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Authors

Chen, Mingfei
Trotter, Valentine V
Walian, Peter J
et al.

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Molecular mechanisms and environmental adaptations of flagellar loss and biofilm growth of *Rhodanobacter* under environmental stress

Running title: Rhodanobacter Biofilm Adaptation to Stress

Authors: Mingfei Chen[1], Valentine V. Trotter[1], Peter J. Walian[1], Yan Chen[1], Romario Lopez[1], Lauren M. Lui[1], Torben N. Nielsen[1], Ria Gracielle Malana[1], Michael P. Thorgersen[2], Andrew J. Hendrickson[1], Heloise Carion[1], Adam M. Deutschbauer[1,3], Christopher J. Petzold[1], Heidi. J. Smith[4], Adam P. Arkin[1,3] Michael W.W. Adams[2], Matthew W. Fields[4], Romy Chakraborty[1*]

Affiliations:

[1] Lawrence Berkeley National Laboratory, Berkeley, CA, United States

[2] University of Georgia, Athens, GA, United States

[3] University of California, Berkeley, CA, United States

[4] Montana State University, Bozeman, MT, United States

Corresponding author:

Romy Chakraborty; rchakraborty@lbl.gov

Mailing address:

1 Cyclotron Road, Building 070A-3317H, Berkeley, CA, 94720, United States

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Abstract

Biofilms aid bacterial adhesion to surfaces via direct and indirect mechanisms, and formation of biofilms is considered as an important strategy for adaptation and survival in sub-optimal environmental conditions. However, the molecular underpinnings of biofilm formation in subsurface sediment/groundwater ecosystems where microorganisms often experience fluctuations in nutrient input, pH, nitrate or metal concentrations is underexplored. We examined biofilm formation under different nutrient, pH, metal, and nitrate regimes of 16 *Rhodanobacter* strains isolated from subsurface groundwater wells spanning diverse pH (3.5 to 5) and nitrate levels (13.7 to 146 mM). Eight *Rhodanobacter* strains demonstrated significant biofilm growth under low pH, suggesting adaptation to survive and grow at low pH. Biofilms intensified under aluminum stress, particularly in strains possessing fewer genetic traits associated with biofilm formation warranting further investigation. Through RB-TnSeq, proteomics, use of specific mutants and transmission electron microscopy analysis, we discovered flagellar loss under aluminum stress, indicating a potential relationship between motility, metal tolerance, and biofilm growth. Comparative genomic analyses revealed absence of flagella and chemotaxis genes, and presence of putative Type VI secretion system in the high biofilm-forming strain FW021-MT20. This study identifies genetic determinants associated with biofilm growth in a predominant environmental genus, *Rhodanobacter*, under metal stress and identifies traits aiding survival and adaptation to contaminated subsurface environments.

Keywords: Biofilm growth, *Rhodanobacter*, Flagella, Aluminum stress, Pangenome

Introduction

Biofilms are structured communities of microorganisms enmeshed in an self-produced, extracellular matrix, typically consisting of exopolysaccharides, proteins, and nucleic acids [1]. Microbial biofilms are found quite commonly on Earth [2], even discovered in ancient geological records [3, 4], suggesting a potential role in adaptation to different environments. As the three-dimensional architecture of biofilms differs significantly from planktonic microorganisms [5, 6], biofilms often have enhanced resilience against environmental perturbation such as UV radiation, temperature, pH, antibiotics and other stressors [7–10]. The architecture of biofilms not only shields the microbial members but also facilitates substrate exchange and cell-cell communication, underscoring biofilms as a vital evolutionary strategy for microbial survival [11]. Although biofilms within the human microbiome, rhizobiome, wastewater treatment, and those in extreme environments is extensively documented [11–14], given sampling constraints, biofilms in shallow, subsurface environments, characterized by low oxygen, limited nutrients, and sometimes high concentrations of contaminants [15, 16] remains largely underexplored despite important implications in carbon, nitrogen and water turnover.

The U.S. Department of Energy's Oak Ridge Reservation (ORR) is characterized by a contamination plume containing high concentrations of mixed waste [17], and previous work identified a significant correlation between presence of *Rhodanobacter* species, low pH (< 4.0), and high levels of metals (UO_2^{2+}, Mn^{2+}, Al^{3+}, Cd^{2+}, Zn^{2+}, Co^{2+}, and Ni^{2+}) [18]. *Rhodanobacter* appear to be versatile, inhabiting diverse environments such as groundwater, sediments, soil, and plant endospheres [19–23]. Many *Rhodanobacter* strains exhibit complete denitrification, positioning them as key players in the environmental nitrogen cycle [21, 22], which is particularly relevant given the increasing global issue of nitrate pollution in surface and groundwater [22, 24].

The pronounced abundance of these species at the likely stems from notable resistance to metals [25, 26] and tolerance to low pH environments [18] in addition to high nitrate. Previous studies at the ORR have illuminated the adaptation mechanisms of *Rhodanobacter* strains including lateral and horizontal gene transfer and negative selection [27, 28]. However, the specifics of *Rhodanobacter sp.* tolerance to stress conditions are not fully understood. Given that biofilms are known to have heightened resistance to various metals [29, 30], it is plausible that biofilm in the shallow subsurface (*e.g.*, sediment-adhered, aggregates) plays a crucial role in *Rhodanobacter* survival under low pH and metal stress and could contribute to the abundance and distribution of *Rhodanobacter* at the ORR.

The overarching goal of this study was to identify the different factors that contribute to *Rhodanobacter* predominance in the contaminated sediment and groundwater of the ORR site. To identify biofilm growth and stress responses in the indigenous *Rhodanobacter* sp., we assessed individual performance across varied nutrient, pH, nitrate, and metal conditions. 13 of 16 strains exhibited significant biofilm growth ($P < 0.05$) compared to the control, and eight showed significant biofilm growth at low pH levels. Aluminum stress, in particular, elicited the most pronounced biofilm responses in six out of eight strains. To identify genes and proteins involved in response to aluminum stress, we incorporated insights from random barcode transposon site sequencing (RB-Tnseq), proteomics, and Transmission Electron Microscopy (TEM). An extensive pangenome analysis, involving 48 *Rhodanobacter* genomes from diverse environments was conducted to identify potential genes central to biofilm growth, particularly emphasizing flagellar roles in the *Rhodanobacter* genus. The findings highlight a mechanism among *Rhodanobacter* strains, in which aluminum stress enhances biofilm growth that can be closely linked to flagella loss, suggesting a strategic adaptation to metal stress.

Materials and Methods

Microbial Strain, growth media, and plate assay preparation

A summary of the genomic characteristics for *Rhodanobacter* strains utilized in this study are detailed in Supplementary Table S1. Isolates were selected based on: (1) origin from groundwater wells with diverse geochemical profiles from the ORR (Table 1), (2) unique phylogenies (Supplementary Table S1), and (3) availability of whole-genome sequences for detailed genetic analysis (Supplementary Table S1). The groundwater wells were sampled to reflect the large geochemical gradient across the site[31]. Initially, bacterial cultures were revived from frozen stocks and grown on R2A agar plates at 30°C. Subsequent liquid cultures of these strains were incubated in R2A broth under shaking conditions (170 r.p.m.) at 30°C. Growth was monitored through optical density measurements at 600 nm (OD600), and cultures harvested for experiments upon reaching OD600 values of 0.2 to 0.3, followed by a single wash in a 30 mM phosphate buffer.

