Title

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Journal

Applied and Environmental Microbiology, 87(21)

ISSN

0099-2240

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Publication Date

2021-10-14

DOI

10.1128/aem.01037-21

Peer reviewed

Applied and Environmental Microbiology

(AEM01037-21: revised version submitted 08/13/2021)

Deciphering Microbial Metal Toxicity Responses using RB-TnSeq and Activity-Based Metabolomics

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Running Title: *Pantoea* strain sp. MT58 metal tolerance mechanisms

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Abstract: 246 words

Text: 5,795 words

Abstract

To uncover metal toxicity targets and defense mechanisms of the facultative anaerobe *Pantoea* strain sp. MT58 (MT58), we used a multi-omic strategy combining two global techniques, random bar code transposon-site sequencing (RB-TnSeq) and activity-based metabolomics. MT58 is a metal-tolerant Oak Ridge Reservation (ORR) environmental isolate that was enriched in the presence of metals at concentrations measured in contaminated groundwater at an ORR nuclear waste site. The effects of three chemically-different metals found at elevated concentrations in the ORR contaminated environment were investigated: the cation Al^{3+}, the oxyanion CrO_4^{2-}, and the oxycation UO_2^{2+}. Both global techniques were applied using all three metals under both aerobic and anaerobic cultures to elucidate metal interactions mediated through the activity of metabolites and key genes/proteins. These revealed that Al^{3+} binds intracellular arginine, CrO_4^{2-} enters the cell through sulfate transporters and oxidizes intracellular reduced thiols, and membrane-bound lipopolysaccharides protect the cell from UO_2^{2+} toxicity. In addition, the Tol outer membrane system contributed to the protection of cellular integrity from the toxic effects of all three metals. Likewise, we found evidence of regulation of lipid content in membranes under metal stress. Individually, RB-TnSeq and metabolomics are powerful tools to explore the impact various stresses have on biological systems. Herein we show that together they can be used synergistically to identify the molecular actors and mechanisms of these pertubations to an organism furthering our understanding of how living systems interact with their environment.

Importance

Studying microbial interactions within their environment can lead to a deeper understanding of biological molecular mechanisms. In this manuscript, two global techniques, RB-TnSeq and activity metabolomics, were successfully used to probe the interactions between a metal resistant microorganism, *Pantoea* strain sp. MT58, and metals contaminating a site where the organism can be located. A number of novel metal to microbe interactions were uncovered including Al^{3+} toxicity targeting arginine synthesis, which could lead to a deeper understanding of the impact Al^{3+} contamination has on microbial communities as well as its impact on higher level organisms including plants for whom Al^{3+} contamination is an issue. Using multi-omic approaches as the one described here is a way to further our understanding of microbial interactions and their impacts on the environment overall.

Introduction

The contamination plume emanating from the former S-3 ponds waste site at the Oak Ridge Reservation (ORR) is a unique environment containing interesting metal-related microbial interactions. Waste from uranium enrichment and other processes containing elevated concentrations of nitrate and mixed metals were deposited into four unlined clay S-3 pond reservoirs for over 30 years before the liquid was neutralized and removed, with contaminated sludge depositing at the bottom of the reservoirs. The S-3 ponds were capped in 1988 and are now used as a parking lot (1). Despite the cleanup effort, groundwater from wells surrounding the S-3 ponds have decreased pH (as low as 3.0) and elevated concentrations of nitrate (up to 230 mM) as well as a variety of potentially toxic metals compared to background groundwater such as aluminum (Al, up to 21 mM), uranium (U, up to 580 μM), and chromium (Cr, up to 8.3 μM) (2, 3). A survey of the geochemical properties and microbial communities (by SSU rRNA gene sequencing) of 93 wells in both non-contaminated and contaminated areas at ORR found that several geochemical properties, including pH and U and nitrate concentrations, could be predicted based on the constituent bacterial community demonstrating a tight link between ORR microbes and their environment (3). Bacterial strains discovered in the ORR S-3 ponds contamination plume likely have molecular mechanisms that protect them from metal toxicity.

Pantoea is a genus of facultative anaerobic bacteria in the class Gammaproteobacteria that contains a number of species studied for their interactions with metals. *P. agglomerans* SP1 is a mesophilic facultative anaerobe that can couple the oxidation of acetate to the reduction of metals, including Fe(III), Mn(IV), and Cr(VI), with electrons being supplied by H_2 oxidation (4). Also, *Pantoea* sp. TEM18, has been shown to accumulate Cr(VI), Cd(II), and Cu(II) (5). Herein we focus on *Pantoea* strain sp. MT58 (MT58), which is closely related to *P. ananatis* by SSU

100 rRNA gene sequence (>99% identical) and was previously isolated from a pristine ORR
101 groundwater sample (6). MT58 was isolated anaerobically on glucose and nitrate in the presence
102 of a contaminated ORR environment metal mixture (COMM), approximating metal
103 concentrations found in highly contaminated groundwater samples taken near the S-3 ponds (6).
104 Analysis of exact sequence variants from SSU rRNA gene sequencing of 93 different ORR non-
105 contaminated and contaminated groundwater wells revealed that MT58 (or relatives with the
106 same SSU rRNA gene sequence) was located in both the pristine area from which it was isolated,
107 and also in highly contaminated groundwater wells adjacent to the S-3 ponds (6).
108 The goal of this study was to identify molecular interactions that allow MT58 to be
109 resistant to and grow in the presence of the range of metals found in the contaminated ORR
110 environment. We used two orthogonal omic approaches that give us different types of
111 information, that when taken together resulted in an enhanced representation of how MT58 is
112 impacted by various metal challenge conditions. Random bar code transposon-site sequencing
113 (RB-TnSeq) is a powerful technique to annotate gene function using a transposon mutant library.
114 With the use of random DNA barcodes, this method has higher throughput than standard
115 transposon mutagenesis with next-generation sequencing (TnSeq) allowing us to probe multiple
116 metal challenges efficiently (7). Compared with other genomics techniques like transcriptomics,
117 gene fitness pinpoints which genes are necessary for survival in a given condition, not just those
118 genes that are expressed. The other technique, activity-based metabolomics, allows for rapid
119 comparison of the metabolomes of various microbial samples grown under different conditions
120 and the identification of metabolites with a condition-specific functional role in the biological
121 process. Defining metabolomes is a critical step in understanding and discovering drivers of

biological processes, as any one protein may perform a number of reactions and the metabolites themselves act to perform numerous functions in cells (8).

Results/Discussion

A complete genome assembly of MT58 was generated using a combination of PacBio and Illumina data. The draft genome of MT58 contained 4,675,847 bp in four contigs with a 54.37 % G+C content (Genbank accession GCA_014495885.1). A total of 4,370 coding sequences were predicted and three plasmids were discovered. A RB-TnSeq fitness library was constructed for MT58 with 447,153 uniquely bar coded single transposon mutations using previously described approaches (7). Using this library, we were able to calculate gene fitness scores for 3,820 out of the 4,370 predicted protein encoding genes in the MT58 genome.

