Electronic Supplementary Material (ESI) for Metallomics.

Supporting Information

for

TiO_2 exposure alters transition metal ion quota in *Rhodococcus ruber* GIN-1

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Rhodococcus ruber GIN-1 Growth Conditions

Reagents were sourced from Sigma-Aldrich (Tris base (≥ 99.0%), $MgSO_4$ (≥ 99.0% Reagent Plus)), JT Baker (K_2HPO_4 (Certified ACS Fisher Chemical), KH_2PO_4 (Certified ACS Fisher Chemical), and sucrose (Baker Analyzed ACS Reagent Grade)), American Bioanalytical ($(NH_4)_2SO_4$ (ACS Reagent Grade) and yeast extract (Ultra Pure)), Fisher Scientific (dextrose (>99.0% anhydrous for molecular biology) and Marine Enterprises International (Crystal Sea Marinemix artificial seawater). *Rhodococcus ruber* GIN-1 cells were obtained from the National Collections of Industrial and Marine Bacteria (NCIMB) in Aberdeen, Scotland, where GIN-1 is deposited under No. 40340.

Procedures for cell growth followed published protocols.[1,2] Cells were grown on LB agar plates at 30°C for one week. A single orange-colored colony was grown in 10 mL artificial sea water media (29.9 g L^{-1} Crystal Sea, 1.68 g L^{-1} K_2HPO_4, 0.72 g L^{-1} KH_2PO_4,

1 g L^{-1} $(NH_4)_2SO_4$, 10 g L^{-1} dextrose, 8 g L^{-1} yeast extract) overnight at 30 °C and 225 rpm. A 100 mL portion of the same media was inoculated with the 10 mL overnight culture and returned to shaking incubator for 24 h. This culture was used to inoculate a 1 L growth in the same media. Cells grew for another 24 h, with growth monitored by the optical density at 660 nm. Cells at OD_{660} ~1.5 were harvested by centrifugation and resuspended in buffer containing 5 mM Tris, 2.5 mM $MgSO_4$, and 20% sucrose.[1]

Titanium exposure

Reagents were sourced from JT Baker (NaCl (Certified ACS Fisher Chemical), KCl (Certified ACS Fisher Chemical), Na_2HPO_4 (Certified ACS Fisher Chemical), and KH_2PO_4 (Certified ACS Fisher Chemical)), Fisher Scientific (urea (99%) and nitric acid (TraceMetal Grade)), and Sigma-Aldrich (sodium dodecyl sulfate (98.5% electrophoresis grade). Sachtopore TiO_2 particles (~40 μM particle size, anatase or rutile) were a gift from Huntsman (Duisburg, Germany).

Procedures for TiO_2 absorption and desorption followed published protocols.[1,2] The 1 L growth was divided into two portions comprising exposed cells and control cells. Both portions were treated in the same manner, with the only difference being that the exposed cells had 1 g of Sachtopore TiO_2 particles (anatase, rutile, or a 1:1 mixture of anatase and rutile) added. The cells were incubated at 30 °C and 225 rpm for 1 h.

To separate the bacteria from the oxide particles, 8 M urea and 1% sodium dodecyl sulfate were added and the suspension was incubated for an additional 30 min. The cells were centrifuged 30 min at 19 x g to remove the TiO_2 particles, and the cells were

transferred to another centrifuge bottle, where they were collected by centrifugation at 24,000 x g, at 4 °C for 20 min. The cells were washed with phosphate-buffered saline solution and centrifuged several times and then were lyophilized. The lyophilized cells were refluxed in 50% nitric acid for 6 h to digest all of the organic matter. Experiments were carried out in four (mixed anatase and rutile) or five (anatase and rutile individually) sequential trials.