We conducted a comprehensive multilevel experiment to investigate the independent impacts of environmental parameters. The influence of organic carbon availability was assessed using a minimal medium (synthetic groundwater, SGW) [32], and a defined broad nutrient medium (NLDM) based upon exo-metabolites of the field site [33]. The detailed composition of SGW medium is listed in supplementary materials. We also evaluated the impacts of pH and nitrate—two critical geochemical parameters in the field—by adjusting a pH range of 4 to 7 (achieved using 10-30 mM HOMOPIPES, with adjustment via 1M sodium dibasic phosphate buffer) and a nitrate gradient from 0 to 300 mM (diluted using a 5 M stock solution of sodium nitrate).

Metal response assays were conducted with six metals, previously identified as relevant to field conditions (Table 1) [18]. Sterile metal stock solutions were serially diluted into deep-well 96-well plates (Costar, Thermo Fisher Scientific, Waltham, MA, USA). An INTEGRA (Integra lifesciences, Princeton, NJ, USA) liquid handling robot was employed for transferring these solutions into 96-well flat-bottom transparent microplates (Corning). The assay included negative controls (by addition of chloramphenicol at 0.2 g/L) and positive controls (MilliQ water). Each well in the assay plates was filled with 40 µL of metal solution discussed above.

Quantitation of biofilm growth with the crystal violet assay

Crystal violet assay for rigid biofilm growth was conducted in 96-well non-treated microtiter plates (Corning), following the procedure previously described [34]. *Rhodanobacter* strains were inoculated into the plates at a 1:10 (v/v) ratio to achieve a final volume of 80 µL (initial OD600 of 0.02) and incubated statically at 30°C in an incubator for 3 days. Cell density was assessed at OD600 using a BioTek plate reader (Synergy H1, Agilent) equipped with Gen5 software. Post incubation, supernatant was discarded, and each well was washed three times with MilliQ water before air-drying the plates upside down on paper towels. For staining, 100 µL of a 0.1% crystal violet solution (0.1% v/v crystal violet, 1% v/v methanol, and 1% v/v isopropanol in MilliQ water) was added to each well, followed by a 30-minute incubation at room temperature. After discarding the stain, wells were rinsed thrice with MilliQ water. The biofilm containing plates were then destained with 100 µL of a 30% acetic acid and 70% MilliQ water solution, incubated at room temperature for 30–60 minutes. Absorbance at 550 nm and 595 nm (OD550 and OD595) of the destaining solution was measured. Statistical significance ($p < 0.05$) of

experimental conditions were evaluated against control using the wilcoxon test and PERMANOVA analysis in R (version 4.3.0) with default parameters (stats v. 3.6.2).

DNA extraction, whole-genome sequencing, and assembly

Six unpublished *Rhodanobacter* strains isolated from the ORR field site or from packed-bed reactor enrichments seeded with ORR site material were sequenced and assembled for this study. FW021-MT20 was isolated from well FW021 groundwater, whereas five other strains (FW106-PBR-R2A-3-15, FW106-PBR-R2A-1-13, FW106-PBR-LB-2-19, FW106-PBR-LB-2-11, FW106-PBR-LB-1-21) were isolated from packed-bed reactors (PBR) that mimic field sediment conditions using well FW106 sediment as seedling inoculum. High-molecular weight (HMW) DNA was extracted from cell pellets using the QIAGEN Genomic-tip 100/G kit according to manufacturer directions. The DNA was needle-sheared before input to library prep via the Illumina DNA Prep kit. Illumina libraries were sequenced by Novogene (California, USA) using 2X150bp reads on the Novaseq 6000 platform. The HMW DNA was used as input to the Oxford Nanopore Technologies Native Barcoding Expansion kit (EXP-NBD104). Adapters for Nanopore sequencing were attached using the Native Ligation kit (SQK-LSK109). Libraries were sequenced on an R9 flow cell (FLO-MIN106) using a MK1C MinION device.

The sequencing data were quality-filtered and trimmed before assembly. For Illumina data, Novogene removed adapters in-house. We then used BBTools v38.86 (sourceforge.net/projects/bbmap/) for quality-filtering and adapter removal. The processing was done in two passes using bbduk.sh as described in [35]. Nanopore data was basecalled, demultiplexed, quality-filtered, and had adapters removed using Guppy 4.0.9. Isolate FW021-MT20 was only sequenced via the Illumina platform and assembled using SPAdes version 3.13.0

[36] with parameters *--only-assembler --careful -k 21,33,55,77,99,127*. The PBR isolates (FW106-PBR-R2A-3-15, FW106-PBR-R2A-1-13, FW106-PBR-LB-2-19, FW106-PBR-LB-2-11, FW106-PBR-LB-1-21) had genomes assembled through hybrid datasets with Unicycler v.0.4.8 [37] using default parameters with Illumina and Nanopore data as input.

Genome assembly, annotation, and metabolic characterization

We utilized the Department of Energy Systems Biology Knowledgebase platform (KBase) [38] for the genome assembly and annotation of 16 *Rhodanobacter* isolates from the ORR field site. The quality of the genome assemblies was assessed using QUAST [39] via contig number, size, total length, and predicted gene count. CheckM v1.4.0 [40] was employed to evaluate assembly quality and completeness. For functional annotation and metabolic profiling, we used DRAM v0.1.2 [41] for interactive functional summaries and comparison of metabolic profiles across the 16 genomes. To construct detailed metabolic overviews, we utilized KEGG Orthology (KO) numbers obtained through DRAM genome annotations. The criteria for selecting particular genes for analysis were based on documented association with biofilm growth and/or metal toxicity, as identified in the KEGG database and supported by literature cited in Supplementary Table S2. To validate presence in the genomes, we first searched for specific gene names within the DRAM annotations to confirm presence and uploaded these KO numbers to the KEGG database to reconstruct metabolic pathways and verify their incorporation.

Reconstructing taxonomy for the genomes

Phylogenetic analyses were conducted using the KBase “Insert Set of Genomes Into SpeciesTree” app (v2.2.0), which uses 49 core COG gene families for species tree construction

[42]. A subset of public KBase genomes closely related to *Rhodanobacter* genomes were selected based upon alignment similarity to these COG domains. The *Rhodanobacter* genomes were then inserted into curated multiple sequence alignments (MSAs) for each COG family. After post-trimming with GBLOCKS to remove poorly aligned sections, the MSAs were concatenated, and FastTree2 (v2.1.10) was used to reconstruct an approximate maximum-likelihood phylogenetic tree [42], which included the 16 ORR *Rhodanobacter* genomes and 20 selected publicly available genomes. Average Nucleotide Identity (ANI)/Average Amino Acid Identity (AAI) values were calculated using a genome-based distance matrix calculator [43].

Pangenome analysis of the Rhodanobacter species

Pangenome analysis encompassing 64 *Rhodanobacter* genomes (16 from this study and 48 publicly available genomes), was performed using anvi'o (version 6.2) [44, 45]. The selection of public genomes from NCBI and Joint Genome Institute databases was confirmed to belong to the *Rhodanobacter* genus through the Ribosomal Database Project (RDP Taxonomy 18) [46]. The circular plot generated by anvi'o was manually binned as core, soft core, shell, and cloud gene clusters. The "core" genes were present in all 64 genomes, "soft core" genes were present in 61 to 63 genomes, "cloud" genes were present in 1 to 3 genomes, and the remaining genes are "shell" genes.