Base and metal challenges fitness experiments were conducted for three metals individually (Al^{3+}, CrO_4^{2-} and UO_2^{2+}) under both aerobic and anaerobic conditions at their half maximal inhibitory concentration (1 mM for Al^{3+}, 5 μM for CrO_4^{2-} and 200 μM for UO_2^{2+}). Strain fitness values were calculated as previously described (7) and are expressed as the normalized $\log_2$ ratio of counts for each strain between the base or challenge growth sample and the reference (time-zero) sample (Data file S1). Gene fitness values were calculated as a weighted average of the strain fitness values for a given gene (7). Changes in gene fitness values were calculated for each challenge condition by subtracting the corresponding base condition gene fitness value from the challenge condition gene fitness value. Genes that had negative changes in gene fitness to the metal challenges $\log_2 \leq -1.5$ (defined as large negative fitness changes) are listed in Table 1 (aerobic) and Table 2 (anaerobic). Large positive gene fitness changes ($\log_2 \geq 1.5$) from the metal challenges are listed in Table S1 (aerobic) and Table S2 (anaerobic). A negative change in gene fitness value indicates that the metal challenge resulted in a decrease in the fitness of library mutants lacking that gene. To give a perspective on the

number of genes with large metal related gene fitness changes, those falling within defined ranges are shown in Table S3. Less than 2% of the 3880 genes in the library had large gene fitness changes ($\log_2 \geq \mid 1.5 \mid$), while over 90% of the genes had a change of gene fitness between -0.5 and 0.5 under any given challenge condition.

A complementary set of global activity -based metabolomic experiments using wild-type MT58 were conducted matching the metal challenge fitness experiments. Extracted metabolite mass features were measured using LC-MS/MS and fold differences in concentrations of the mass features were determined between the six metal challenge samples and their corresponding base conditions using the XCMS online platform (9). Compound identification was performed by comparing the experimental MS2 spectra with those recorded in the METLIN library (10). A summary of the number of dysregulated features with fold changes $\geq$ 1.5 ($p < 0.01$) is shown in Table 3, while identified compounds with dysregulated features for each metal challenge aerobically and anaerobically are listed in Table 4 and Table 5, respectively. The relative number of significantly dysregulated metabolites was small (< 251 of the several thousands of features detected) under any condition. Comparing the results of the fitness and related metabolomics experiments uncovered a number of potential interaction points between MT58 and the metal challenges, which are described in the following sections.

Al^{3+} Impacts Multiple Amino Acids Resulting in an Arginine Auxotrophy

The aerobic and anaerobic Al^{3+} challenges of MT58 uncovered a link between Al^{3+} toxicity and arginine metabolism using both fitness and metabolomics data (Fig 1). In the aerobic Al^{3+} fitness challenge, genes encoding six of the eight enzymes involved in converting glutamate to arginine had large negative gene fitness changes (ranging from -1.7 to -2.3) and two

of the arginine synthetic pathway intermediate metabolites (ornithine (2.2 fold down) and citrulline (1.6 fold down)) were negatively dysregulated (Fig.1). Arginine metabolism was also affected in the anaerobic Al^{3+} challenge with four intermediate metabolites (all amino acids) plus arginine itself being negatively dysregulated (2.0 to 25.0 fold down) (Fig. 1). In contrast to the aerobic Al^{3+} challenge, while negative (ranging from -0.2 to -1.1), none of the arginine synthetic genes had large negative gene fitness changes in the anaerobic Al^{3+} challenge. Therefore, while Al^{3+} affects arginine metabolism under both oxygenation conditions, the observation is more pronounced using fitness data for the aerobic challenge, and metabolomics data for the anaerobic challenge, illustrating how making use of both techniques was essential in making this observation.

The metabolism of other amino acids were impacted by the aerobic and anaerobic Al^{3+} challenges as well. In the aerobic Al^{3+} challenge a gene encoding one of the two enzymes involved in converting homoserine to threonine and genes encoding three enzymes involved in branch chain amino acid (BCAA) synthesis from threonine had large negative gene fitness changes, ranging from -1.6 to -2.3 (Fig. S1, Table 1). In addition, genes encoding glutamate-5-semialdehyde dehydrogenase (-1.7) and phosphoserine phosphatase (-1.5), involved in proline and serine synthesis respectively, had large negative gene fitness changes (Table 1). For the anaerobic Al^{3+} challenge, the metabolomics data showed that in addition to the amino acids involved in arginine synthesis discussed above (Fig. 1), several other amino acids were also negatively dysregulated several fold, including threonine (2.9 down), histidine (15.0 down), phenylalanine (2.6 down), and glutamine (2.4 down) (Table 5).

It is interesting that we observed large fitness changes in amino acid synthesis genes while conducting the experiments in a minimal medium as these strains are often auxotrophic for

193 the amino acids in question. One explanation is that amino acid carry over from the LB recovery
194 growth (despite washing) could have allowed limited growth of amino acid auxotrophic strains.
195 This small amount of growth in turn could be negatively impacted by the Al^{3+} challenge. The
196 fitness data supports this hypothesis with several amino acid synthesis genes having negative
197 fitness values that decress further upon Al^{3+} challenge (Fig. 2A, Table 1).

198 Under environmentally relevant conditions, Al^{3+} is not redox active. The Al^{3+} cation has
199 a slow ligand exchange rate, and although it is largely insoluble at neutral pH forming
200 amorphous $Al(OH)_3$ and mineralizing as Gibbsite (also $Al(OH)_3$), it can remain in solution
201 interacting with numerous anions. Al^{3+} has a strong preference for negatively charged oxygen
202 atoms including those of SO_4^{2-} and CO_3^{2-} and this also includes the carboxylic acid groups of
203 amino acids (11). In human serum, Al^{3+} was shown to interact with several amino acids, with its
204 affinity for lysine being the greatest followed by affinity for ornithine, tyrosine, glutamate, and
205 aspartate, respectively, although arginine was not reported (12).

206 To further test the impact of Al^{3+} on arginine metabolism, wild-type MT58 was grown
207 aerobically in the presence and absence of 3 mM Al^{3+} at pH 6.5 in the presence and absence of
208 several amino acids (5 mM) (Fig. 2B). Of the amino acids tested, only arginine supplementation
209 was able to correct the growth defect caused by Al^{3+} (Fig. 2B). Taken together, the data support
210 a model in which, Al^{3+} binds to multiple amino acids intracellularly resulting in their
211 dysregulation, with the primary observable toxic effect being an arginine auxotrophy. Both the
212 RB-TnSeq and metabolomics data indicate that Al^{3+} has a wider impact on amino acid
213 metabolism in MT58 than just arginine synthesis causing multiple large negative gene fitness
214 changes in BCAA synthetic genes (Table 1) and the downward dysregulation of threonine
215 among several other amino acids (Table 5). However, only the addition of arginine was able to

correct growth in the presence of Al^{3+}. The most likely explanation for the specificity of the arginine auxotrophy is that arginine synthesis involves four amino acids in addition to arginine that are dysregulated by Al^{3+}, resulting predominantly in severe arginine limitation. We can also conclude that the Al^{3+} toxicity is mediated intracellularly as the fitness and metabolomics data were gathered using minimal medium growth conditions without arginine present. It follows that Al^{3+} binding arginine in the growth medium cannot be part of the toxicity mechanism. Additionally, if Al^{3+} toxicity could be prevented with extracellular binding, other amino acids would be expected to corrected growth. Particularly ornithine, which is chemically similar to arginine (with an amine rather than a guanidino side group at the end of the C_3 aliphatic chain) and is known to bind Al^{3+} with high affinity (12).

