair end reads were assembled using the default setting of the assembly programs Velvet (version 1.2.10) (Zerbino and Birney, 2008) and AbySS (version 1.3.6) (Simpson et al., 2009) at a range of Kmer surrounding the estimated Kmer size from KmerGenie. A third assembly was completed using the raw pair-end reads and the intergrated a5 pipeline assembly (Tritt et al., 2012). This pipeline automates the processes of data cleaning, error correction, contig assembly, scaffolding, and quality control. The draft assemblies were compared and the best assembly picked for each isolate based on their total contigs, N50, genome size, max contig length, and mean contig length (Table S1). Although the average coverage of each genome was high (> 200X) , the best assemblies still contained 30 – 81 contigs

due to the high overall GC content ( ~68%) which resulted in small gaps in the Illumina read coverage whenever the GC content exceeded 80%. The contigs from the selected assembly were annotated and analyzed using the Departments of Energy's Joint Genome Institutes IMG program (Markowitz et al., 2012). Utilizing IMG's annotation data, Pfam categories and their broad category gene counts for the respective genomes were extracted and normalized based on the sum of each row. Principle Components analysis (PCA) was then performed using PAST3 (Harper and Ryan, 2001). All protein coding genes from the annotated draft isolate genomes were submitted to the Pacific Northwest National Laboratory's Species Parallel and Orthology Solver (SPOCS) for analysis to determine pairs of orthologous and paraorthologous proteins between the closely related isolates (Curtis et al., 2013).

*Manual Annotation*

Assembled contigs from the four representative isolate genomes were searched against a protein database of chromate related genes acquired from the UniprotKB database (http://www.uniprot.org/). Three databases were downloaded containing the chromate related protein sequences of ChrA (efflux pump), ChrR (reductase), and YieF (reductase). Assembled contigs were searched against individual databases with the BLASTX algorithm using an e-value cutoff of 1e-05. Nucleotide sequences with corresponding hits were translated using the translation tool from ExPAsy (http://expasy.org/) and examined to see if the sequences were found within a larger open reading frame (ORF). The resultant amino acid sequence was searched using the protein blast tool from NCBI. For each sequence, the top sequence corresponding to an annotated protein with the highest bit score was selected. Sequences were further scrutinized by aligning known chromate related proteins with the resultant blast protein hits from the isolate genomes using ClustalW (default settings) in the Biology Workbench

(version 3.2) from the San Diego Supercomputer Center (http://sdsc.edu/). Isolate sequences were examined for alignment of the protein sequences and conserved regions from the corresponding blast protein hit.

**Results**

*Bacterial isolation and characterization of chromate resistant and reduction*

Four bacterial strains isolated from chromium contaminated soil in Seymour, IN were studied for their ability to resist and reduce Cr(VI). Each isolate was able to grow and survive in 2 mM chromate but differences in their growth rates were noted. Overall, three isolates (Cr-K20, Cr-K29, and Cr-K32) grew to relatively dense cultures (OD $\geq$ 4.0). Although Cr-K1W reached exponential growth earlier than the other isolates, it grew to a significantly lower maximum density (OD $\leq$ 1.0) (Figure 1). Isolates Cr-K29 and Cr-K32 all entered exponential growth after 24 hours of inoculation and reached stationary phase around 48 hours. Cr-K20 also started exponential phase after 24 hours but this strain did not reach stationary phase until after 72 hours. Cr-K1W was able to maintain stationary growth during over 100 hours of stationary phase while the other isolates did encounter declines in growth in stationary phase.

The isolates also had diverse abilities to reduce and resist various concentrations of chromate. Cr-K29 and Cr-K32 reduced all the available Cr(VI) (2 mM) within 48 hours (Figure 1B). In contrast, Cr-K20 reduced 0.5 mM Cr(VI) and Cr-K1W reduced 1.2 mM of Cr(VI) after 120 hours (Figure 1B). Variation in chromate tolerance was also observed between the isolates (Table 1). Isolates Cr-K29 and Cr-K32 were able to grow in up to 100 mM chromate. Conversely, Cr-K20 (the lowest reducer) and Cr-K1W were only able to grow in 5 mM and 10 mM chromate, respectively.

*General genome information*

The four *Microbacterium* sp. draft genomes had a total length between 3.79 - 3.91 Mbp with a GC content of ~ 68% (Table 2). The assemblies were comprised of 30-81 contigs with a N50 (contigs cover 50% of the genome) between 5 and 10 (Table S1). Gene annotation of the four genomes documented between 3,616 and 3,806 predicted protein coding genes (Table 2). Analysis of partial 16S rRNA gene fragments indicated that all four isolates had identical 16S rRNA sequences, and that they are *Microbacterium* sp. (*Actinobacteria* phylum), specifically members of the *Microbacterium oxydans* clade (Figure 2). Additionally, the rRNA gene (1,405 bp) identified for each isolate was identical for all four isolates, which confirmed the initial 16S rRNA identification. Genomes were assessed to be nearly complete with all genomes containing the 35 universal single copy marker genes (Table S2) as used by Raes *et al*. (Raes et al., 2007).