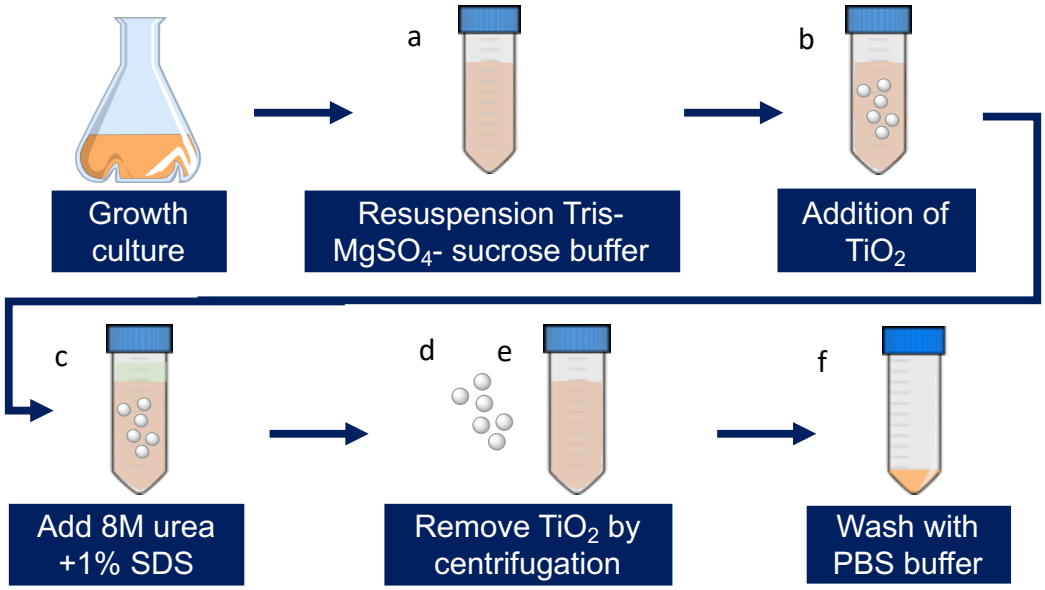

Figure S1. Overview of the exposure of *Rhodococcus ruber* GIN-1 cells to, and subsequent desorption from, TiO_2 particles. The letters correspond to the samples imaged by SEM in Figure S2.

SEM images

Samples were prepared for SEM analysis by pipetting 50 μL of the suspension onto 12 mm carbon tabs (SPI Supplies) that were attached to an aluminum specimen mount, ½ inch slotted head (Ted Pella, Inc.). SEM images were obtained with an FEI quanta FEG450 scanning electron microscope with an acceleration voltage of 10.00 kV and a spot size of 3.0, equipped with an Everhart Thornley Detector (ETD). Horizontal field widths are a) 103 μm; b) 136 μm; c) 95.3 μm; d) 6.5 mm; e) 6.8 mm; f(top)) 6.66 mm; f(bottom)) 88.8 μm.