Mutant library construction and genome-wide fitness assays

Given the varied biofilm growth responses among strains under our test conditions, a deeper investigation into the genetic and molecular mechanisms is warranted. FW104-10B01 was selected to facilitate this analysis due to its substantial response to aluminum. The FW104-10B01

RB-TnSeq library was generated via conjugation with an *E. coli* donor carrying a DNA barcoded *mariner* transposon delivery vector using previously established methods [47–49]. Details on mutant library construction will be described elsewhere (V.V. Trotter et al., in preparation). Library stocks, frozen at −80°C with 10% glycerol, were revived in R2A media containing 5 μg/mL kanamycin, achieving an optical density (OD600) of 0.4 after approximately 20 hours. Biofilm fitness growth experiments were performed in 96-well plates, where each well received 100 μL of SGW or NLDM media, with or without 1 mM aluminum as a stress factor. Initially, mutant library stock cultures were washed with 30 mM phosphate buffer and resuspended in media to an OD of 0.04, with 100 μL of this suspension added to the wells. Cultures in NLDM were incubated for 2 days and in SGW for 4 days before measuring the OD600. Planktonic fractions were processed by pooling media from 10 wells (4 replicates), and the attached fraction by washing, resuspending in phosphate buffer, sonicating for 5 minutes in an ice bath, and pooling from 10 wells (4 replicates). Both culture fractions (planktonic and attached) were then centrifuged at 15,000 rpm for 5 minutes to concentrate samples. These, along with time-zero reference samples, were flash-frozen and stored at -80°C for subsequent DNA extraction. We performed DNA barcode sequencing [50] and calculated gene fitness scores as previously described [47]. To validate our RB-TnSeq results, we also examined individual transposon mutants (knockouts) arrayed from the pooled RB-TnSeq library, focusing on genes exhibiting significant fitness changes (>2.5) between biofilm and planktonic fractions.

Proteomics culture growth and analysis

Cultures of wild-type FW104-10B01 were grown in R2A to a final OD600 of 0.4 to be consistent with RB-Tnseq experiments and washed once using 30 mM phosphate buffer (pH 7.0).

Cultures were grown in 96-well deep-well plates using the same stress variables tested for the RB-TnSeq experiments. Each well contained 500 µL of stress/control medium and 500 µL of original inoculum resuspended in corresponding media (SGW, NLDM) with a starting OD = 0.02. After 2 days (NLDM) and 4 days (SGW) of growth, the planktonic fraction was harvested by transferring the supernatants of a deep-well plate to another sterile 96-well deep well plate and centrifuged at 3,250 x g for 10 min (JS 5.9). The original plate was considered containing sessile/biofilm fraction. Protein was extracted from cell pellets and tryptic-digested peptides were prepared by following established proteomic sample preparation protocol [50]. The detailed protocol for protein analysis can be found in the supplementary materials. The generated mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [51] partner repository with the dataset identifier PXD049428.

Transmission Electron Microscopy (TEM)

FW104-10B01 (characterized by RB-Tnseq and proteomics) and FW021-MT20 (observed strongest biofilm growth) were grown in NLDM and SGW under identical conditions as described above for fitness growth for TEM imaging. To prepare specimens for electron microscopy, 4 µL aliquots of each strain were applied to glow-discharged carbon-coated grids (Formvar-carbon, 200 mesh copper, Electron Microscopy Sciences). After five minutes, grid samples were partially blotted to leave about 1 µL. Grids were then placed sample-side down on a drop of DI water for five seconds and removed. Water-treated grids were oriented sample-side up and again partially blotted to about 1 µL. For negative staining, 2 µL of a 1% uranyl acetate solution was then added to the remaining liquid on each grid. After ten seconds, grids were blotted to dryness. Completed grids were examined in a JEOL JEM-1200EX electron microscope operating at 80 kV and over a

magnification range of 5,000X to 30,000X. Images were recorded using a 2k x 2k pixel CCD camera (UltraScan, Gatan) controlled by the DigitalMicrograph software package.

Results

Variable response in biofilms formation among Rhodanobacter strains

Using high-throughput crystal violet assay we evaluated biofilm growth of 16 *Rhodanobacter* strains, chosen for their unique geochemical origins, diverse phylogeny, and available genomes (Supplementary Table S1). We first assessed the impact of nutrients on biofilm formation by comparing growth in a minimal (SGW) and broad-nutrient (NLDM) media representing general in-situ conditions and occasional nutrient pulses, SGW contains substantially less total dissolved Organic Carbon (OC) (232 mg/L) than NLDM (1260 mg/L) (Supplementary Table S3). We then investigated the effect of pH and nitrate, given the notable variability at the ORR field site where these strains predominate (Table 1). There were no significant differences in biofilm growth under the same pH or nitrate levels in the two media types (Supplementary Figure S1) despite variable OC concentrations. Besides, Wilcoxon tests show no significant differences in individual strains' biofilm growth between media types at the same pH or nitrate levels (Supplementary Table S4). Non-uniform response to environmental factors was observed across isolates from identical wells or species. Where strains from wells FW106 and FW510 (pH < 4) showed a significant negative correlation with nitrate levels in NLDM medium ($P = 0.009$ and $P = 0.005$, respectively), a pattern not replicated in SGW medium ($P = 0.155$ and $P = 0.419$, respectively) (Supplementary Figure S2, Supplementary Table S5). Additionally, strain-specific biofilm growth in response to environmental stresses is observed. Six strains had a significant ($P < 0.05$) positive correlation with pH (Supplementary Figure S3) and nine strains showed a

significant negative correlation with nitrate. Only FW104-T7 displayed a positive correlation (Supplementary Figure S3) with nitrate. FW021-MT20 formed robust biofilms under varying OC conditions and across a broad pH and nitrate range, demonstrating exceptional resilience (Figure 1). These findings highlight the complex, strain-specific responses of *Rhodanobacter* biofilm growth to environmental factors.

Aluminum can trigger biofilm growth in select Rhodanobacter strains

From a group of 16 Rhodanobacter isolates, we chose eight strains for metal testing based on their diverse origins, which included environments with pH levels ranging from 3.43 to 5.22, nitrate concentrations between 13 and 146 mM, aluminum levels from 0.2 to 4.2 mM, and manganese levels from 0.16 to 2.39 mM (Table 1). These selected strains also represented five distinct species, ensuring genetic diversity (Supplementary Table S1), and observed strain-specific patterns (Figure 2). Across 480 experimental setups (combining different strains, metals, and concentrations), 93 conditions significantly enhanced biofilm growth (log2FC > 0.5 compared to control conditions without metal stress). Strain FW510-T8 showed the most robust response with enhanced growth under 46 metal conditions. In contrast, FW104-R8 responded positively only to three specific aluminum concentrations. Among six tested metals, aluminum boosted biofilm growth in most conditions (25 conditions across six strains) and showed the most pronounced effects (OD550 as high as 1.0). However, high aluminum concentrations (> 10 mM) severely inhibited biofilm growth in several strains, illustrating complex, strain-dependent responses (Figure 2). Although other metals do have some effect, for the scope of this work we focused on the metal (aluminum) that caused the most pronounced effect.

Strain FW104-10B01 demonstrated a pronounced ability to thrive under a wide range of aluminum concentrations, highlighting a particular resilience to aluminum stress (Figure 2). To determine the underlying molecular mechanisms influencing biofilm growth under aluminum stress, we have used a combination of BarSeq-based fitness analysis [47], point mutants, and proteomics. We used 1 mM aluminum for BarSeq-based fitness tests due to its significant biofilm-inducing effect on this strain and compared the harvested biofilm with the planktonic fraction. Strain fitness values were calculated as the normalized log2 ratio of counts for each individual mutant between growth samples and a time-zero reference [47]. Gene fitness values were derived as a weighted average of strain fitness values (Supplementary File S1). Under aluminum stress, many flagellar genes (e.g., *flh*, *fli*, *flg*) in *Rhodanobacter* sp. FW104-10B01 showed significant (>1.5) positive fitness changes in the biofilm fraction, a response not observed without aluminum stress or in planktonic fraction. This suggests that flagellar loss and/or down-expression is a specific physiological response to aluminum stress in biofilms. Proteomic analysis of biofilm fraction confirmed this finding, flagella-related genes (e.g., *fliA*) were significantly downregulated in biofilms under aluminum exposure (Supplementary Table S6). Transmission electron microscopy (TEM) also showed notable morphological changes that included flagella/pili loss (Figure 3). Additionally, genes involved in secondary metabolite metabolism, like c-di-GMP and cAMP, were significantly altered by aluminum, with RB-TnSeq highlighting *Crp* (cAMP metabolism) and a GGDEF domain-containing phosphodiesterase (c-di-GMP metabolism) with positive fitness changes greater than 3.5 (Table 2). Proteomic data corroborated these findings, showing significant downregulation of *Crp* and *pdeB* (c-di-GMP) in biofilms, suggesting roles for cAMP and c-di-GMP for biofilm conditions under aluminum stress in *Rhodanobacter* as well as

highlighting the impact of aluminum on cellular morphology and biofilm dynamics (Supplementary Table S5).