Chromate Enters MT58 through a Sulfate Transporter and Oxidizes Reduced Intracellular Sulfur Species

The hexavalent oxyanion chromate (CrO_4^{2-}, Cr(VI)) is stable and highly soluble, but in contrast to Al^{3+}, is redox active under typical environmental conditions and can be reduced to the Cr(V), Cr(IV) and Cr(III) oxidation states. CrO_4^{2-} is known to be toxic to biological systems and is both mutagenic and carcinogenic (13-18). CrO_4^{2-} has similar chemical properties to sulfate (SO_4^{2-}) and has previously has been shown to enter bacterial cells through sulfate transporters (13, 19). When the MT58 library was exposed to CrO_4^{2-} aerobically, the component genes of the ABC-type sulfate/thiosulfate transporter (*cysT* (2.1), *cysW* (2.2), and *cysA* (2.3)) all had large positive fitness changes, indicating that CrO_4^{2-} enters the cell using this sulfate transporter as inactivation would prevent a key step in the CrO_4^{2-} toxicity mechanism (Fig. 3). The family of ABC-type transporters that CysTWA belongs to requires a periplasmic substrate binding protein

(in the case of CysTWA a periplasmic sulfate binding protein) (20). In MT58, a gene encoding a periplasmic sulfate binding protein (*cysP*) is in the same operon as the *cysTWA* genes. Under the aerobic CrO_4^{2-} challenge *cysP* has a large positive gene fitness change (2.3) of similar magnitude to the *cysTWA* genes. Interestingly, MT58 encodes a second periplasmic sulfate binding protein (Sbp) whose gene has a large negative gene fitness change (-4.1) upon aerobic CrO_4^{2-} challenge, opposite of the fitness changes observed for the *cysTWAP* genes. It was previously shown in *Escherichia coli* that the two periplasmic sulfate binding proteins (CysP and Sbp) have overlapping functions (21). In *E. coli*, single *cysP* and *sbp* mutants are able to use sulfate and thiosulfate as sole sources of sulfur, but when both genes are inactivated, sulfate transport is blocked and a different sulfur source like cysteine is required for growth (21). The negative gene fitness change for *sbp* then would indicate that sulfate transport using Sbp (unlike CysP) as the periplasmic sulfate binding protein results in some level of protection form CrO_4^{2-} toxicity.

Once sulfate is transported into the cell, a series of biochemical steps take place (Fig. 3) resulting in the synthesis of reduced sulfur species. When exposed to CrO_4^{2-} aerobically, a number of sulfate assimilation genes were found to have large negative changes in gene fitness (Table 1) including the genes of both subunits of sulfate adenylyltransferase (-2.2, -1.7) and of cysteine synthase (-3.7, -1.9). One previously proposed mechanism of CrO_4^{2-} toxicity is that CrO_4^{2-} oxidizes intracellular reduced sulfur species. Chromate is known to oxidize sulfide *in vitro* (22). Also, intracellular CrO_4^{2-} reduction has been demonstrated *in vivo* using EPR spectroscopy in metabolically active rat thymocytes in which reduced forms of Cr (Cr(V) or Cr(III)) were detected upon CrO_4^{2-} exposure (23). In addition, in our previous RB-TnSeq study characterizing the anaerobic effects of CrO_4^{2-} toxicity on *Pseudomonas stutzeri* RCH2 (RCH2), fitness results combined with physiological growth studies were used to propose oxidation of

reduced sulfur species as a mechanism of CrO_4^{2-} toxicity (24). In that study, however, quantitation of the reduced thiol pool of RCH2 cells grown with and without CrO_4^{2-} revealed similar reduced thiol concentrations. It was concluded that the cells maintained a consistent intracellular concentration of reduced thiols, and that oxidation of the thiols by CrO_4^{2-} only had the observable effect of decreasing growth (24). Hence, the metabolomics analysis reported herein of the samples both exposed and not exposed to CrO_4^{2-} allow us to further test the hypothesis that CrO_4^{2-} oxidation of reduced sulfur compounds intracellularly is a mechanism of toxicity. Indeed, the concentrations of multiple reduced sulfur species were found to be decreased intracellularly upon MT58 exposure to CrO_4^{2-} including cysteine (2.7 down), gamma-glutamylecysteine (2.8 down), and glutathione (2.5 down) (Table 4).

One CrO_4^{2-} toxicity mechanism previously observed is the generation of reactive oxygen species that damage DNA when CrO_4^{2-} is reduced by species such as ascorbic acid and glutathione (25-27). For the aerobic CrO_4^{2-} challenge, five DNA repair and/or division genes had large negative gene fitness changes (*recA* (-2.2) and *ruvB* (-1.7) involved in homologous recombination, *xerD* (-2.5) and *xerC* (-2.1) involved in chromosomal segregation during cell division, and *ybiB* (-2.6) a DNA binding protein induced in the DNA damage SOS response) (Table 1) (28-31). No DNA repair and/or division genes had large negative gene fitness changes in the anaerobic CrO_4^{2-} challenge (Table 2) indicating for MT58 this mechanism of CrO_4^{2-} toxicity is either elevated in or exclusive to aerobic growth. So, does CrO_4^{2-} target sulfate transport and assimilation under anaerobic conditions as seen above in the aerobic CrO_4^{2-} challenge? Although none of the reduced sulfur species that were negatively dysregulated under the aerobic CrO_4^{2-} challenge were dysregulated anaerobically, several sulfate transport and assimilation genes had negative fitness changes with the anaerobic CrO_4^{2-} challenge, although to a lesser degree

285 including Sbp (-1.8) and the two subunits of sulfate adenylyltransferase (-1.8, -1.5) (Table 2).
286 The lower magnitude of the anaerobic CrO_4^{2-} fitness response targeting sulfate assimilation
287 compaired to aerobic conditions may reflect a lower requirement for reduced sulfur species like
288 gluthathione, which is needed to repair oxidative stress damage among other uses.
289
290 **Lipopolysaccharide Protects MT58 from Anaerobic Uranium Challenge**
291 Like Cr, U can be redox active under physiological conditions and is present in
292 environmental groundwaters in three different oxidation states (U(IV), U(V), and U(VI)) and
293 these form different soluble and insoluble complexes depending on the pH, oxygen level and
294 geochemical composition of the environment (32). In oxic groundwater, U is present in the
295 U(VI) oxidation state as the soluble uranyl oxycation (UO_2^{2+}). In anoxic groundwater, U(V) and
296 U(IV) predominate, and U(V) usually forms soluble complexes, but U(IV) tends to precipitate as
297 uraninite (UO_2) (32). However, uraninite is easily oxidized to soluble UO_2^{2+} by O_2, NO_3^-, and
298 Fe(III) (33-35). In the present experiments, soluble UO_2^{2+} was added to both the aerobic and
299 anaerobic U challenge cultures.
300 The anaerobic (but not aerobic) UO_2^{2+} challenge resulted in negative fitness changes for
301 multiple genes involved in lipopolysaccharide (LPS) synthesis. LPS is a major component of the
302 outer membrane in gram negative bacteria, and is composed of three different sections, a lipid A
303 hydrophobic membrane anchor, a core region made up of phosphorylated oligosaccharides, and
304 an O-antigen composed of variable polysaccharides in repeating units. The genes encoding two
305 enzymes involved in synthesis of the LPS core region (*waaG* (-1.5) and lipolysaccharide 1,6-
306 galactosyltransferase (-1.5)) had large negative fitness changes in the anaerobic UO_2^{2+} challenge
307 (Table 2). The LPS core region could be key to protecting microbes from UO_2^{2+} stress as several

