

Rapid Metabolism of 1,4-Dioxane to below Health Advisory Levels by Thiamine-Amended *Rhodococcus ruber* Strain 219

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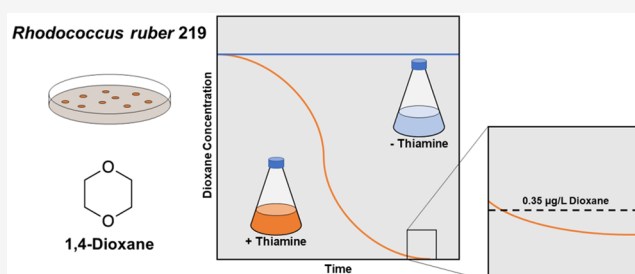


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Supporting Information

ABSTRACT: Bioremediation is a promising treatment technology for 1,4-dioxane-contaminated groundwater. However, metabolic dioxane-degrading bacteria identified to date are limited by their slow kinetics and inability to sustain growth at low dioxane concentrations ($<100 \mu\text{g/L}$). Furthermore, strains may underperform because of missing growth factors, such as amino acids or vitamins. In this work, we reevaluate *Rhodococcus ruber* strain 219 as a dioxane-degrading strain with bioaugmentation potential. We report rapid growth and metabolic dioxane degradation by *R. ruber* 219 when supplemented with thiamine (vitamin B1). We also discern that the strain lacks a complete *de novo* thiamine synthesis pathway, indicating that *R. ruber* 219 is a probable thiamine auxotroph. However, when supplemented with thiamine, the strain's Monod kinetics ($K_s = 0.015 \pm 0.03 \mu\text{g/L}$) and exceedingly low S_{\min} ($0.49 \pm 1.16 \mu\text{g/L}$) suggest this strain can maintain growth at very low dioxane concentrations ($<100 \mu\text{g/L}$). Accordingly, we demonstrate that thiamine-grown *R. ruber* 219 sustains degradation of dilute dioxane ($<100 \mu\text{g/L}$) to below health advisory levels. This is the first study to report sustained metabolic dioxane biodegradation to below health advisory levels of $0.35 \mu\text{g/L}$. Overall, our findings solidify *R. ruber* 219 as a promising candidate for bioremediation of dioxane-contaminated groundwater.



INTRODUCTION

1,4-Dioxane (dioxane) is a probable human carcinogen and prevalent groundwater pollutant.^{1,2} Because of its high mobility in water, dioxane plumes are often large and dilute ($<100 \mu\text{g/L}$).^{3,4} On the basis of a one-in-one million cancer risk assessment, the US EPA has established a Health Reference Level (HRL) for dioxane of $0.35 \mu\text{g/L}$.⁵ Treatment of dilute dioxane plumes to this low concentration presents a significant challenge and financial burden for many stakeholders. Because of high capital and operating costs, aggressive *ex situ* technologies (e.g., pump and treat, advanced oxidation) are poorly suited for large dilute plumes.^{6,7} Therefore, ongoing research has aimed to develop low-cost *in situ* technologies for dioxane-contaminated groundwater.

Bioremediation is a promising treatment strategy for dilute dioxane plumes. In recent years, a number of metabolic^{8–16} and cometabolic^{17–24} dioxane-degrading microorganisms have been isolated and characterized. However, these strains have significant limitations that may hinder their bioremediation efficacy. For example, metabolic strains isolated to date are limited by their slow kinetics and inability to sustain growth at low dioxane concentrations ($<100 \mu\text{g/L}$) commonly found at contaminated sites.^{4,25} Specifically, relatively high half-saturation Monod constants (K_s) and minimum dioxane concentrations required for growth prevent metabolic strains from degrading dioxane to meet proposed drinking water stand-

ards.^{26,27} In contrast, cometabolic strains can degrade dioxane to very low concentrations provided sufficient primary substrate is present.^{26,28} However, these primary substrates can be relatively expensive or potentially harmful to humans (e.g., tetrahydrofuran).²⁹ Because of these limitations, there is a need to identify metabolic dioxane-degrading bacteria that thrive in dilute dioxane conditions.