To validate the above results and confirm the role of specific genes in biofilm growth, we tested transposon mutants for selected genes exhibiting large fitness changes (log2 ≥ 2.5) from biofilm fractions. In NLDM, no individual mutants (knock-out) surpassed wild-type biofilm growth (Supplementary Figure S4), regardless of aluminum stress. However, in SGW, several mutants, including LRK54_RS11585 (*Crp*), LRK54_RS13565 (*FlhA*), LRK54_RS13580 (*FliA*), and LRK54_RS13485 (Fis family transcriptional regulator), demonstrated significantly higher biofilm growth compared to the wild type (Supplementary Figure S4). Furthermore, TEM imaging of individual transposon mutants (knock-out) in SGW without aluminum stress showed an absence of flagella but the presence of multiple pili, leading to increased cell self-association (Supplementary Figure S5).

Genomic insights into biofilm growth and phylogenetic relationships in Rhodanobacter *strains*

To identify genetic features linked to the biofilm phenotype across the *Rhodanobacter* genus, we used comparative genomic and pangenomic analysis. We analyzed genomes of 16 field-derived *Rhodanobacter* strains (six sequenced and assembled from this study and ten previously published [27, 28]) from the ORR sediment and groundwater. Phylogenetic analysis, based on 49 highly conserved COG domains [42], placed our 16 isolates into six distinct clusters (Figure 4A) within the *Rhodanobacter* genus. ANI and AAI metrics confirmed these isolates as belonging to six different species (same species cutoff for ANI: 95% and AAI: 96%) [52, 53], with distinct phylogenetic affiliations even among isolates from the same well (Figure 4B). The genomes

(Supplementary Table S1) generally showed similar GC content (from 67.11% to 67.9%), except for FW021-MT20 (64.52%), which also has a slightly larger genome size (4.33 Mbp).

Typical biofilm matrix proteins reported in literature (e.g. *Pel*, *Psl*, *VPS*, *RbmA*, *Bap1*, and *RbmC*) [54] were absent in the genomes of 16 *Rhodanobacter* strains tested in this research, including FW021-MT20, which demonstrated the most robust biofilm growth overall (Figure 1). However, FW021-MT20 does possess several other genes annotated as related to biofilm such as *epsG* (EPS biosynthesis enzymes) [55] which is absent in other *Rhodanobacter* genomes (Supplementary Table S2). Another notable distinction of FW021-MT20 is the complete absence of flagella and most chemotaxis genes (Supplementary Table S2) compared to other *Rhodanobacter* genomes.

Pangenomic analysis of 64 *Rhodanobacter* genomes (23 of them are from the deep subsurface), including our 16 ORR genomes and all other 48 publicly available genomes, identified 22,671 gene clusters, categorized into core (n = 1,363), soft core (n = 253), shell (n = 6,311), and cloud clusters (n = 14,744) (Figure 5). As expected, core and soft-core clusters primarily include genes essential for basic cellular functions and central metabolism, along with proteins for pilus assembly. Flagella and chemotaxis genes, typically vital for movement and environmental response, predominantly appear in shell clusters. This categorization is supported by the presence of nearly complete flagella and chemotaxis gene sets in 48 of 64 genomes, according to the KEGG database (Supplementary Table S7). Further analysis integrating genotype with phylogenetic, and the isolation sources based on the ANI index, revealed a distinct clustering of *Rhodanobacter* isolates from deeper subsurface environments, marked by complete flagella and chemotaxis gene sets (Supplementary Figure S6). Conversely, genomes without flagellar genes,

363 often isolated from soil or rhizosphere environments, showed significant phylogenetic divergence
364 from other *Rhodanobacter* strains (ANI < 85%).
365
366 **Discussion**
367 We examined biofilm growth in 16 environmental *Rhodanobacter* strains isolated from
368 field samples with distinct geochemistry from the ORR field site. We observed varied biofilm
369 responses to environmental stresses - namely pH, nitrate and metals (Figure 1). Although
370 availability of nutrients are generally known to impact biofilm growth [56], our results show
371 insignificant effects of higher OC load on biofilms (Supplementary Figure S1). Eight
372 *Rhodanobacter* strains produced more biofilm mass under low pH (Supplementary Figure S3),
373 which align with the fact that strains FW106-PBR-R2A-3.15 and FW106-PBR-LB-2.19 were
374 isolated from a low-pH (4.0) packed-bed reactor run with inocula derived from groundwater well
375 FW106 (3.88-4.21), suggesting that these *Rhodanobacter* strains are adapted to survive and grow
376 at low pH. Producing biofilms likely facilitates this adaptation, providing microniches with
377 different pH and/or metal concentrations [15]. Similarly, many pathogenic bacteria enhance
378 extracellular matrices when challenged with acidic environments such as the gut [57, 58]. Nine
379 strains also showed higher biofilm growth under lower nitrate concentrations (Supplementary
380 Figure S3), corroborating previous findings that nitrate can impede biofilm growth in some
381 bacteria as in *Pseudomonas aeruginosa*, *Burkholderia pseudomallei*, and *Escherichia coli* [59–
382 62].
383 Our study demonstrated that five nearly identical *Rhodanobacter* strains (ANI > 99.9,
384 Supplementary Table S1, Figure 4B) exhibit varied responses to stress, despite their close
385 phylogenetic relationship. Strain FW104-R8 isolated from well FW104 with pH 5.22, showed a

significant inverse relationship ($P < 0.001$) between biofilm formation and pH, whereas strains FW106-PBR-LB-1-21, FW106-PBR-LB-2-11 from wells FW106 (pH 4.01) and FW510-R10, FW510-T8 from FW510 (pH 3.55), were unperturbed by pH (Supplementary Table S8). In fact, these latter strains exhibited increased overall growth (as indicated by OD600 readings) at higher pH levels (data not shown), ruling out the notion that enhanced biofilm formation was simply a consequence of increased microbial growth. Phenotypic differences among near-identical strains can potentially be linked to niche adaptation [63], where strains isolated from higher pH environment form biofilm as a defense mechanism when exposed to acidic conditions, and conversely, strains from lower pH environments naturally adapt to the niche, eliminating the need for biofilm growth. Future work is needed to delineate the relationships between biofilm capacity and microbial distribution along physical and chemical gradients in shallow, subsurface environments, particularly using a multifactorial design to explore the interactions between pH, nitrate, and metal conditions on biofilm growth.