studies have shown that the positively charged UO_2^{2+} ion binds to negatively charged phosphate residues in the LPS core region (36, 37). In *E. coli*, deletion of *waaG* resulted in a truncated LPS core section lacking most of the negatively charged phosphate groups where the UO_2^{2+} cation binds (38). A large negative fitness change was also observed for the gene encoding O-antigen ligase (*waaL*, -1.5). This protein ligates the O-antigen to the already constructed core region. Previously, a *waaL* deletion mutant in *Pseudomonas aeruginosa* was shown to result in cells with "rough" LPS that lacked the O-antigen (39). Additionally, two hypothetical genes next to each other in the MT58 genome IAI47_00675 (-2.1) and IAI47_00670 (-1.6) had negative fitness changes upon anaerobic UO_2^{2+} challenge. Both genes are part of glycosyltransferase families that are involed in several steps of LPS synthesis (40) and the same is presumbably true for LPS synthesis in MT58. LPS may also protect MT58 from aerobic CrO_4^{2-} stress as *waaG* (-1.5) has a large negative fitness change in this challenge experiment (Table 1). Although it has previously been reported that the UO_2^{2+} cation binds to LPS (36, 37), our fitness experiments show that this interaction is part of a defense mechanism that protects the organism from UO_2^{2+} toxicity. LPS molecules were not significantly dysregulated in the metabolomics analysis and this is consistent with the proposed mechanism because the UO_2^{2+} cation binding to LPS would not nessisarily alter the concentration of LPS as detected by mass spectrometry.

The Tol Outer Membrane Integrity System Protects MT58 from the Challenge of Multiple Metals

Our results show that the Tol proteins are important for MT58 fitness under multiple different metal challenges both aerobically and anaerobically (Tables 1 and 2). These proteins

include an inner membrane complex composed of TolQ, TolR and TolA along with the periplasmic protein TolB, which is known to interact with both TolA and the outer membrane lipoporotein anchor Pal (41). Large negative fitness changes were observed for TolB in five out of six (not the aerobic CrO_4^{2-} challenge) metal challenge conditions ranging from -2.1 (anaerobic Al^{3+} challenge) to -5.0 (aerobic Al^{3+} challenge) (Table 1, Table 2). In addition, TolR had large negative fitness changes in the anaerobic CrO_4^{2-} (-1.5) challenge, while TolQ had large negative fitness changes in both the aerobic Al^{3+} (-1.9) and anaerobic UO_2^{2+} (-1.6) challenges. There are multiple theories for the exact physiological role of the Tol system, although it is likely that the Tol proteins are connected to several outer membrane processes. The Tol proteins are important for outer membrane integrity since mutant strains lacking these proteins are osmosensitive (42). It has been suggested that the Tol proteins are used to bring the inner and outer membranes together in certain locations to help transport outer membrane components, such as the abundant outermembrane porin OmpA (41). The reverse of this process has been shown whereby filamentous phage DNA and specific colicin antibiotic proteins are known to require components of the Tol system to enter the cell through outer membrane porins. For example, ColE9' enters the cell through the porin OmpF, and the import process is dependent on TolB (43). OmpA, is one of the most abundant outer membrane proteins, and has been extensively studied as it is a site for phage attachment. OmpA mutants are sensitive to several stresses including sodium dodecyl sulfate, acid, and high osmolarity (44). Herein, aerobic Al^{3+} (-2.1) and anaerobic UO_2^{2+} (-1.8) challenges resulted in large negative gene fitness changes for *ompA*. The fitness data herein show that the outer membrane integrity Tol system as well as the abundant outer membrane porin OmpA are critical for defense against multiple types of metal stresses (cation,

oxyanion, and oxycation) in addition to previously observed environmental stresses including pH and osmolarity changes.

Membrane Phosphatidylethanolamine Metabolites are Dysregulated in Multiple Metal Challenges

While fitness data were instrumental in determining the critical role of the outer membrane in metal stress, metabolomics data indicate that phospholipid molecules used to make both the inner and outer membranes are potential targets of general metal stress as well. A variety of metabolites associated with the bacterial cell membranes changed significantly under multiple metal challenge conditions (Table 6). Phosphatidylethanolamine (PE) is the principal phospholipid in bacterial membranes (45) and we found that several PEs or PE-associated metabolites, including PE(16:0/0:0), PE(17:1(9Z)/0:0), PE(18:1(9Z)/0:0), 1-palmitoyl-2-linoleoyl PE, and lysoPE(16:1(9Z)/0:0), were dysregulated significantly under anaerobic Al^{3+}, anaerobic UO_2^{2+}, and aerobic CrO_4^{2-} challenges. Differences in the direction of dysregulation between aerobic and anaerobic conditions were observed: up dysregulated in aerobic CrO_4^{2-} challenge, while down dysregulated in anaerobic Al^{3+} and UO_2^{2+} challenge (1-palmitoyl-2-linoleoyl PE is the only exception in anaerobic Al^{3+} challenge). A mono-unsaturated fatty acid, cis-7-hexadecenoic acid, known as a component of the cellular membranes of autotrophic bacteria (46), was significantly up dysregulated for aerobic CrO_4^{2-} (1.9 up) challenge, while serine, used for the synthesis of sphingolipids and phospholipids in cellular membranes (47), was significantly down dysregulated in anaerobic UO_2^{2+} (1.7 down) challenge. Lysine, which is critical to the stability of bacterial cell walls (48), was also significantly down dysregulated in anaerobic UO_2^{2+} (23 down) challenge. Dysregulation of the various forms of PE may indicate

direct damage done to the outer membrane by the various metals, or it could be due to the organism sensing the metal stress and changing the regulation of genes involved at different points in PE synthesis. This could account for the dysregulation in different directions of the aerobic and anaerobic metal stresses. Many genes involved in PE synthesis and differentiation are either essential, or temperature sensitive, which may account for why we did not observe any large fitness effects for them in the metal challenges (49). The different sensitivities and limitations of the two global techniques demonstrates the need for both to provide information the other may miss that can lead to a deeper understanding of the molecular processes driving microbial interactions with their environment.

Challenges in Combining Metabolomics and Fitness Data

The challenge in combining metabolomics with genomics-based experiments such as fitness, transcriptomics, and metagenomics functional prediction come from limitations of both technology types. Metabolomics aims to profile all of the metabolites present in biofluids, cells and tissues (50). However, it is not yet possible to obtain all metabolite classes simultaneously, as many factors affect the metabolome coverage such as sample pretreatment method, separation column, mobile phase composition, and ionization mode. To capture the majority of the compounds in a complex mixture, several complementary analytical techniques are often needed in a metabolomics experiment. Here we used both reverse phase and HILIC LC columns to facilitate the separation of both non-polar and polar molecules, however, only positive ionization mode was used in the mass spectrometry analysis in both cases. Thus, those molecules that can only be measured in negative ionization mode or separated using gas chromatography would be missed. For example, LPS that was shown from the fitness data to be critical in the anaerobic

398 UO_2^{2+} challenge, is often measured via the detection of β-hydroxy fatty acids in negative
399 ionization mode (51). Further, the instrument MS acquisition range of 40-1000 m/z was used in
400 the analysis, and those molecules with precursor ions outside of this mass range cannot be
401 detected including LPS. Also, molecules at concentration levels below the limits of detection for
402 the QTOF instrument used in this experiment would also be missing in the processed data. More
403 importantly, only a subset of acquired metabolite features from any experiment can be
404 confidently identified because of the limitations in metabolic feature analysis algorithms (52) and
405 availability of reference molecule spectra in metabolite databases (53). However, through
406 advancements in instrument technology, automated data processing, increased coverage in
407 spectral databases and better metabolite identification workflows, metabolomics is starting to
408 overcome these challenges and have throughput to match genome-based omic technologies.
409 The challenges with fitness or genomics lie in the ability to have a genetic system in
410 diverse microbes and in gene annotation (54, 55). In particular, limitations exist in the functional
411 annotation of genes and the existence of uncharacterized metabolic pathways. We may see genes
412 or gene products respond together with metabolites, but the connection between a particular
413 metabolite and gene may not be described in the literature or databases (56, 57). This could be
414 from unknown gene product activities or from unknown or uncharacterized pathways. This
415 problem is increased when using non-model organisms as we did in this study. Trying to
416 overcome these issues was part of our rationale for this study in selecting fitness profiling, as it
417 confirms a functional role in response to a specific condition just as changes in the metabolome
418 can be attributed as a direct response to the condition of interest. There are also limitations in
419 being able to generate transposon libraries in many different types of organisms, although, like

improvements to metabolite identification databases and automated workflows, there have been recent demonstrations of new methods that extend fitness profiling to many organisms (58, 59).