Identification and isolation of novel dioxane-degraders remain incremental and often fail to account for many factors influencing culturability or performance. For example, minimal microbial media commonly used during enrichment often lack various required growth factors (i.e., amino acids or vitamins).¹¹ To obtain these growth factors, bacteria may salvage them from the environment or form syntrophic relationships with the commensal microbial community.^{30,31} Identifying these growth factors represents a challenge that limits attempts to enrich and isolate novel dioxane-degrading strains. Furthermore, previously identified dioxane-degrading bacteria may underperform in lab-scale experiments because of missing growth factors.

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In this work, we reevaluate *Rhodococcus ruber* strain 219 as a dioxane-degrading candidate strain. *R. ruber* 219 was initially reported as a slow dioxane metabolizer⁸ and later as capable only of dioxane cometabolism.³² Because of issues replicating metabolic growth on minimal media, we hypothesized that *R. ruber* 219 requires an essential growth factor to metabolize dioxane. Through screening tests, we identified thiamine (vitamin B1) as a limiting nutrient for strain 219. We report rapid growth and metabolic dioxane degradation by *R. ruber* 219 in minimal media supplemented with thiamine. In addition, through bioinformatics analyses, we reveal that the strain possesses an incomplete thiamine synthesis pathway. Finally, Monod model data and semibatch experiments indicate the strain can maintain degradation in dilute dioxane concentrations ($\sim 100 \mu\text{g/L}$) and degrade dioxane to below the HRL ($< 0.35 \mu\text{g/L}$). These findings suggest *R. ruber* 219 is a promising bioaugmentation candidate for dioxane-contaminated groundwater.

MATERIALS AND METHODS

Chemicals. ACS grade ($\geq 97\%$) 1,4-dioxane (anhydrous), biotin, calcium pantothenate, folic acid, nicotinic acid, *p*-aminobenzoic acid, pyridoxine hydrochloride, riboflavin, thiamine hydrochloride, thiocetic acid, and vitamin B12 were purchased from MilliporeSigma (Burlington, MA). Analytical standards (2000 $\mu\text{g/mL}$ in methanol) for 1,4-dioxane and 1,4-dioxane-d8 were purchased from Restek Corporation (Bellefonte, PA).

Strain Cultivation. *Rhodococcus ruber* 219 (DSM #44190) was purchased from DSMZ (Braunschweig, Germany). Initial cultures grew readily on R2A media.³³ Contrary to previous reports,⁸ no growth was observed on ammonium mineral salt (AMS)⁹ cultures amended with 500 mg/L dioxane as the sole carbon source. However, rapid growth and dioxane degradation occurred when the AMS was supplemented with MD-VS vitamins (ATCC; Manassas, VA). Therefore, subsequent cultures were pregrown in AMSV (AMS with vitamins supplements, Table S1). All cultures were maintained on 500 mg/L dioxane and incubated at 30 °C in an orbital shaker set to 150 rpm.

Growth/Depletion Curve Experiments in Vitamin Mixtures. To identify the limiting cofactor for *R. ruber* 219, a screening test was conducted in 50 mL centrifuge tubes. AMS liquid cultures (10 mL) were amended with 500 mg/L dioxane and a single vitamin component from the ATCC formulation. Screening tests were initiated by inoculating tubes with 100 μL of active *R. ruber* 219 culture. After 7 days, growth was observed only in tubes amended with thiamine.

To confirm thiamine is a limiting cofactor for *R. ruber* 219, a growth/depletion curve experiment was conducted in batch reactors (100 mL liquid culture in 500 mL Erlenmeyer flasks). Culture volume was limited to 20% of the total volume to ensure aerobic conditions. Treatments included AMSV, AMS with thiamine (50 $\mu\text{g/L}$, per ATCC formulation), AMS with all vitamins except thiamine, and AMS without vitamins. All treatments had a starting dioxane concentration of 500 mg/L and were conducted in triplicate.