We discovered that aluminum substantially enhances biofilm growth in *Rhodanobacter* (Figure 2). The concentration of aluminum at which certain *Rhodanobacter* strains exhibited notable biofilm enhancement (Figure 2, log2FC > 1) aligns with the naturally occurring aluminum concentrations found in the groundwater wells from which these strains were originally isolated (Table 1), suggesting an adaptive response to the environmental presence of aluminum and perhaps other metals. Aluminum generally interferes in biological processes by mimicking essential metals for arginine synthesis and calcium-mediated signaling [64, 65], both known to affect biofilm formation [66, 67] as demonstrated in *Pseudomonas* and *Escherichia coli* [68, 69]. However, the significance and mechanism of aluminum regulating biofilm growth remains largely unknown in environmental strains. Our study reveals that aluminum exposure leads to flagellar loss and

increased biofilm in *Rhodanobacter* sp. FW104-10B01, challenging the conventional view that flagellar genes are vital for biofilm formation [70–75], as flagellar is an important structure to reach substratum for attachment and initiate biofilm formation [76]. Instead, pili might play an important role in biofilm formation for *Rhodanobacter* strains, as a high density of relatively long pili was observed on the cell surface of FW021-MT20 and in flagellar mutants of FW104-10B01. Nevertheless, both flagella and pili disappear under extreme stress (such as aluminum), which reinforces the biofilm's stability and defense mechanisms. Beyond flagellar and pili genes, we highlight the potential role of cAMP and c-di-GMP regulatory proteins in *Rhodanobacter* biofilm growth using both RB-TnSeq and proteomics data. c-di-GMP can act as a pivotal secondary messenger mediating the transition from planktonic to sessile life forms [77–79], and the cAMP-activated regulator *Crp* is known to influence biofilm regulation in *Pseudomonas aeruginosa* and *Escherichia coli* [80–82]. *Rhodanobacter* mutants lacking *Crp* or a presumptive GGDEF domain-containing phosphodiesterase showed positive fitness values for biofilm formation, particularly under aluminum stress (Table 2), and *Crp* mutants demonstrated a marked increase in biofilm production coinciding with the lack of flagella in stress-free conditions (Supplementary Figure S4). Moreover, the interaction between c-di-GMP and cAMP appears to modulate flagellar expression, with higher c-di-GMP levels inhibiting flagellar motion and promoting adhesin and EPS production [83] (Supplementary Figure S5). Moreover, *Rhodanobacter* FW104-10B01 response to aluminum stress involved *crp* and *pdeB* down-expression and secondary messenger accumulation, appearing to promote a shift towards biofilm growth correlated with the downregulation of flagellar genes. Together, these findings enhance our understanding of the interaction among c-di-GMP, cAMP and flagellar pathways in *Rhodanobacter* under aluminum

stress, clarifying previously conflicting reports regarding the influence of metal stress on secondary messenger levels and associated biofilm growth [84, 85].

Comparative genomic analyses across strains of the *Rhodanobacter* genus enabled the identification of genes linked to biofilm formation under environmentally relevant conditions. Although no typical biofilm matrix genes are found in the genomes, *Rhodanobacter* species might have evolved distinct biofilm formation mechanisms due to specific ecological niches or environmental pressures, differing from biofilm model organisms [86, 87], and like *Pseudomonas*, they may use varied and yet-to-be-characterized sets of proteins or polysaccharides for biofilm formation [88]. Future work involving proteomic and transcriptomic analyses will help identify and characterize the biofilm matrix components in *Rhodanobacter*. *Rhodanobacter* strain FW021-MT20, characterized by robust biofilm formation under diverse conditions (Figure 1), completely lacks flagellar and most chemotaxis genes (Supplementary Table S2), suggesting that lack of flagella (either genetically or phenotypically) is potentially linked to increased biofilm production. FW021-MT20 also possesses unique genes related to the Type VI secretion system (T6SS), pivotal in bacterial virulence and interactions, including biofilm formation [89]. Specifically, previous studies have shown that certain T6SS genes, especially *hcp* genes, are essential for the ability of bacteria to form mature biofilms [90–92]. Many of the genomes (n=31) showed presence of *hcp* genes from our pangenomic analysis, and we identified a unique hcp sequence in strain FW021-MT20 that exhibited less than 40% similarity to those of 28 other *Rhodanobacter* strains (Supplementary Figure S8). This suggests that *hcp* gene in FW021-MT20 potentially performs a different function compared to other strains. Recent studies also highlighted the role of T6SS in the transport of metal ions [93], which could be relevant to FW021-MT20's responses to aluminum stress. *Rhodanobacter glycinis* T01E-68, a biofilm producing strain in rhizosphere environments

[23], has high genetic similarity with FW021-MT20 (high ANI values, both lack flagellar genes, and have identical *hcp* genes) (Supplementary Figures S6, S8). The identification of two genetically similar *Rhodanobacter* strains exhibiting potentially analogous behaviors in distinctly different environments underscores the critical role of gene conservation in microbial adaptation and ecological versatility, especially for those genes linked to biofilm growth. In nutrient-limited subsurface environments, we propose that these findings can be extended beyond *Rhodanobacter* to provide a broader understanding of microbial adaptation and survival.

In summary, our comprehensive analysis of 16 *Rhodanobacter* strains revealed a previously unknown pathway of biofilm growth significantly influenced by environmental factors - low pH and aluminum stress. These conditions not only increase biofilm development but also lead to a reduction in flagella, as supported by RB-TnSeq, proteomic and TEM analyses. Additionally, our results indicate a suppression of the *crp* and *pde* genes under stress, suggesting that an accumulation of secondary messengers like cAMP and c-di-GMP may be involved in the transition from a planktonic to a sessile lifestyle and impact flagella expression. Furthermore, we show that strain FW021-MT20, exhibiting highest biofilm formation, lacks flagella and chemotaxis genes but possesses a specific secretion system and quorum sensing genes. Together, these results not only underscore resilience and adaptation in *Rhodanobacter* in metal-stressed environments but also provide fresh perspectives on mechanisms of biofilm growth in this genus.

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Data availability

The six unpublished *Rhodanobacter* genomes sequenced and analyzed for this study are available are available in the NCBI under the BioProject ID PRJNA1141476.

Competing interests

The authors declare no competing financial interests.

References:

1. Flemming H-C, Wingender J. The biofilm matrix. *Nat Rev Microbiol* 2010; **8**: 623–633.

2. Stoodley P, Sauer K, Davies DG, Costerton JW. Biofilms as complex differentiated communities. *Annu Rev Microbiol* 2002; **56**: 187–209.

3. Westall F, de Wit MJ, Dann J, van der Gaast S, de Ronde CEJ, Gerneke D. Early Archean fossil bacteria and biofilms in hydrothermally-influenced sediments from the Barberton greenstone belt, South Africa. *Precambrian Res* 2001; **106**: 93–116.

4. Rasmussen B. Filamentous microfossils in a 3,235-million-year-old volcanogenic massive sulphide deposit. *Nature* 2000; **405**: 676–679.

5. Van Houdt R, Michiels CW. Biofilm Formation and the Food Industry, a Focus on the Bacterial Outer Surface. *J Appl Microbiol* 2010; **109**: 1117–1131.

6. Lewis K. Persister cells. *Annu Rev Microbiol* 2010; **64**: 357–372.

7. Hostacká A, Ciznár I, Stefkovicová M. Temperature and pH affect the production of bacterial biofilm. *Folia Microbiol (Praha)* 2010; **55**: 75–78.

8. Harrison JJ, Ceri H, Turner RJ. Multimetal resistance and tolerance in microbial biofilms. *Nat Rev Microbiol* 2007; **5**: 928–938.

9. de Carvalho CCCR. Biofilms: microbial strategies for surviving UV exposure. *Adv Exp Med Biol* 2017; **996**: 233–239.

10. Hathroubi S, Mekni MA, Domenico P, Nguyen D, Jacques M. Biofilms: microbial shelters against antibiotics. *Microb Drug Resist* 2017; **23**: 147–156.

11. Yin W, Wang Y, Liu L, He J. Biofilms: the microbial "protective clothing" in extreme environments. *Int J Mol Sci* 2019; **20**.