More generally, when we consider the alignment of metabolomics with fitness in systems biology analysis frameworks, we must take into account the differences in the techniques, both the biological dynamics they represent and the analytical limitations of the method or instrumentation. Metabolomics captures a single time point snapshot of the state of overall metabolism in a cell. We also know that compared to transcription and translation, the metabolome responds fastest and most directly to changing environmental conditions (8). Gene fitness on the other hand is a measure of strain survival during growth that spans several doubling times before harvest (60). To maximize overlapping observations from the two different techniques, we measured the metabolites from the same time point that the RB-TnSeq was measured and examined the data from both the perspective of starting with top fitness hits and then looking for metabolites related to those pathways, and in parallel, identifying the top dysregulated features in the metabolomics data and then looking for related genes in the RB-TnSeq data with large fitness changes. The majority of significantly dysregulated metabolites identified in this study aligned well with the Rb-TnSeq results. However, we did identify a few significantly dysregulated metabolites which were not on known metabolic pathways identified by the RB-TnSeq technique. For example, the aerobic Al^{3+} challenge induced significant downregulation of cis-Δ2-11-methyl-dodecenoic acid (1.7 down), which is responsible for extracellular microbial communication a response not noted from the fitness results. In other systems, studies we previously conducted combining transcriptomic and metabolomic profiling, we typically identified more metabolites that did not have a direct correlation with gene expression values (61). This appears to be an advantage of gene fitness over transcriptomic

results because expression does not always imply activity or function of the gene product, whereas fitness phenotypes do provide a direct connection from gene product to a functional role in phenotype presentation. By analyzing data from the metabolomics and genomics sides and following up on the generated hypotheses, we are currently able to use these different techniques on similar footages.

Summary

Multi-omic approaches are useful for uncovering complex biological interactions. For example, metabolomics and TnSeq were previously combined to probe the interaction of the plant pathogen *Agrobacterium tumefaciens* and its host to discover plant metabolites exploited as nutrients by *A. tumefaciens* central metabolism (62). Herein we use RB-TnSeq and activity-based metabolomics to probe the interaction of the metal tolerant ORR strain MT58 to toxic metals found in the ORR S-3 ponds contamination plume. In some cases, like LPS protecting the cell from UO_2^{2+} stress, only one of the techniques, in this case RB-TnSeq, provided evidence of the interaction. However, in most cases both RB-TnSeq and metabolomics provided complementary evidence for the metal interactions we observed with MT58. Fitness changes in five different genes, and dysregulation of five metabolites uncovered that arginine is a primary target of Al^{3+} stress (Fig. 1). Also, fitness changes in nine different genes (some encoding multi-subunit proteins) and 3 dysregulated metabolites resulted in new observations on CrO_4^{2-} toxicity impacting SO_4^{2-} transport and assimilation, including that Sbp allows MT58 to differentiate CrO_4^{2-} from SO_4^{2-} (Fig. 3). Both techniques also led to insights into how critical the outer membrane is for resistance to metals in general, whether in the form of cations, oxyanions or oxycations. All three tested metals, with these different chemistries, under either aerobic or

anaerobic conditions, had large negative fitness changes for protein(s) in the Tol system involved in several outer membrane processes, while several PE-related metabolites were dysregulated in the metal challenge experiments. Therefore, the use of multi-omic techniques provides a way to probe complex molecular interactions surrounding metal toxicity and resistance, key factors driving microbial survival in contaminated environments.

Materials and Methods

Genome Sequencing

The MT58 genome (Genbank accession GCA_014495885.1) was sequenced using both the Illumina and PacBio platforms. Illumina reads were cleaned and trimmed using BBtools (https://jgi.doe.gov/data-and-tools/bbtools) with default parameters. The resulting clean and trimmed Illumina reads were then assembled as a hybrid assembly with the raw PacBio reads using Unicycler with default parameters (63). This resulted in 4 circular contigs. These were then polished using the Illumina reads and Pilon until two successive passes produced no further changes (64). The 3 longest contigs were rotated to start with UniRef90_C9P927 (dnaA), UniRef90_A0A0E9C431 (repA) and UniRef90_A0A0F5F2F9 (repA), respectively. For the fourth, no suitable initiation replication match could be found. The assembly was uploaded to NCBI and was annotated using thier prokaryotic genome assembly pipeline (GCA_014495885.1).

Growth Conditions

Base medium containing 20 mM glucose, 4.7 mM NH_4Cl, 1.3 mM KCl, 2 mM $MgSO_4$, 0.2 mM NaCl, 1.2 mM $NaHCO_3$, 5 mM NaH_2PO_4, and 0.1 mM $CaCl_2$ with 1X vitamins and 1X trace elements was used to grow strains. The trace elements were prepared at 1,000X and contained 65.4 mM nitrilotriacetic acid, 25.3 mM $MnCl_2 \cdot 4H_2O$, 7.4 mM $FeCl_3 \cdot 6H_2O$, 7.7 mM $CoCl_2 \cdot 6H_2O$, 9.5 mM $ZnCl_2$, 0.4 mM $CuCl_2 \cdot 2H_2O$, 0.4 mM $AlK(SO_4)_2 \cdot 12H_2O$, 1.6 mM H_3BO_3, 1.0 mM $(NH_4)_2MoO_4 \cdot 4H_2O$, 1.9 mM $NiCl_2 \cdot 6H_2O$, 0.8 mM $Na_2WO_4 \cdot 2H_2O$, and 1.1 Na_2SeO_3. The vitamin mix was prepared at 1,000X and contained 0.08 mM d-biotin, 0.05 mM folic acid, 0.49

mM pyridoxine HCl, 0.13 mM riboflavin, 0.19 mM thiamine, 0.41 nicotinic acid, 0.23 mM pantothenic acid, 0.74 µM vitamin B12, 0.36 mM p-amino benzoic acid, and 0.24 mM thiotic acid. Unless otherwise stated, 5 M HCl was used to pH the medium to 7.0, and the medium was filter sterilized before use. For anaerobic growth, 20 mM KNO_3 was added to the growth medium. Growths were performed at 23ºC with shaking (150 rpm). For growth curves, growth was monitored in a Bioscreen C (Thermo Labsystems, Milford, MA) by monitoring OD600. For anaerobic growth, the Bioscreen C was placed in an anaerobic chamber (Plas Labs, Lansing, MI) with an atmospheric composition of 95% Ar and 5% H_2. Growth curves were performed in biological triplicate with error bars representing the standard deviation. For metal challenge experiments; $KAl(SO_4)_2{\cdot}12H_2O$, $K_2Cr_2O_7$ and $C_4H_6O_6U$ metal compounds were used.