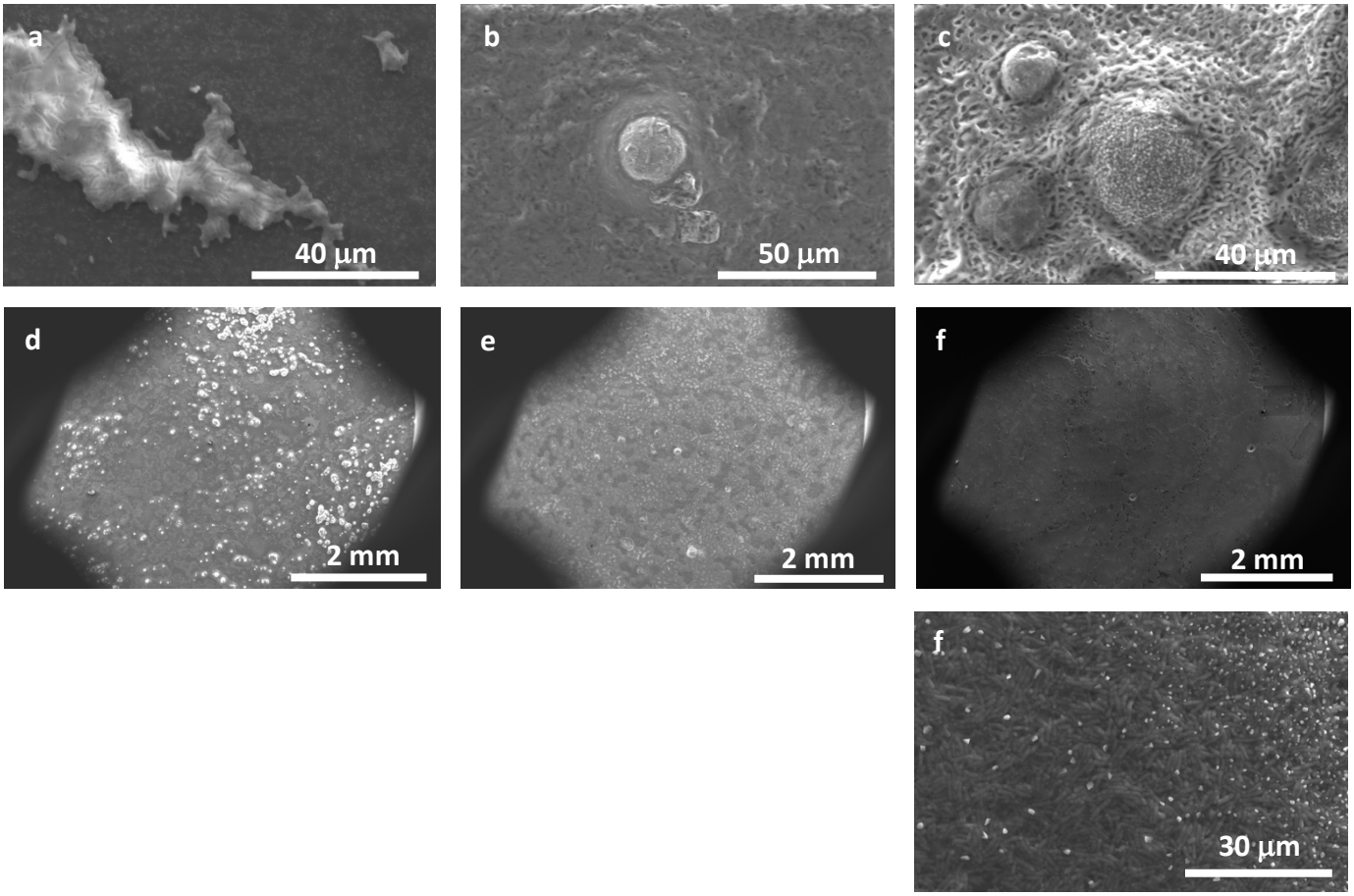

Figure S2. Scanning electron micrographs of samples corresponding to the stages of treatment shown in Figure S1. Sample a) shows cells in the absence of TiO_2 particles. Sample b) shows a single TiO_2 particle with adhesive bacteria. Sample c) shows particles and bacteria after 30 min urea/SDS treatment but before separation. Sample d) shows the TiO_2 particles and e) the cells after slow centrifugation and separation. Sample f) shows the washed cells before metal quantitation. The particulates in sample f) are consistent with salt crystals from the PBS buffer.

ICP-OES

Reagents were sourced from Fisher Scientific (trace metal grade nitric acid), Sigma-Aldrich (titanium atomic absorption standard solution), High-Purity Standards (zinc atomic absorption standard solution, Ricca Chemical Company (iron atomic absorption standard solution), and Thermo-Scientific (iCAP 6000 multi-element test solution).

Inductively coupled plasma optical emission spectroscopy was done on a Thermo-Scientific iCAP 7400-ASX520 inductively coupled plasma optical emission spectrometer operating in axial mode. Wavelengths (nm) were used for each metal were: aluminum, 396.152; copper, 324.754; iron, 238.204; manganese, 257.610; titanium, 336.121; zinc, 213.856. Values were routinely confirmed at a second wavelength to guard against interference.

The standard curves were matrix-matched to the samples. Undiluted samples had 50% nitric acid, so the corresponding standard curve had 50% nitric acid. Typical samples were diluted 1:10 and 1:100, with 5% nitric acid, which was matrix matched with its standard curve. Trace metal grade nitric acid was used for all samples. The standard curve samples contained Ti AA standard solution, Zn AA standard solution, Fe AA standard solution, and iCAP 6000 multi-element test solution.

Table S1. Average concentrations of various metals in cells exposed to titanium dioxide determined by ICP-OES. Averages and standard errors of means were over 14 individual cell growths.