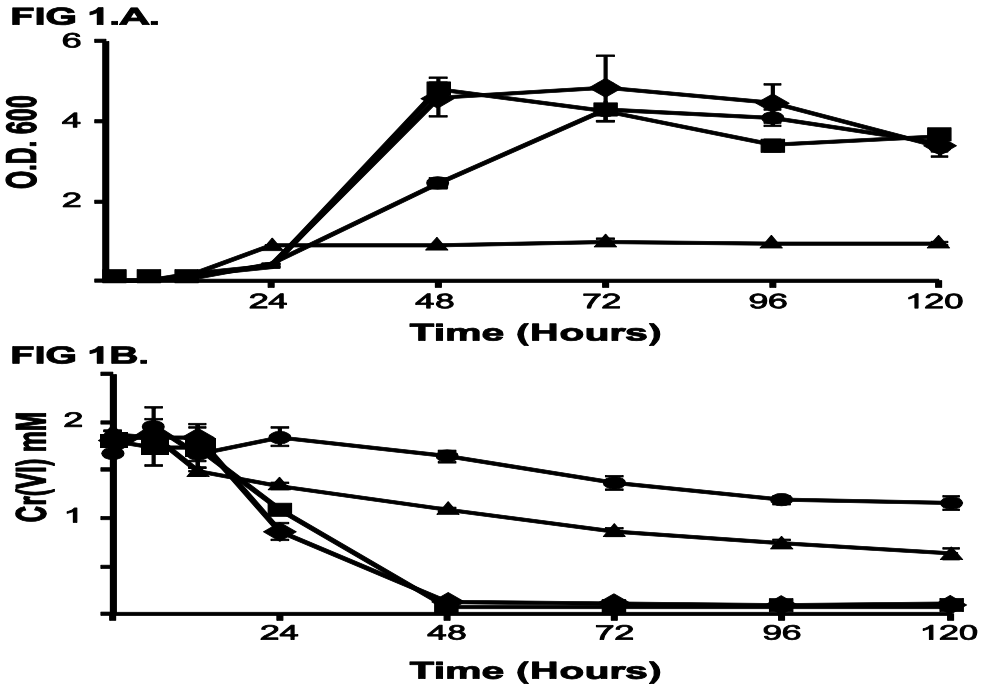


Figure 1(A) Growth and (B**)** reduction of the four Microbacterium sp. isoaltes (Circle=Cr-K230, Triangle=Cr-K1W, Diamond=Cr-K29, and Square=Cr-K32 in 2mM chromate and 50% TSB (n=3, error bars are SD).

| Table 1. Chromate resistance of the four *Microbacterium spp.* isolates | |
|---|---|
| **Isolate** | **Highest Detected Cr(VI) Growth** |
| Cr-K1W | 10mM |
| Cr-K20 | 5mM |
| Cr-K29 | 100mM |
| Cr-K32 | 100mM |

Broad level proteomic comparisons between the isolates showed that most proteins are well conserved at the amino acid level with a smaller subset of unique proteins shared by the quick and slower reducers, respectively. Using reciprocal best hit BLAST, SPOCS determined a total of 2,810 shared orthologous proteins between the four isolates. The quick reducers, Cr-K29 and Cr-K32, shared an additional 602 orthologous proteins, and the slower reducers, Cr-K1W and Cr-K20, shared an additional 854 orthologous proteins). Broad scale analysis of the 21 Pfam categories was conducted using principle components analysis (PCA). Of the 21 Pfam categories, Carbohydrate Transport and Metabolism, Transcription, and Inorganic Ion Transport and Metabolism, General Function, and Function Unknown were shown to create the greatest separation among the annotated draft genomes (Figure 4). Of these differences, Cr-K1W and Cr-K20 were separated from Cr-K29 and Cr-K32 by genes belonging to the categories of Carbohydrate Transport and Metabolism and Transcription while Cr-K29 was differentiated from Cr-K32 by genes belonging to the category of Inorganic Ion transport and Metabolism (Figure S1).