12. Shahot K, Idris A, Omar R, Yusoff HM. Review on biofilm processes for wastewater treatment. *Life Sci J* 2014.

13. Nayak SK, Nayak S, Patra JK. Rhizobacteria and its biofilm for sustainable agriculture: A concise review. *New and future developments in microbial biotechnology and bioengineering: microbial biofilms*. 2020. Elsevier, pp 165–175.

14. Zijnge V, van Leeuwen MBM, Degener JE, Abbas F, Thurnheer T, Gmür R, et al. Oral biofilm architecture on natural teeth. *PLoS ONE* 2010; **5**: e9321.

15. Smith HJ, Zelaya AJ, De León KB, Chakraborty R, Elias DA, Hazen TC, et al. Impact of hydrologic boundaries on microbial planktonic and biofilm communities in shallow terrestrial subsurface environments. *FEMS Microbiol Ecol* 2018; **94**.

16. Meckenstock RU, Elsner M, Griebler C, Lueders T, Stumpp C, Aamand J, et al. Biodegradation: Updating the concepts of control for microbial cleanup in contaminated aquifers. *Environ Sci Technol* 2015; **49**: 7073–7081.

17. Fields MW, Bagwell CE, Carroll SL, Yan T, Liu X, Watson DB, et al. Phylogenetic and functional biomakers as indicators of bacterial community responses to mixed-waste contamination. *Environ Sci Technol* 2006; **40**: 2601–2607.

18. Carlson HK, Price MN, Callaghan M, Aaring A, Chakraborty R, Liu H, et al. The selective pressures on the microbial community in a metal-contaminated aquifer. *ISME J* 2019; **13**: 937–949.

19. Cho G-Y, Lee J-C, Whang K-S. Rhodanobacter rhizosphaerae sp. nov., isolated from soil of ginseng rhizosphere. *Int J Syst Evol Microbiol* 2017; **67**: 1387–1392.

20. Dahal RH, Kim J. Rhodanobacter humi sp. nov., an acid-tolerant and alkalitolerant gammaproteobacterium isolated from forest soil. *Int J Syst Evol Microbiol* 2017; **67**: 1185–1190.

21. Green SJ, Prakash O, Gihring TM, Akob DM, Jasrotia P, Jardine PM, et al. Denitrifying bacteria isolated from terrestrial subsurface sediments exposed to mixed-waste contamination. *Appl Environ Microbiol* 2010; **76**: 3244–3254.

22. Prakash O, Green SJ, Jasrotia P, Overholt WA, Canion A, Watson DB, et al. Rhodanobacter denitrificans sp. nov., isolated from nitrate-rich zones of a contaminated aquifer. *Int J Syst Evol Microbiol* 2012; **62**: 2457–2462.

23. Lee SA, Kanth BK, Kim HS, Kim T-W, Sang MK, Song J, et al. Complete genome sequence of the plant growth-promoting endophytic bacterium *Rhodanobacter glycinis* T01E-68 isolated from tomato (*Solanum lycopersicum* L.) plant roots. *The Microbiological Society of Korea* 2019.

24. Bijay-Singh, Craswell E. Fertilizers and nitrate pollution of surface and ground water: an increasingly pervasive global problem. *SN Appl Sci* 2021; **3**: 518.

25. Caldeira JB, Chung AP, Morais PV, Branco R. Relevance of FeoAB system in Rhodanobacter sp. B2A1Ga4 resistance to heavy metals, aluminium, gallium, and indium. *Appl Microbiol Biotechnol* 2021; **105**: 3301–3314.

26. Lui LM, Majumder EL-W, Smith HJ, Carlson HK, von Netzer F, Fields MW, et al. Mechanism across scales: A holistic modeling framework integrating laboratory and field studies for microbial ecology. *Front Microbiol* 2021; **12**: 642422.

27. Peng M, Wang D, Lui LM, Nielsen T, Tian R, Kempher ML, et al. Genomic Features and Pervasive Negative Selection in Rhodanobacter Strains Isolated from Nitrate and Heavy Metal Contaminated Aquifer. *Microbiol Spectr* 2022; **10**: e0259121.

28. Hemme CL, Green SJ, Rishishwar L, Prakash O, Pettenato A, Chakraborty R, et al. Lateral Gene Transfer in a Heavy Metal-Contaminated-Groundwater Microbial Community. *MBio* 2016; **7**: e02234-15.

29. Teitzel GM, Parsek MR. Heavy metal resistance of biofilm and planktonic Pseudomonas aeruginosa. *Appl Environ Microbiol* 2003; **69**: 2313–2320.

30. Chien C-C, Lin B-C, Wu C-H. Biofilm formation and heavy metal resistance by an environmental Pseudomonas sp. *Biochem Eng J* 2013; **78**: 132–137.

31. Smith MB, Rocha AM, Smillie CS, Olesen SW, Paradis C, Wu L, et al. Natural bacterial communities serve as quantitative geochemical biosensors. *MBio* 2015; **6**: e00326-15.

32. Martinez RJ, Wu CH, Beazley MJ, Andersen GL, Conrad ME, Hazen TC, et al. Microbial community responses to organophosphate substrate additions in contaminated subsurface sediments. *PLoS ONE* 2014; **9**: e100383.

33. de Raad M, Li YV, Kuehl JV, Andeer PF, Kosina SM, Hendrickson A, et al. A defined medium for cultivation and exometabolite profiling of soil bacteria. *Front Microbiol* 2022; **13**: 855331.

34. Haney EF, Trimble MJ, Hancock REW. Microtiter plate assays to assess antibiofilm activity against bacteria. *Nat Protoc* 2021; **16**: 2615–2632.

35. Lui LM, Nielsen TN, Arkin AP. A method for achieving complete microbial genomes and improving bins from metagenomics data. *PLoS Comput Biol* 2021; **17**: e1008972.

36. Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, et al. SPAdes: A new genome assembly algorithm and its applications to single-cell sequencing. *J Comput Biol* 2012; **19**: 455–477.

37. Wick RR, Judd LM, Gorrie CL, Holt KE. Unicycler: Resolving bacterial genome assemblies from short and long sequencing reads. *PLoS Comput Biol* 2017; **13**: e1005595.

38. Arkin AP, Cottingham RW, Henry CS, Harris NL, Stevens RL, Maslov S, et al. KBase: the United States Department of Energy Systems Biology Knowledgebase. *Nat Biotechnol* 2018; **36**: 566–569.

39. Gurevich A, Saveliev V, Vyahhi N, Tesler G. QUAST: quality assessment tool for genome assemblies. *Bioinformatics* 2013; **29**: 1072–1075.

40. Parks DH, Imelfort M, Skennerton CT, Hugenholtz P, Tyson GW. CheckM: assessing the quality of microbial genomes recovered from isolates, single cells, and metagenomes. *Genome Res* 2015; **25**: 1043–1055.

41. Shaffer M, Borton MA, McGivern BB, Zayed AA, La Rosa SL, Solden LM, et al. DRAM for distilling microbial metabolism to automate the curation of microbiome function. *Nucleic Acids Res* 2020; **48**: 8883–8900.

42. Price MN, Dehal PS, Arkin AP. FastTree 2 — approximately maximum-likelihood trees for large alignments. *PLoS ONE* 2010; **5**: e9490.

43. Rodriguez-R LM, Konstantinidis KT. The enveomics collection: a toolbox for specialized analyses of microbial genomes and metagenomes. 2016.

44. Eren AM, Esen ÖC, Quince C, Vineis JH, Morrison HG, Sogin ML, et al. Anvi'o: an advanced analysis and visualization platform for 'omics data. *PeerJ* 2015; **3**: e1319.

45. Delmont TO, Eren AM. Linking pangenomes and metagenomes: the Prochlorococcus metapangenome. *PeerJ* 2018; **6**: e4320.