Mutant Library Construction and Growth

The MT58 RB-TnSeq library was generated by conjugation using the *E. coli* donor strain WM3064 carrying the barcoded *mariner* transposon vector pHLL250 (strain AMD290) (65). Mid-log phase MT58 was conjugated with mid-log phase AMD290 at a 1:1 ratio for 6 hours at 30ºC on an LB agar plate supplemented with diaminopimelate. Mutant colonies were selected by plating on LB plates supplemented with 100 µg/mL kanamycin. Resistant colonies were pooled to make the final mutant library. To map transposon insertions and to associate these insertions to the random DNA barcodes, we used a TnSeq-like protocol (PMID 25968644). The MT58 RB-TNSeq mutant library contains 447,153 single mapped transposon mutations (7). Frozen (-80ºC) library 10% glycerol stocks were recovered aerobically in LB with 50 µg/mL kanamycin to an OD600 of 1.0. Samples of the recovery culture were saved as the reference (time-zero)

517 samples. Base and challenge fitness growths were carried out in triplicate in 5 mL cultures of
518 base medium with and without the indicated challenge metals (1 mM for Al^{3+}, 5 μM for CrO_4^{2-}
519 and 200 μM for UO_2^{2+}) were inoculated to an OD600 of 0.02 from the recovery culture that had
520 been washed once with basal medium and were grown for 5 -10 h to an OD600 of approximately
521 1.0 for aerobic growths and 0.5 for anaerobic growths. For anaerobic fitness growths, sealed
522 Hungate tubes with an argon atmosphere were used. At the end of growth, the OD_{600} of each
523 culture was recorded, and the cells were harvested by centrifugation (7,500 rpm for 10 min) as
524 postgrowth samples. Both pregrowth and postgrowth samples were flash frozen and kept at -
525 80ºC until used for DNA extraction.
526
527 **Metabolomics Culture Growth**
528 Cultures of wild-type MT58 for metabolomics experiments were grown in quintuplicate
529 under identical conditions as described above for fitness growths. At the end of growth, a 1 mL
530 sample was taken to determine the protein content of each culture using Bradford reagent
531 (Millipore St. Louis, MO), and the remaining cells were harvested by centrifugation (7,500 rpm
532 for 10 min), flash frozen in liquid nitrogen, and kept at 80ºC until used for global metabolomics
533 analysis.
534
535 **DNA Preparation, Sequencing, and Fitness Analysis**
536 Frozen pellets from the fitness growths were processed for DNA isolation, sequencing,
537 and sequence analysis as previously described using the BarSeq98 method (7). PCR products
538 were sequenced with the Illumina HiSeq system. Fitness of each strain, defined as the binary

logarithm of the ratio of postgrowth to pregrowth relative abundances, were calculated for individual transposon insertion strains before gene fitness values were calculated as previously described (7). Data quality control and normalization were performed as previously reported (7).

Global metabolomics

Global metabolites were extracted from bacterial cell pellets using a mixture of acetonitrile:methanol:water (2:2:1; v/v) as previously describe (66). Bacteria cellular metabolites were profiled using an electrospray ionization quadrupole time-of-flight mass spectrometry (ESI-QTOF-MS) (BRUKER impact II) in positive mode, and separated in both reverse phase (RP) and HILIC conditions using ultra-high-performance liquid chromatography (UPLC) columns ACQUITY BEH C18 column (2.1 × 100 mm, 1.7 μm) and ACQUITY BEH Amide column (2.1 × 100 mm, 1.7 μm, Waters Corp.), respectively. The mobile phases were comprised of water containing 0.1% formic acid (A) and acetonitrile containing 0.1% formic acid (B). In the RP analysis, the gradient started with 1% B (0-1 min), increased to 99% B in 9 min, and held for 3 min, followed by a 4-min re-equilibration time. In the HILIC analysis, the gradient started with 1% A (0-1 min), increased to 35% A in 13 min, then further increased to 60% A in 3 min, held for 1 min, and re-equilibrated for 5 min. The flow rate was 150 μL/min, and the sample injection volume was 3 μL. ESI source parameters were set as follows: dry temperature, 200 °C; dry gas, 8 L/min; nebulizer, 29 psi; capillary voltage, 4000 V; transfer isCID energy, and 0 eV. The instrument acquisition range was set at 40-1000 *m/z* and the MS acquisition rate was 2 spectra/s. A data-dependent auto MS/MS workflow was performed to acquire the MS/MS spectra of metabolite features with the following settings: intensity threshold of 55; cycle time of 3 s; a smart exclusion after two spectra; a precursor reconsideration if the

intensity difference is > 4. The MS/MS spectra were acquired at multiple collision energies (10, 20, and 50 eV) in an acquisition rate of 2 spectra/s over the m/z range 25–1,000.

After manually checking the LC–MS data using the vendor software (Bruker Compass Data Analysis 4.3), XCMS Online web platform was used for data processing (https://xcmsonline.scripps.edu). Pairwise jobs were performed to examine the differences of metabolite features between bacteria cell samples grown under different conditions, with a *p*-value of <0.05 set as the statistical significance threshold cut-off. Metabolite identification was done using accurate mass comparison and matching MS2 spectra, through the METLIN metabolite repository. Additionally, many metabolite identities were validated through comparison with authentic reference standards.

Acknowledgments

This material by ENIGMA (Ecosystems and Networks Integrated with Genes and Molecular Assemblies) (http://enigma.lbl.gov), a Scientific Focus Area Program at Lawrence Berkeley National Laboratory, is based upon work supported by the U.S. Department of Energy, Office of Science, Office of Biological and Environmental Research, under contract number DE-AC02-05CH11231. The authors wish to thank Astrid Terry for project management.

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Figure Legends

Figure 1: Aerobic and anaerobic Al^{3+} challenge results in negative fitness changes for multiple arginine synthesis genes and the decrease of key arginine synthetic pathway metabolites. Arginine synthesis pathway enzymes and metabolites that are impacted by Al^{3+} challenge are shown in red. Proteins encoded by genes with negative fitness changes are indicated in boxes with the change in parentheses while metabolite fold changes are indicated in parentheses above or below the metabolite with the negative sign indicating downward dysregulation. Aerobic Al^{3+} challenge effects are shown in blue while anaerobic Al^{3+} challenge effects are shown in purple.

Figure 2: Arginine corrects growth defect caused by Al^{3+} in MT58. A) Gene fitness value comparisons between aerobic base and aerobic Al^{3+} challenge conditions. Data points shown in red correspond to arginine synthetic genes with large changes in fitness values upon Al^{3+} challenge, while those in black correspond to other amino acid synthetic genes with large changes in fitness values upon Al^{3+} challenge. **B)** Endpoint growth at 22hr of MT58 grown in the absence (black) and presence (grey) of 3 mM Al^{3+} with and without the addition of various amino acids (5 mM).

Figure 3: Aerobic CrO_4^{2-} challenge impacts the sulfate assimilatory pathway. The genes encoding enzymes in red have negative fitness changes with the metal challenge while those in green have positive fitness changes with gene fitness change values in parentheses. Multiple

809 fitness change values indicate the genes encoding multiple subunits of the protein. Metabolites
810 in red are dysregulated in a downward fashion with the metal challenge, and the fold change is in
811 parentheses below the metabolite with the negative sign indicating downward dysregulation.
812

Tables and Figures

Table 1: Genes with large negative fitness changes ($log_2 \leq -1.5$) with aerobic metal challenges.