*Genomic analysis for genes related to chromate reduction and resistance*

The automated IMG annotation of the isolate draft genomes failed to find any homologies to known chromate reducing or resistant genes. Consequently, custom searches were performed to identify putative proteins implicated in Cr(VI) reduction. Manual BLASTX searches were performed using the UniprotKB databases for ChrA, ChrR, YieF and a NCBI database of nucleic sequences of chromate reducase genes (Table 2A). BLASTX searches of known chromate transporter ChrA proteins still returned no hits within any of the four assembled draft genomes. However, ChrR BLASTX searches (against the ChrR UniprotKB database) indicated homology (50-51% identity) to an annotated *Thermus scotoductus* ChrR reducase (Old Yellow Enzyme homolog) (GenBank accession number AM902709 (Opperman et al., 2008)). CLUSTALW alignment between the four ChrR-like proteins found in the four *Microbacterium* isolates gave alignments between 35 – 100% (Table 2B). Cr-K29 and Cr-K32, the two fastest reducers, shared 100% match of the resultant protein, while Cr-K1W resulted in a 35% alignment to Cr-K20, Cr-K29, and Cr-K32 (Table 2B).

BLAST searches using the YieF and general chromate reductase databases returned a hit to a predicted flavoprotein for each of the individual isolates. The resultant hit was to a chromate reductase belonging to *Legionella longbeachae* serogroup 1 (43-46% query identity) and *Providencia burhodogranariea* (46-48% query identity), for the YieF and general chromate databases, respectively. CLUSTALW alignment of the four individual isolate sequences revealed high degrees of sequence similarity with Cr-K1W and Cr-K20 having 100% alignment and Cr-K29 and Cr-K32 having 98% alignment (Table 2C).