46. Wang Q, Garrity GM, Tiedje JM, Cole JR. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl Environ Microbiol* 2007; **73**: 5261–5267.

47. Wetmore KM, Price MN, Waters RJ, Lamson JS, He J, Hoover CA, et al. Rapid quantification of mutant fitness in diverse bacteria by sequencing randomly bar-coded transposons. *MBio* 2015; **6**: e00306-15.

48. Price MN, Wetmore KM, Waters RJ, Callaghan M, Ray J, Liu H, et al. Mutant phenotypes for thousands of bacterial genes of unknown function. *Nature* 2018; **557**: 503–509.

49. Liu H, Price MN, Waters RJ, Ray J, Carlson HK, Lamson JS, et al. Magic pools: parallel assessment of transposon delivery vectors in bacteria. *mSystems* 2018; **3**.

50. Chen Y, Gin JW, Wang Y, de Raad M, Tan S, Hillson NJ, et al. Alkaline-SDS cell lysis of microbes with acetone protein precipitation for proteomic sample preparation in 96-well plate format. *PLoS ONE* 2023; **18**: e0288102.

51. Perez-Riverol Y, Bai J, Bandla C, García-Seisdedos D, Hewapathirana S, Kamatchinathan S, et al. The PRIDE database resources in 2022: a hub for mass spectrometry-based proteomics evidences. *Nucleic Acids Res* 2022; **50**: D543–D552.

52. Jain C, Rodriguez-R LM, Phillippy AM, Konstantinidis KT, Aluru S. High throughput ANI analysis of 90K prokaryotic genomes reveals clear species boundaries. *Nat Commun* 2018; **9**: 5114.

53. Konstantinidis KT, Ramette A, Tiedje JM. The bacterial species definition in the genomic era. *Philos Trans R Soc Lond B Biol Sci* 2006; **361**: 1929–1940.

54. Fong JNC, Yildiz FH. Biofilm Matrix Proteins. *Microbiol Spectr* 2015; **3**.

55. Peng N, Cai P, Mortimer M, Wu Y, Gao C, Huang Q. The exopolysaccharide-eDNA interaction modulates 3D architecture of Bacillus subtilis biofilm. *BMC Microbiol* 2020; **20**: 115.

56. Bowden GH, Li YH. Nutritional influences on biofilm development. *Adv Dent Res* 1997; **11**: 81–99.

57. Petrin S, Mancin M, Losasso C, Deotto S, Olsen JE, Barco L. Effect of ph and salinity on the ability of salmonella serotypes to form biofilm. *Front Microbiol* 2022; **13**: 821679.

630 58. D'Urzo N, Martinelli M, Pezzicoli A, De Cesare V, Pinto V, Margarit I, et al. Acidic pH
631 strongly enhances in vitro biofilm formation by a subset of hypervirulent ST-17
632 Streptococcus agalactiae strains. *Appl Environ Microbiol* 2014; **80**: 2176–2185.

633 59. Mangalea MR, Plumley BA, Borlee BR. Nitrate Sensing and Metabolism Inhibit Biofilm
634 Formation in the Opportunistic Pathogen Burkholderia pseudomallei by Reducing the
635 Intracellular Concentration of c-di-GMP. *Front Microbiol* 2017; **8**: 1353.

636 60. Park J-S, Choi H-Y, Kim W-G. The Nitrite Transporter Facilitates Biofilm Formation via
637 Suppression of Nitrite Reductase and Is a New Antibiofilm Target in Pseudomonas
638 aeruginosa. *MBio* 2020; **11**.

639 61. Van Alst NE, Picardo KF, Iglewski BH, Haidaris CG. Nitrate sensing and metabolism
640 modulate motility, biofilm formation, and virulence in Pseudomonas aeruginosa. *Infect*
641 *Immun* 2007; **75**: 3780–3790.

642 62. Martín-Rodríguez AJ, Rhen M, Melican K, Richter-Dahlfors A. Nitrate Metabolism
643 Modulates Biosynthesis of Biofilm Components in Uropathogenic Escherichia coli and
644 Acts as a Fitness Factor During Experimental Urinary Tract Infection. *Front Microbiol*
645 2020; **11**: 26.

646 63. Zhu B, Ibrahim M, Cui Z, Xie G, Jin G, Kube M, et al. Multi-omics analysis of niche
647 specificity provides new insights into ecological adaptation in bacteria. *ISME J* 2016; **10**:
648 2072–2075.

649 64. Thorgersen MP, Xue J, Majumder ELW, Trotter VV, Ge X, Poole FL, et al. Deciphering
650 Microbial Metal Toxicity Responses via Random Bar Code Transposon Site Sequencing
651 and Activity-Based Metabolomics. *Appl Environ Microbiol* 2021; **87**: e0103721.

652 65. Quarles LD, Hartle JE, Middleton JP, Zhang J, Arthur JM, Raymond JR. Aluminum-
653 induced DNA synthesis in osteoblasts: mediation by a G-protein coupled cation sensing
654 mechanism. *J Cell Biochem* 1994; **56**: 106–117.

655 66. Kolodkin-Gal I, Parsek MR, Patrauchan MA. The roles of calcium signaling and calcium
656 deposition in microbial multicellularity. *Trends Microbiol* 2023; **31**: 1225–1237.

657 67. Barrientos-Moreno L, Molina-Henares MA, Ramos-González MI, Espinosa-Urgel M.
658 Arginine as an environmental and metabolic cue for cyclic diguanylate signalling and
659 biofilm formation in *Pseudomonas putida*. *Sci Rep* 2020; **10**: 13623.

660 68. Cui X, Huo M, Chen C, Yu Z, Zhou C, Li A, et al. Low concentrations of Al(III)
661 accelerate the formation of biofilm: Multiple effects of hormesis and flocculation. *Sci*
662 *Total Environ* 2018; **634**: 516–524.

663 69. Booth SC, George IFS, Zannoni D, Cappelletti M, Duggan GE, Ceri H, et al. Effect of
664 aluminium and copper on biofilm development of Pseudomonas pseudoalcaligenes
665 KF707 and P. fluorescens as a function of different media compositions. *Metallomics*
666 2013; **5**: 723–735.

667 70. Liu W, Sun Y, Shen R, Dang X, Liu X, Sui F, et al. A Chemotaxis-Like Pathway of
668 Azorhizobium caulinodans Controls Flagella-Driven Motility, Which Regulates Biofilm

669 Formation, Exopolysaccharide Biosynthesis, and Competitive Nodulation. *Mol Plant*
670 *Microbe Interact* 2018; **31**: 737–749.

671 71. Subramanian S, Kearns DB. Functional regulators of bacterial flagella. *Annu Rev*
672 *Microbiol* 2019; **73**: 225–246.

673 72. Pratt LA, Kolter R. Genetic analysis of Escherichia coli biofilm formation: roles of
674 flagella, motility, chemotaxis and type I pili. *Mol Microbiol* 1998; **30**: 285–293.

675 73. Wang F, Deng L, Huang F, Wang Z, Lu Q, Xu C. Flagellar Motility Is Critical for
676 Salmonella enterica Serovar Typhimurium Biofilm Development. *Front Microbiol* 2020;
677 **11**: 1695.

678 74. Li Y, Chen N, Wu Q, Liang X, Yuan X, Zhu Z, et al. A Flagella Hook Coding Gene flgE
679 Positively Affects Biofilm Formation and Cereulide Production in Emetic Bacillus
680 cereus. *Front Microbiol* 2022; **13**.

681 75. Clark ME, Edelmann RE, Duley ML, Wall JD, Fields MW. Biofilm formation in
682 Desulfovibrio vulgaris Hildenborough is dependent upon protein filaments. *Environ*
683 *Microbiol* 2007; **9**: 2844–2854.