Locus Tag	Gene Product Function	Δ Fitness[a]
Al^{3+} Challenge		
Periplasm and Outer Membrane Related		
IAI47_13685	Tol-Pal system protein, TolB	-5.0
IAI47_09245	Putative membrane protein, YmiA (family)	-2.2
IAI47_12645	Outer membrane porin, OmpA	-2.1
IAI47_13700	Tol-Pal system protein, TolQ	-1.9
IAI47_08355	Flagellar protein, FlhE	-1.6
Arginine Synthesis		
IAI47_00915	Argininosuccinate synthase, ArgG	-2.3
IAI47_00910	Acetylglutamate kinase, ArgB	-2.1
IAI47_03975	Amino-acid N-acetyltransferase, ArgA	-2.0
IAI47_16830	Ornithine carbamoyltransferase, ArgF	-1.9
IAI47_00920	Argininosuccinate lyase, ArgH	-1.7
IAI47_00905	N-acetyl-gamma-glutamyl-phosphate reductase, ArgC	-1.7
Other Amino Acid Synthesis		
IAI47_16015	3-isopropylmalate dehydratase small subunit	-2.3
IAI47_01040	Threonine ammonia-lyase	-2.2
IAI47_16210	Homoserine kinase	-2.0
IAI47_15115	Glutamate-5-semialdehyde dehydrogenase	-1.7
IAI47_01030	Branched-chain amino acid transaminase	-1.6
IAI47_01035	Dihydroxy-acid dehydratase	-1.6
IAI47_16350	Phosphoserine phosphatase	-1.5
DNA Division and Repair		
IAI47_03820	Site-specific tyrosine recombinase, XerD	-2.3
Other and Hypothetical		
IAI47_08085	Helix-turn-helix domain-containing protein	-1.6
IAI47_09305	Cyclic di-GMP phosphodiesterase	-1.6
IAI47_03550	Membrane-bound lytic murein transglycosylase, MltC	-1.5
IAI47_08705	Carboxy terminal-processing peptidase	-1.5
CrO_4^{2-} Challenge		
Periplasm and Outer Membrane Related		

IAI47_05420	PTS glucose transporter, IIA	-2.9
IAI47_05070	Outer membrane protein assembly, BamB	-2.8
IAI47_11830	Muramoyltetrapeptide carboxypeptidase	-2.0
IAI47_06395	NADH-quinone oxidoreductase, NuoI	-1.9
IAI47_05465	Bile acid:sodium symporter	-1.6
IAI47_00655	Glycosyltransferase, WaaG	-1.5
Sulfate Assimilation		
IAI47_00785	Periplasmic sulfate binding protein, Sbp	-4.1
IAI47_05405	Cysteine synthase, CysM	-3.7
IAI47_04180	Sulfate adenylyltransferase, CysD	-2.2
IAI47_05440	Cysteine synthase, CysK	-1.9
IAI47_04185	Sulfate adenylyltransferase, CysN	-1.7
Iron Transport		
IAI47_15605	Fe^{3+}-hydroxamate ABC Transporter, FhuC	-4.3
IAI47_15595	Fe^{3+}-hydroxamate ABC Transporter, FhuB	-3.2
IAI47_15600	Fe^{3+}-hydroxamate ABC Transporter, FhuD	-2.7
DNA Division and Repair		
IAI47_13290	DNA-binding protein YbiB	-2.6
IAI47_03820	Site-specific tyrosine recombinase, XerD	-2.5
IAI47_04300	DNA recombinase, RecA	-2.2
IAI47_18400	Site-specific tyrosine recombinase, XerC	-2.1
IAI47_08450	Holliday junction branch migration DNA helicase, RuvB	-1.7
Other and Hypothetical		
IAI47_18830	tRNA synthesis GTPase, MnmE	-2.1
IAI47_08705	Carboxy terminal-processing peptidase	-2.1
IAI47_09350	Oxidoreductase	-1.9
IAI47_15970	16S rRNA (cytosine(1402)-N(4))-methyltransferase RsmH	-1.8
IAI47_07015	ABC transporter ATP-binding protein	-1.8
IAI47_06680	LysR family transcriptional regulator	-1.7
IAI47_21665	DUF333 domain-containing protein	-1.7
IAI47_00905	N-acetyl-gamma-glutamyl-phosphate reductase, ArgC	-1.6
IAI47_04175	Uroporphyrinogen-III C-methyltransferase	-1.6
IAI47_06245	tRNA pseudouridine(38-40) synthase, TruA	-1.5
UO_2^{2+} Challenge		
Periplasm and Outer Membrane Related		
IAI47_13685	Tol-Pal system protein, TolB	-3.3

816 [a] The Δ fitness is the difference between the average gene fitness value (of biological triplicate
817 samples) in the metal challenge condition to the control condition with no metal addition for
818 each gene.
819

820 **Table 2: Genes with large negative fitness changes ($log_2 \leq -1.5$) with anaerobic metal**

821 **challenges.**

Locus Tag	Gene Product Function	Δ Fitness[a]
Al^{3+} Challenge		
Periplasm and Outer Membrane Related		
IAI47_13685	Tol-Pal system protein, TolB	-2.1
DNA Division and Repair		
IAI47_12790	Chromosome partition protein, MukE	-2.4
Other and Hypothetical		
IAI47_09580	FNR family transcription factor	-1.5
CrO_4^{2-} Challenge		
Periplasm and Outer Membrane Related		
IAI47_13685	Tol-Pal system protein, TolB	-2.7
IAI47_13695	Tol-Pal system protein, TolR	-1.5
Sulfate Assimilation		
IAI47_04160	Phosphoadenylyl-sulfate reductase	-2.0
IAI47_00785	Periplasmic sulfate binding protein, Sbp	-1.8
IAI47_04180	Sulfate adenylyltransferase, CysD	-1.8
IAI47_04185	Sulfate adenylyltransferase, CysN	-1.5
Other and Hypothetical		
IAI47_18530	Thioredoxin, TrxA	-2.1
IAI47_13745	2-oxoglutarate dehydrogenase E1 component	-1.7
IAI47_03725	Phosphoglycerate dehydrogenase	-1.7
UO_2^{2+} Challenge		
Periplasm and Outer Membrane Related		
IAI47_13685	Tol-Pal system protein, TolB	-2.2
IAI47_00675	Glycosyltransferase	-2.1
IAI47_12645	Outer membrane porin, OmpA	-1.8
IAI47_13700	Tol-Pal system protein, TolQ	-1.6
IAI47_00670	Glycosyltransferase	-1.6
IAI47_03645	O-antigen ligase, WaaL	-1.5
IAI47_00655	Glycosyltransferase, WaaG	-1.5
IAI47_03640	Lipopolysaccharide 1,6-galactosyltransferase	-1.5
Other and Hypothetical		
IAI47_07070	HisA	-1.8
IAI47_09040	NAD-dependent epimerase	-1.7

822 [a] The Δ fitness is the difference between the average gene fitness value (of biological triplicate
823 samples) in the metal challenge condition to the control condition with no metal addition for
824 each gene.
825

826 **Table 3: Number of dysregulated features with fold changes ≥ 1.5 (p < 0.01) for aerobic**

827 **(A) and anaerobic (An) metal challenges.**

	Al (A)	Cr (A)	U (A)	Al (An)	Cr (An)	U (An)
Reverse Phase	17	11	0	30	1	5
Hilic	46	195	46	221	9	105
Total	63	206	46	251	10	110
Annotated	8	14	6	22	15	15

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829

Table 4: Annotated dysregulated features with changes ≥ 1.5 ($p < 0.01$) for aerobic metal challenges.