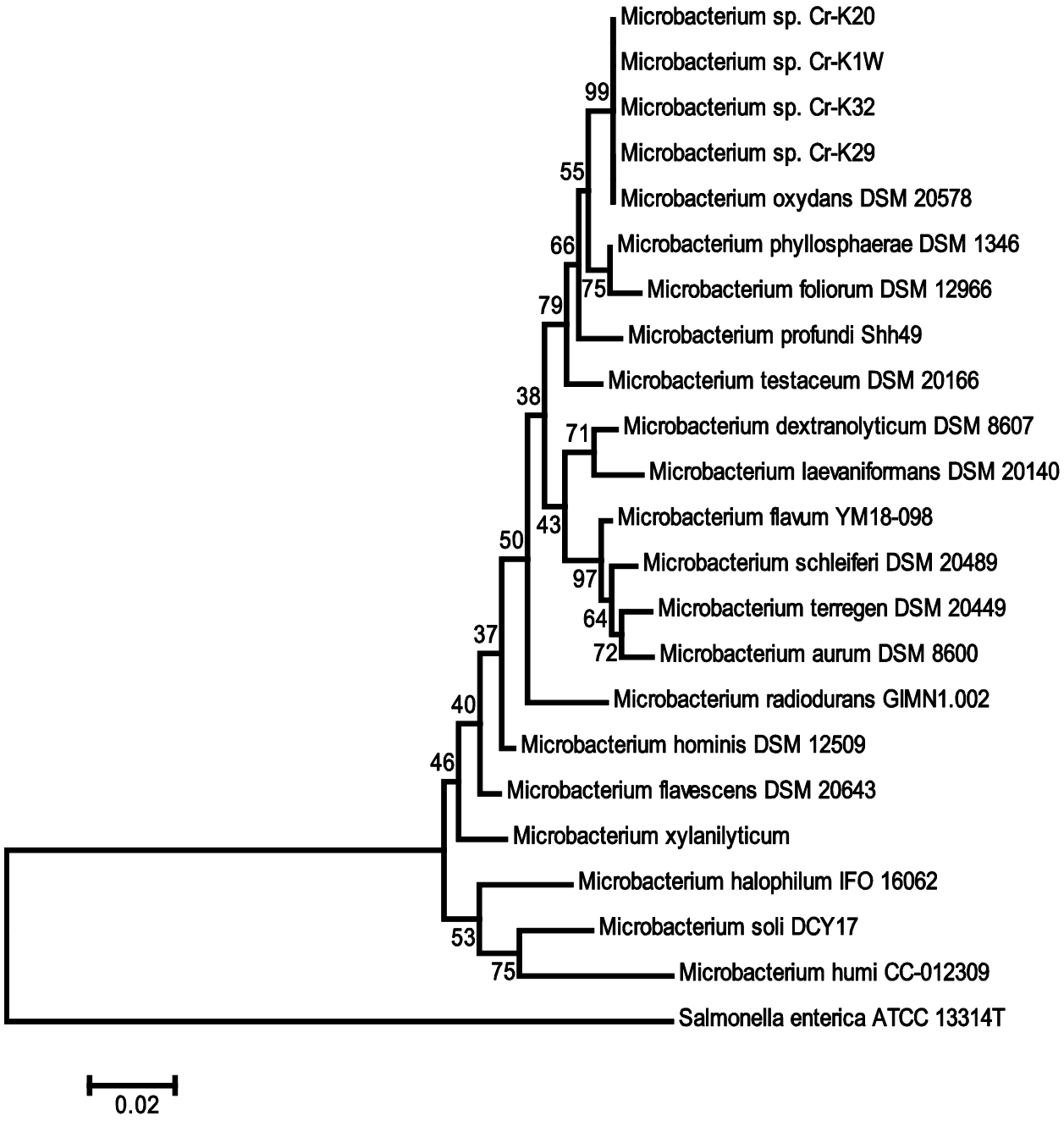


Figure 2. Maximum likelihood phylogenetic tree (bootstrap=500) of four *Microbacterium* sp. isolates and representative *Microbacterium* species.

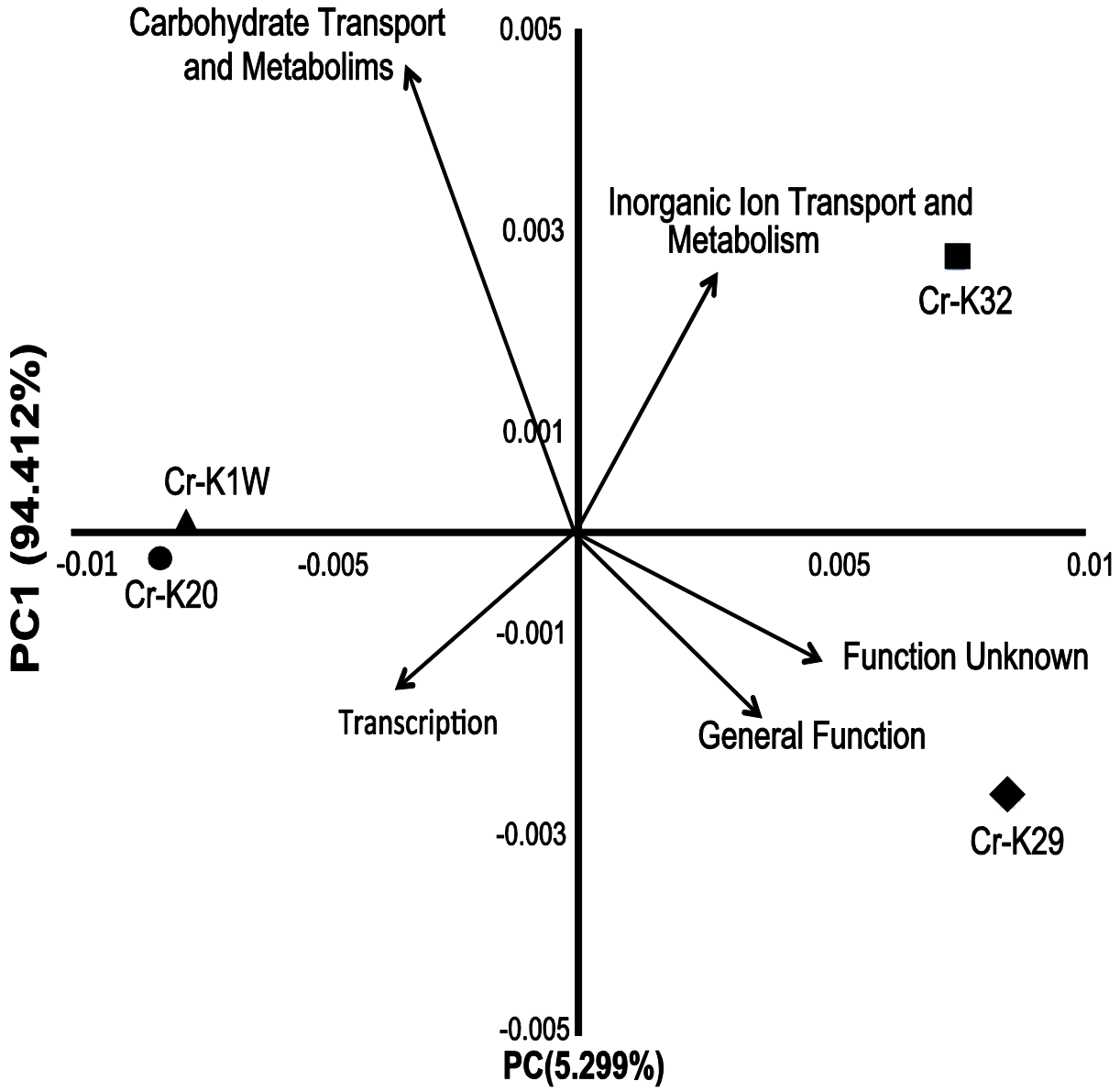


Figure 3. Principle Components analysis of 21 Pfam catagories from IMG annotations of the four *Microbacterium* sp. genomes. Top five eigenvalues were then plotted as vectors after analysis.