	Artificial seawater medium (mg L^{-1})	Control cells (mg kg^{-1})	Exposed cells (mg kg^{-1})
Ti	[a]	0.29 ± 0.05	2.2 ± 0.2
Fe	0.045 ± 0.001	20 ± 2	12 ± 1
Zn	0.22 ± 0.03	50 ± 2	40 ± 10
Mn	0.009 ± 0.002	1.8 ± 0.1	1.51 ± 0.06
Cu	[a]	1.8 ± 0.2	1.8 ± 0.2
Al	[a]	7 ± 1	8 ± 2

[a] Below standard curve, < 0.005 mg L^{-1}.

Statistical analysis

Given that 14 Ti-exposed cell growths and 14 controls were run in parallel, we first performed a basic nonparametric analysis treating each pair of Ti-exposed and unexposed as a pair. This nonparametric analysis eliminates the need to assume that the population is normally distributed. To do this, we performed a **Wilcoxon signed-rank test** to test the null hypothesis that the Ti-exposed cells were statistically the same as the Ti-unexposed cells with respect to their content of each of the 6 metals described in the paper. The P-value represents the probability of falsely rejecting the null hypothesis when the null hypothesis is true in the population. To address the issue of making multiple comparisons, we can adjust the P value for the total number of comparisons using Bonferroni correction. We used 7 comparisons: titanium (diluted), titanium (undiluted), Fe, Zn, Mn, Cu, Al. The z-score indicates the direction of the effect. We used Stata 15.1 for the analysis. This treatment yielded the following results:

Code:
bys metal: signrank exposed_metal_value=control_metal_value

Results of **Wilcoxon signed-rank test:**

Metal	**z-score**	**Uncorrected P value**	**P value corrected for 7 comparisons**
titanium (diluted)	3.296	.0010	.0070
titanium(undiluted)	3.296	.0010	.0070
Fe	-3.296	.0010	.0070
Zn	-3.170	.0015	.0105
Mn	-2.856	.0043	.0301
Cu	-0.471	.6378	>.9999
Al	0.031	.9750	>.9999

We performed an additional analysis that allowed us to model the effect sizes for each metal using a logarithmic function as well as preserve the integrity of the pairings,

and Bonferroni-correct the P values for multiple comparisons. We did this using individual **mixed-effects generalized linear models** for each metal, with a random intercept for the "run ID" which was the same ID number for each control-exposed pair (i.e. in this analysis every metal had 28 rows, 14 control and 14 exposed, and the 14 run IDs indicated the paired control and exposed readings). This random intercept approach allowed the baseline control value to vary across runs. The model used a Gaussian distribution with a logarithmic link function and an unstructured covariance matrix. We then obtained predictive margins and contrasts of marginal predictions from the model. We manually Bonferroni-corrected the P values for 7 comparisons. We used Stata 15.1 for the analysis.

Code (run separately for each measured metal):
meglm mg L^{-1} i.exposed ll run_id: , cov(uns) family(gaussian) link(log) intpoints(15)
margins i.exposed
margins r.exposed

Results of **mixed-effects generalized linear models:**

Metal	**Modeled control value**	**Modeled exposed value**	**Modeled contrast**	**Uncorrected P value for contrast**	**P value corrected for 7 comparisons**
titanium (diluted)	0.2250	1.8463	+1.6214	<.0001	<.001
titanium(undiluted)	0.2400	2.2038	+1.9638	<.0001	<.001
Fe	20.1390	11.5065	-8.6324	<.0001	<.001
Zn	49.8306	38.2331	-11.5975	<.0001	<.001
Mn	1.7825	1.5058	-0.2766	<.0001	<.001
Cu	1.8261	1.7678	-0.0583	.6225	>.9999
Al	6.5451	8.6804	2.1353	.2081	>.9999

References

1. Y. Shabtai and G. Fleminger, *Appl. Environ. Microbiol.*, 1994, **60**, 3079-3088.
2. G. Gertler, I. Brudo, R. Kenig and G. Fleminger, *Materialwiss. Werkstofftech.*, 2003, **34**, 1138-1144.