Annotated Feature	m/z	RT(min)	Fold Change[a]	p value	Direction
Al^{3+} Challenge					
Arginine Synthesis					
Ornithine	133.0971	14.4	2.2	3E-03	down
Citrulline	176.1027	11.0	1.6	3E-03	down
Amino Acid Synthesis					
Tyrosine	182.0810	8.4	1.6	6E-03	up
Other					
Met Glu	279.1008	9.8	2.1	2E-02	down
Xanthine	153.0408	5.8	1.7	9E-03	up
Cis-Δ2-11-methyl-dodecenoic acid	227.2005	9.6	1.7	3E-02	down
Glutathione, oxidized	613.1583	14.4	1.5	4E-02	down
Pantothenic acid	220.1177	2.0	1.5	9E-03	down
CrO_4^{2-} Challenge					
Sulfate Assimilation and Oxidative Stress					
Gamma-glutamylcysteine	251.0696	9.3	2.8	3E-04	down
Cysteine	122.0271	9.3	2.7	1E-04	down
Glutathione, oxidized	613.1554	14.2	2.5	1E-03	down
Glutathione	308.0912	10.0	2.5	4E-03	down
Fatty acid/membrane					
Ethyl oleate	311.2575	7.9	2.5	1E-03	up
LysoPE(16:1(9Z)/0:0)	452.2775	6.5	2.3	1E-03	up
PE(18:1(9Z)/0:0)	480.3079	6.4	2.2	4E-05	up
PE(16:0/0:0)	454.2922	8.7	2.0	5E-04	up
Cis-7-hexadecenoic acid; 11Z-hexadecenoic acid	255.2317	9.9	1.9	8E-04	up
Other					
DL-2-aminoadipic acid; N-Methyl-L-glutamate	162.0743	9.1	33.6	1E-04	down
Pantothenic acid	220.1174	1.9	11.9	3E-04	down
N-acetyl-L-lysine	189.1231	9.3	4.2	4E-04	down
DL-phenylalanine	166.0855	1.7	3.1	1E-02	down
DL-o-tyrosine	182.0809	8.2	2.6	2E-04	down
UO_2^{2+} Challenge					
Lysine	147.1128	14.8	4.6	3E-03	down
Glutathione, oxidized	613.1586	14.4	2.7	1E-03	down
Ornithine	133.0971	14.4	2.4	2E-03	down

1-octadecanamine	270.3153	9.0	1.8	4E-02	down
Glutamate	148.0604	9.7	1.7	3E-03	down
Nicotinamide adenine dinucleotide	664.1159	14.2	1.7	2E-02	down

[a]Pairwise jobs were performed in the XCMS platform to examine the fold change in metabolite features between bacteria cell samples grown under metal challenge and not metal added control conditions in biological quintuplicate samples. A *p*-value of <0.05 was set as the statistical significance threshold cut-off.

837 **Table 5: Annotated dysregulated features with changes ≥ 1.5 (p < 0.01) for anaerobic**
838 **metal challenges.**
839

Annotated Feature	m/z	RT(min)	Fold Change[a]	p value	Direction
Al^{3+} Challenge					
Arginine Synthesis					
Aspartic acid	134.0448	9.9	25.0	2.00E-03	down
Ornithine	133.0971	14.4	20.0	1.00E-04	down
Arginine	175.1187	14.2	14.0	2.00E-03	down
Glutamate	148.0604	9.8	5.2	3.00E-04	down
Citrulline	176.1028	11.1	2.0	5.00E-03	down
Amino Acid Synthesis					
Histidine	156.0766	14.1	15.0	8.00E-04	down
Threonine	120.0656	9.8	2.9	8.00E-05	down
Phenylalanine	166.0859	1.7	2.6	1.70E-02	down
Glutamine	147.0764	10.4	2.4	5.00E-04	down
Fatty acid/membrane					
LysoPE(16:1(9Z)/0:0)	452.2770	6.5	20.0	3.00E-02	down
PE(18:1(9Z)/0:0)	480.3083	6.5	16.1	3.00E-03	down
PE(17:1(9Z)/0:0)	466.2926	6.5	11.2	3.00E-03	down
PE(16:0/0:0)	454.2920	8.5	7.2	3.00E-03	down
Cis-7-Hexadecenoic Acid	255.2313	9.9	3.0	6.00E-04	down
Oleic acid	283.2629	10.6	2.6	2.00E-04	down
1-palmitoyl-2-linoleoyl PE	716.5216	5.3	2.0	3.00E-02	up
Other					
Betaine; 5-Aminopentanoic acid	118.0865	6.8	37.0	9.00E-03	down
Adenosine; vidarabine	268.1040	7.0	11.0	4.00E-03	down
Glutathione, oxidized	613.1588	14.4	9.0	9.80E-07	down
Deoxyadenosine	252.1092	6.6	4.9	3.00E-03	down
Glutathione	308.0909	10.2	3.3	3.00E-04	down
Nicotinamide adenine dinucleotide	664.1155	14.2	3.0	7.00E-04	down
CrO_4^{2-} Challenge					
Ophthalmic acid	290.1347	10.1	3.2	4.00E-02	up
DL-phenylalanine	166.0855	1.7	3.1	1.00E-02	down
Betaine dimer	235.1654	7.3	2.3	8.00E-03	down
Adenosine; vidarabine	268.1041	7.0	2.2	1.00E-02	down
Riboflavin	377.1458	4.0	2.0	3.00E-02	down
UO_2^{2+} Challenge					
Fatty acid/membrane					

PE(16:0/0:0)	454.2921	8.5	2.4	1.30E-02	DOWN
PE(18:1(9Z)/0:0)	480.3074	8.8	1.9	8.00E-03	DOWN
1-palmitoyl-2-linoleoyl-PE	716.5216	5.2	1.7	4.00E-02	down
Other					
Methylhistamine	126.1025	5.1	59.0	5.00E-03	down
Lysine	147.1128	15.0	23.0	3.00E-03	down
Ornithine	133.0971	14.4	20.7	1.00E-04	down
S-nitroso-l-glutathione	337.0814	9.2	9.9	8.00E-03	up
Arginine	175.1187	14.0	6.8	2.00E-03	down
Histidine	156.0765	13.9	4.8	8.00E-04	down
Nicotinamide adenine dinucleotide	664.1158	14.1	3.6	1.00E-04	down
2-pyrrolidone-5-carboxylic acid	130.0498	9.2	2.9	2.60E-02	up
Glutathione, oxidized	613.1586	14.4	2.8	8.00E-03	down
Glutathione	308.0911	10.0	2.2	2.00E-03	down
Niacin	124.0393	1.9	2.2	3.00E-03	UP
Serine	106.0499	10.2	1.7	2.30E-02	down

[a]Pairwise jobs were performed in the XCMS platform to examine the fold change in metabolite features between bacteria cell samples grown under metal challenge and not metal added control conditions in biological quintuplicate samples. A p-value of <0.05 was set as the statistical significance threshold cut-off.

845 **Table 6: Cellular membrane related metabolites dysregulated under aerobic (A) and**

846 **anaerobic (An) metal challenges.**

Metabolite	CrO_4^{2-} (A)	Al^{3+} (An)	UO_2^{2+} (An)
LysoPE(16:1(9Z)/0:0)	2.3	-20	
PE(16:0/0:0)	2.0	-7.2	-2.4
PE(17:1(9Z)/0:0)		-11	
PE(18:1(9Z)/0:0)	2.2	-16	-1.9
1-palmitoyl-2-linoleoyl PE		2.0	-1.7
Cis-7-hexadecenoic acid	1.9		
Serine			-1.7
Lysine			-23

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