*Table 2A. Manual annotations of Cr reducing and resistance genes*

| ***Database Type*** | ***Resultant Blast Hit*** | ***Annotation in IMG*** | ***Cr-K1W*** | **Cr-K29** | **Cr-K32** | **Cr-K20** |
|---|---|---|---|---|---|---|
| ***ChrA*** | *No resultant hits* | *No annotations* | | | | |
| ***ChrR*** | *NADH-dependent Oxidoreductase* | *NADH:flavin oxidoreductases, Old Yellow Enzyme family* | *X* | X | X | X |
| ***YieF*** | *NADPH-dependent FMN reductase* | *Predicted flavoprotein* | *X** | X* | X* | X* |
| ***General*** | *NADPH-dependent FMN reductase* | *Predicted flavoprotein* | *X** | X* | X* | X* |

** represent that the Blast hit was the same gene within the representative genome*

*Table 2B. ClustalW alignment of the four chrR genes from the best Blast hit*

| | | *Alignment* | | | |
|---|---|---|---|---|---|
| *Gene #* | | *Cr-K1W* | *Cr-K20* | *Cr-K29* | *Cr-K32* |
| *2529445967* | *Cr-K1W* | | | | |
| *2529450446* | *Cr-K20* | *35* | | | |
| *2529452261* | *Cr-K29* | *35* | *89* | | |
| *2529442617* | *Cr-K32* | *35* | *89* | *100* | |

*Table 2C. ClustalW alignment of the four yieF genes from the best Blast hit*

| | | *Alignment* | | | |
|---|---|---|---|---|---|
| *Gene #* | | *Cr-K1W* | *Cr-K20* | *Cr-K29* | *Cr-K32* |
| *2529446915* | *Cr-K1W* | | *100* | *86* | *87* |
| *2529451669* | *Cr-K20* | *100* | | *86* | *87* |
| *2529452855* | *Cr-K29* | *86* | *86* | | *98* |
| *2529440805* | *Cr-K32* | *87* | *87* | *98* | |

**Discussion**

Here, we show that four taxonomically related chromium reducing *Microbacterium* isolates have a surprising amount of inter-strain genomic and physiological variation. Specifically, the isolates share a high degree of 16S rRNA gene sequence identity yet they did not have similar physiological responses to chromate. Cr-K29 and Cr-K32 were the quickest

reducers (2 mM chromate within 48 hours), while resisting 100 mM of chromate. Conversely, Cr-K1W and Cr-K20 were only able to resist up to 10 mM and reduce less than 1.2 mM of chromate after 120 hours (Figure 1B, Table 1). We noted a correlation between reduction and resistance, as the faster reducers were able to resist higher amounts of Cr(VI). While past studies have focused on chromate resistance and the presence of the efflux pump gene chrA (e.g. (Diaz-Perez et al., 2007; Ramirez-Diaz et al., 2008; Henne et al., 2009a)), the lack of a putative *chrA* in each of the genomes, and the link between reduction and resistance point to the importance of Cr(VI) reduction to communities that are viable in these contaminated environments.

Full genome analysis showed various differences among these four *Microbacterium* strains that may be linked to the observed physiological variation. While we found a large orthologous protein core among the four strains, the isolates with the faster rates of chromate reduction (Cr-K29 and Cr-K32) and the slower chromate reduction rates (Cr-K1W and Cr-K20) had additional groups of unique orthologous proteins (Figure S1). PCA analysis of the genomes, using the annotated Pfam proteins, also suggests a similar grouping pattern. Specifically, two of the major factors that separate the four isolate genomes were carbohydrate and inorganic metabolism and transport (Figure 3). These broad metabolic pathways are important factors in differentiating microbial communities and have been highlighted in other studies examining microbial response to chromate. For example, a proteomics study of *Arthobacter* FB 24 demonstrated that, when the bacterium was exposed to chromate, a large number of proteins involved in metabolism (i.e., carbohydrate, energy production, and amino acid transport and metabolism) altered their expression (Henne et al., 2009b). This connection between metabolism and chromate was also documented in *Shewanella oneidensis* MR-1 using microarrays (Brown et al., 2006). All together, the genomic variation among the isolates may explain the various

abilities to resist and reduce chromate. Fundamentally, this functional variation between isolates may help explain the importance of functional diversity within the environment and from an ecological standpoint, the importance of the greater community over individual species.