684 76. Guttenplan SB, Kearns DB. Regulation of flagellar motility during biofilm formation.
685 *FEMS Microbiol Rev* 2013; **37**: 849–871.

686 77. Valentini M, Filloux A. Biofilms and Cyclic di-GMP (c-di-GMP) Signaling: Lessons
687 from Pseudomonas aeruginosa and Other Bacteria. *J Biol Chem* 2016; **291**: 12547–12555.

688 78. Floyd KA, Lee CK, Xian W, Nametalla M, Valentine A, Crair B, et al. c-di-GMP
689 modulates type IV MSHA pilus retraction and surface attachment in Vibrio cholerae. *Nat*
690 *Commun* 2020; **11**: 1549.

691 79. Hengge R. Principles of c-di-GMP signalling in bacteria. *Nat Rev Microbiol* 2009; **7**:
692 263–273.

693 80. Liu C, Sun D, Zhu J, Liu J, Liu W. The Regulation of Bacterial Biofilm Formation by
694 cAMP-CRP: A Mini-Review. *Front Microbiol* 2020; **11**: 802.

695 81. Almblad H, Rybtke M, Hendiani S, Andersen JB, Givskov M, Tolker-Nielsen T. High
696 levels of cAMP inhibit Pseudomonas aeruginosa biofilm formation through reduction of
697 the c-di-GMP content. *Microbiology (Reading, Engl)* 2019; **165**: 324–333.

698 82. Kalivoda EJ, Brothers KM, Stella NA, Schmitt MJ, Shanks RMQ. Bacterial cyclic AMP-
699 phosphodiesterase activity coordinates biofilm formation. *PLoS ONE* 2013; **8**: e71267.

700 83. Chao L, Rakshe S, Leff M, Spormann AM. PdeB, a cyclic Di-GMP-specific
701 phosphodiesterase that regulates Shewanella oneidensis MR-1 motility and biofilm
702 formation. *J Bacteriol* 2013; **195**: 3827–3833.

703 84. Alviz-Gazitua P, Fuentes-Alburquenque S, Rojas LA, Turner RJ, Guiliani N, Seeger M.
704 The Response of Cupriavidus metallidurans CH34 to Cadmium Involves Inhibition of the
705 Initiation of Biofilm Formation, Decrease in Intracellular c-di-GMP Levels, and a Novel
706 Metal Regulated Phosphodiesterase. *Front Microbiol* 2019; **10**: 1499.

85. Chua SL, Sivakumar K, Rybtke M, Yuan M, Andersen JB, Nielsen TE, et al. C-di-GMP regulates Pseudomonas aeruginosa stress response to tellurite during both planktonic and biofilm modes of growth. *Sci Rep* 2015; **5**: 10052.

86. Lee CS, Kim KK, Aslam Z, Lee S-T. Rhodanobacter thiooxydans sp. nov., isolated from a biofilm on sulfur particles used in an autotrophic denitrification process. *Int J Syst Evol Microbiol* 2007; **57**: 1775–1779.

87. Zhang W, Wang Y, Bougouffa S, Tian R, Cao H, Li Y, et al. Synchronized dynamics of bacterial niche-specific functions during biofilm development in a cold seep brine pool. *Environ Microbiol* 2015; **17**: 4089–4104.

88. Mann EE, Wozniak DJ. Pseudomonas biofilm matrix composition and niche biology. *FEMS Microbiol Rev* 2012; **36**: 893–916.

89. Silverman JM, Brunet YR, Cascales E, Mougous JD. Structure and regulation of the type VI secretion system. *Annu Rev Microbiol* 2012; **66**: 453–472.

90. Gallique M, Decoin V, Barbey C, Rosay T, Feuilloley MGJ, Orange N, et al. Contribution of the Pseudomonas fluorescens MFE01 Type VI Secretion System to Biofilm Formation. *PLoS ONE* 2017; **12**: e0170770.

91. Fei N, Ji W, Yang L, Yu C, Qiao P, Yan J, et al. Hcp of the Type VI Secretion System (T6SS) in Acidovorax citrulli Group II Strain Aac5 Has a Dual Role as a Core Structural Protein and an Effector Protein in Colonization, Growth Ability, Competition, Biofilm Formation, and Ferric Iron Absorption. *Int J Mol Sci* 2022; **23**.

92. Chen L, Zou Y, Kronfl AA, Wu Y. Type VI secretion system of Pseudomonas aeruginosa is associated with biofilm formation but not environmental adaptation. *Microbiologyopen* 2020; **9**: e991.

93. Yang X, Liu H, Zhang Y, Shen X. Roles of type VI secretion system in transport of metal ions. *Front Microbiol* 2021; **12**: 756136.

94. Letunic I, Bork P. Interactive Tree of Life (iTOL) v6: recent updates to the phylogenetic tree display and annotation tool. *Nucleic Acids Res* 2024; **52**: W78–W82.

Figure and Table Captions

Figure 1: Heatmap results highlighting the biofilm growth capabilities of 16 *Rhodanobacter* isolates under varying pH conditions (ranging from pH 4 to pH 7) and nitrate conditions (ranging from 0 mM to 300 mM), carried out across two different media (SGW, NLDM). (A): Nitrate, SGW, (B): Nitrate, NLDM, (C): pH, SGW, (D): pH, NLDM. The OD550 values are calculated from the mean values observed in the corresponding isolates for each medium. The row is clustered using euclidean distance in between the values measured from each sample.

Figure 2: Heatmap showing biofilm growth capacities of eight selected *Rhodanobacter* strains under various metal stress conditions (aluminum, cobalt, copper, manganese, nickel, zinc). Biofilm quantification was performed using crystal violet staining, with readings taken at OD550. The mean value of the triplicates for individual isolates under certain metal stress was calculated against the "positive control" (no stress condition) to get the log2FC values shown in the heatmap.

Figure 3: TEM images of *Rhodanobacter* (A-D) FW104-10B01 and (E) FW021-MT20 cultured in synthetic groundwater media supplemented with 1 mM Aluminum Chloride (B-D), and in the

absence of any metal additions (A, E). Key features observed include (B) loss of pili and/or flagellar, (C) blebbing, cell morphology change and (D) particle adsorption for FW104-10B01 strains under aluminum stress conditions, and the lack of flagella for FW021-MT20 strains under normal conditions. Scale bar: 1 μm.

Figure 4: Phylogenetic relationship of the 16 *Rhodanobacter* strains analyzed in this study. (A) The 16 Rhodanobacter genomes (highlighted in red color) were compared with a set of related, publicly available genomes (in black). Tree scale: 0.1. The phylogenetic tree was made from Interactive Tree of Life (iTOL) v6 [94]. (B) A heatmap of ANI (green) and AAI (pink) values of the 16 Rhodanobacter genomes. The cluster was calculated using the *hclust* function in R.

Figure 5: Pangenome of *Rhodanobacter*. The 16 *Rhodanobacter* genomes obtained in this study are shown in orange, and the other 48 publicly available genomes are shown in blue. Core genes are genes present in all 64 genomes, soft core genes are genes present in 61 to 63 genomes, cloud genes are genes present in 1 to 3 genomes, and the remaining genes are shell genes. Hierarchical clustering was performed on the ANI index calculated from fastANI. SCG, single-copy genes.

Table 1: Geochemistry information of ORR wells where sixteen *Rhodanobacter* were isolated from.

Table 2: Genes with large fitness changes ($|\log2| \geq 1.5$) with and without 1 mM Al stress. The Δfitness is the difference between the average gene fitness value (of biological triplicate samples) of the biofilm fraction to the planktonic fraction for each gene.