Automated annotations of the isolates' genomes failed to provide any chromate reductases. However, custom database searches did find putative chromate reductases. Each genome contained genes with homology to the *chrR* and *yieF genes* of non-model organisms. The putative *chrR* found in our isolates is homologous to a *chrR* gene found in *Thermus scotoductus,* which has been shown to reduce chromate (Opperman et al., 2008). The isolates with the faster rates of reduction (Cr-K32 and Cr-K29) shared the highest degree of putative *chrR* sequence similarity, while Cr-K20 and Cr-K1W, the slowest reducers, shared lower degrees of similarity (89% and 35%, respectively, Table 3B). Currently, this putative *chrR* is the best candidate for driving the differences in chromate reduction in these isolates. However, the reasons for the variable ability for each isolate to reduce chromate are still not well understood. Reduction differences may be related to sequence similarity, overall expression, or expression during specific stages of growth. Concomitantly, these differences may help explain why Cr-K1W reduces at a different stage of growth than the other three isolates (Figure 1B). Cr-K1W has the least sequence similarity of its putative *chrR* and was capable of chromate reduction through its stationary phase (up to 100 hours), while the other three isolates reduced chromate mainly during exponential growth phase (Figure 1B and Table 3C).

Further, the putative *yieF* genes found in our isolates were homologous to strains that have not been shown to reduce chromate; however, a conserved domain within these genes has been implicated in chromate reduction in other bacteria (Barak et al., 2006). Overall, the putative *yieF* has similar sequence identity (86-100%) when comparing them between the four

isolates. We hypothesize that the broadly conserved sequence similarity of the putative *yieF* suggests that it may function as a general reductase, capable of providing low levels of resistance. YeiF has been shown to provide some levels of reduction in *E. coli* (Barak et al., 2006). Taken together, the putative *chrR* may be the main gene governing chromate reduction, while the putative *yieF* may be providing low levels of both chromate reduction and resistance.

Defining the mechanisms of bacterial chromate reduction and resistance is vital for the systematic understanding of environmental Cr cycling. Even with the high number of chromate resistance bacteria identified, a broadly conserved environmental chromate reduction mechanisms has yet to be fully defined (Cheung and Gu, 2007; Ramirez-Diaz et al., 2008; Viti et al., 2013). While oxidoreductases may be involved in chromate reduction, they only account for a portion of the known pathways. This would explain the lack of annotated classic chromate related genes and why previous gene knockout experiments have not shown complete loss of resistance and reduction (Mugerfeld et al., 2009). While there are different genes implicated in chromate reduction (e.g. NemA, YieF, ChrR), their function in reduction is shared (Park et al., 2000; Barak et al., 2006; Robins et al., 2013). Thus, it is possible that bacteria may not have a specific gene that is uniquely responsible for chromate reduction but may employ a broad reductive response. Continued research using comparative genomics integrated with physiological data, such as this study, offers an avenue to examine the connection between genomic potential and function. Although no direct function was determined, we provided data showing strain-level variation of putative chromate reductases within environmental isolates from contaminated soil that may account for the discrepancies among the four isolates. Further studies from this research will focus on the functional role of the putative *chrR* and *yieF* as potential sources of increased chromate reduction and tolerance. The analysis of these genes may

help define the operative Cr(VI) reduction mechanism within these isolates, and help further define how bacteria resist the toxic effect of chromate in a contaminated environment and lead to bioremediation practice.

## APPENDIX

### *SUPPLEMENTAL TABLES*

Table S1. General genome information and statistics from IMG annotation

| | Genome Status | Coding DNA bases (bp) | DNA scaffolds | GC Content (%) | Protein coding genes | Genes with function prediction | Accession Number |
|---|---|---|---|---|---|---|---|
| **Cr-K20** | Draft | 3648231 | 81 | 68.60 | 3804 | 3096 | JARE00000000 |
| **Cr-K32** | Draft | 3585911 | 37 | 68.27 | 3669 | 3009 | JARD00000000 |
| **Cr-K1W** | Draft | 3650798 | 36 | 68.61 | 3763 | 3083 | JARF00000000 |
| **Cr-K29** | Draft | 3517029 | 44 | 68.32 | 3616 | 2957 | JARC00000000 |

Table S2**.** Genomic stats from the AbySS assemblies

| | Genome Status | Genome Size (mBP) | Num. of Contigs | Mean Contig Size (kBP) | Max Contig Size (kBP) | N50 |
|---|---|---|---|---|---|---|
| **Cr-K1W** | Draft | 3.91 | 46 | 85 | 875 | 5 |
| **Cr-K20** | Draft | 3.91 | 92 | 42.5 | 488 | 10 |
| **Cr-K29** | Draft | 3.79 | 86 | 44 | 485 | 7 |
| **Cr-K32** | Draft | 3.86 | 69 | 56 | 570 | 5 |

Table S3. 35 specific single copy orthologs used for examining genome completeness and the numbers of copies found in each genome.

| Function ID | Name | K1W | K20 | K29 | K32 |
|---|---|---|---|---|---|
| COG0012 | Predicted GTPase, probable translation factor | 1 | 1 | 1 | 1 |
| COG0016 | Phenylalanyl-tRNA synthetase alpha subunit | 1 | 1 | 1 | 1 |
| COG0048 | Ribosomal protein S12 | 1 | 1 | 1 | 1 |
| COG0049 | Ribosomal protein S7 | 1 | 1 | 1 | 1 |
| COG0052 | Ribosomal protein S2 | 1 | 1 | 1 | 1 |
| COG0080 | Ribosomal protein L11 | 1 | 1 | 1 | 1 |

| | | | | | |
|---|---|---|---|---|---|
| COG0081 | Ribosomal protein L1 | 1 | 1 | 1 | 1 |
| COG0085 | DNA-directed RNA polymerase, beta subunit | 1 | 1 | 1 | 1 |
| COG0087 | Ribosomal protein L3 | 1 | 1 | 1 | 1 |
| COG0088 | Ribosomal protein L4 | 1 | 1 | 1 | 1 |
| COG0090 | Ribosomal protein L2 | 1 | 1 | 1 | 1 |
| COG0091 | Ribosomal protein L22 | 1 | 1 | 1 | 1 |
| COG0092 | Ribosomal protein S3 | 1 | 1 | 1 | 1 |
| COG0093 | Ribosomal protein L14 | 1 | 1 | 1 | 1 |
| COG0094 | Ribosomal protein L5 | 1 | 1 | 1 | 1 |
| COG0096 | Ribosomal protein S8 | 1 | 1 | 1 | 1 |
| COG0097 | Ribosomal protein L6P/L9E | 1 | 1 | 1 | 1 |
| COG0098 | Ribosomal protein S5 | 1 | 1 | 1 | 1 |
| COG0099 | Ribosomal protein S13 | 1 | 1 | 1 | 1 |
| COG0100 | Ribosomal protein S11 | 1 | 1 | 1 | 1 |
| COG0102 | Ribosomal protein L13 | 1 | 1 | 1 | 1 |
| COG0103 | Ribosomal protein S9 | 1 | 1 | 1 | 1 |
| COG0124 | Histidyl-tRNA synthetase | 1 | 1 | 1 | 1 |
| COG0184 | Ribosomal protein S15P/S13E | 1 | 1 | 1 | 1 |
| COG0185 | Ribosomal protein S19 | 1 | 1 | 1 | 1 |
| COG0186 | Ribosomal protein S17 | 1 | 1 | 1 | 1 |
| COG0197 | Ribosomal protein L16/L10E | 1 | 1 | 1 | 1 |
| COG0200 | Ribosomal protein L15 | 1 | 1 | 1 | 1 |
| COG0201 | Preprotein translocase subunit SecY | 1 | 1 | 1 | 1 |
| COG0256 | Ribosomal protein L18 | 1 | 1 | 1 | 1 |
| COG0495 | Leucyl-tRNA synthetase | 1 | 1 | 1 | 1 |
| COG0522 | Ribosomal protein S4 and related proteins | 1 | 1 | 1 | 1 |
| COG0525 | Valyl-tRNA synthetase | 1 | 1 | 1 | 1 |
| COG0533 | Metal-dependent proteases with possible chaperone activity | 1 | 1 | 1 | 1 |
| COG0541 | Signal recognition particle GTPase | 1 | 1 | 1 | 1 |

Table S4. Pfam categories from IMG annotations used for the PCA

| | Cr-K32 | Cr-K20 | Cr-K1W | Cr-K29 |
|---|---|---|---|---|
| Amino acid transport and metabolism | 133 | 128 | 128 | 127 |
| Carbohydrate transport and metabolism | 127 | 135 | 135 | 121 |

| | | | | |
|---|---|---|---|---|
| Cell cycle control, cell division, chromosome partitioning | 14 | 16 | 16 | 12 |
| Cell motility | 20 | 20 | 20 | 19 |
| Cell wall/membrane/envelope biogenesis | 86 | 91 | 91 | 86 |
| Coenzyme transport and metabolism | 80 | 86 | 85 | 80 |
| Defense mechanisms | 12 | 15 | 14 | 11 |
| Energy production and conversion | 101 | 107 | 106 | 99 |
| Function unknown | 177 | 169 | 169 | 176 |
| General function prediction only | 129 | 123 | 123 | 129 |
| Inorganic ion transport and metabolism | 96 | 90 | 90 | 93 |
| Intracellular trafficking, secretion, and vesicular transport | 29 | 27 | 27 | 27 |
| Lipid transport and metabolism | 43 | 49 | 48 | 43 |
| Nucleotide transport and metabolism | 39 | 43 | 43 | 39 |
| Posttranslational modification, protein turnover, chaperones | 45 | 46 | 46 | 45 |
| Replication, recombination and repair | 79 | 82 | 81 | 80 |
| RNA processing and modification | 3 | 3 | 3 | 3 |
| Secondary metabolites biosynthesis, transport and catabolism | 16 | 12 | 12 | 16 |
| Signal transduction mechanisms | 31 | 36 | 36 | 32 |
| Transcription | 111 | 122 | 122 | 110 |
| Translation, ribosomal structure and biogenesis | 125 | 122 | 122 | 125 |
| unclassified | 2130 | 2092 | 2204 | 2092 |

*SUPPLEMENTAL FIGURE*

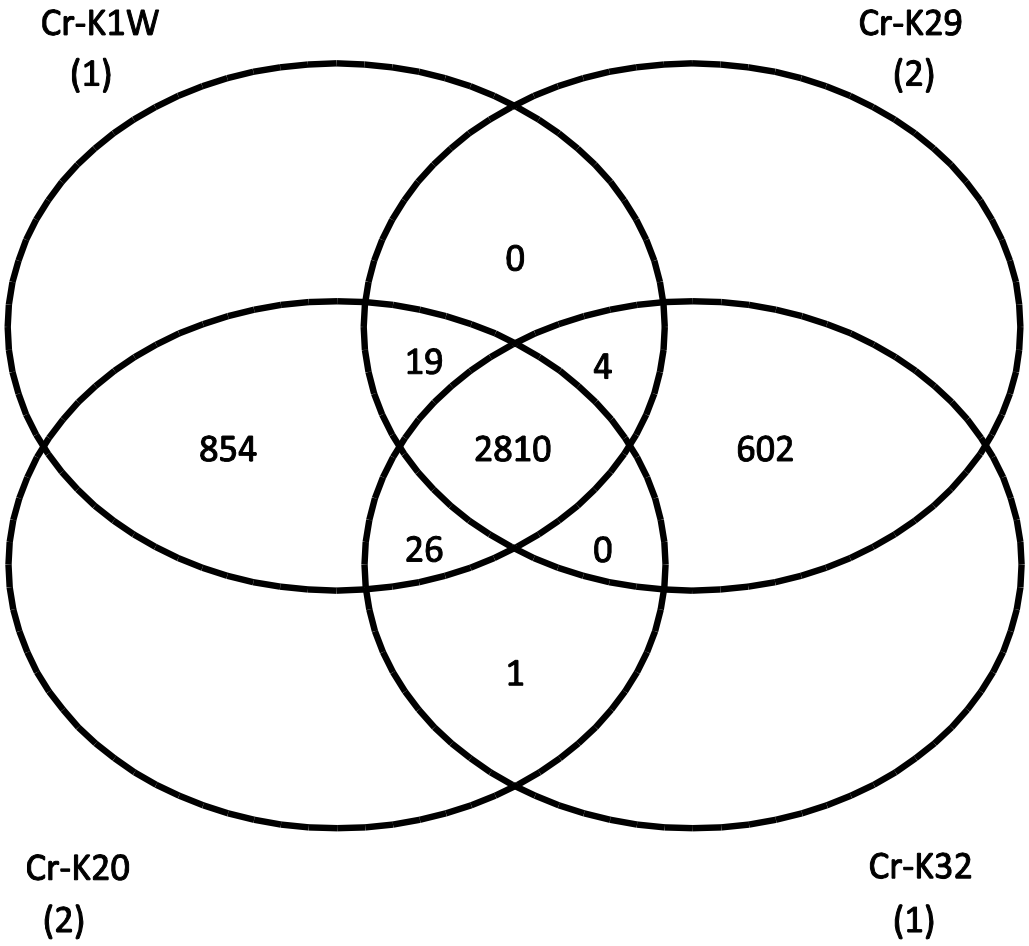


Figure S1. Venn diagram (4-way comparison) illustrating similarities between the four *Microbacterium* spp. genomes. Intersections show the number of shared proteins between two or more organisms based on reciprocal best BLAST hits. Numbers in parentheses depict the missing overlap sectors due to circular drawing and represent proteins shared between opposite genomes but absent in the other two genomes.

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