R. ruber 219 was harvested in exponential phase and washed three times with 20 mM phosphate buffer (pH 6.8). Reactors were inoculated with 1 mL (1% total culture volume) of washed cells. Samples (3 mL) were taken regularly *via* serological pipet, sterile filtered (0.2 μm), and analyzed for dioxane (detailed below) and biomass as protein, as previously described.³⁴

Following the experiment, the culture yield was calculated from the change in biomass per unit dioxane consumed during exponential growth in treatment reactors.

Genomic Sequencing and Bioinformatic Analyses. To determine if *R. ruber* 219 possesses the molecular machinery to synthesize thiamine *de novo*, the strain's genome was sequenced with both long (Oxford Nanopore Technology, ONT) and short (Illumina) read technology, assembled, and annotated. ONT sequencing was conducted using a MinION sequencer with a Flongle adapter. Illumina sequencing was performed by Novogene (Sacramento, CA) using an Illumina Novaseq 6000 (paired-end, 150 bp). A hybrid assembly of the *R. ruber* genome was performed with Unicycler, and the assembly quality was examined with Quast.^{35,36} Pathways of interest were visualized with the KEGG Mapper Reconstruct tool.³⁷ Detailed descriptions of sequencing and bioinformatics are discussed in the Supporting Information.

Kinetics. Monod's maximum specific dioxane degradation rate and half-velocity constant were evaluated using resting cell experiments to maintain constant biomass. Cells were harvested in the exponential phase and washed three times with 20 mM phosphate buffer. Washed cells were diluted to an initial optical density (OD_{600}) of 1.0 ($8.4 \pm 0.60 \text{ mg/L}$ protein). The experiment was initiated by adding 100 mL of washed cells to triplicate 2 L flasks containing 900 mL of 20 mM phosphate buffer with an initial dioxane concentration of 5 mg/L. Dioxane samples (30 mL) were taken at regular intervals over 5 h and analyzed as described below. Monod kinetics were modeled for each replicate using Aquasim 2.1, as previously described.²⁶ Final model parameters were averaged between triplicate bottles. The S_{min} was then calculated as described by McCarty *et al.* (1981).³⁸ A Monte Carlo analysis was used to estimate uncertainty in the S_{min} calculation using Microsoft Excel v2103 (Redmond, WA).³⁹ Further details on kinetic models and S_{min} calculations are discussed in the Supporting Information.

Semibatch Experiment. To confirm that *R. ruber* 219 sustains degradation of dilute dioxane ($< 100 \mu\text{g/L}$) to health advisory levels ($< 0.35 \mu\text{g/L}$), a semibatch experiment was conducted in quadruplicate Erlenmeyer flasks (2 L). *R. ruber* cells were harvested in the exponential phase and washed three times with 20 mM phosphate buffer. Washed cells (100 mL) were added to 900 mL of AMSV media with 100 $\mu\text{g/L}$ initial dioxane. The initial culture biomass was approximately $1.94 \pm 0.28 \text{ mg/L}$ protein ($\text{OD}_{600} = 0.0086 \pm 0.0008$). Samples (25–30 mL) were taken by serological pipet, sterile filtered (0.22 μm), and analyzed for dioxane. Once the dioxane concentration fell below 1 $\mu\text{g/L}$, the cultures were redosed with $\sim 100 \mu\text{g/L}$ dioxane from a 100 mg/L aqueous stock, and OD_{600} was measured. Protein was also measured at the final time point.

Analytical Procedures. Dioxane samples were extracted using a Teledyne Tekmar AQUATek 100 autosampler and Lumin purge and trap concentrator (Teledyne Tekmar, Mason, OH). 1,4-Dioxane-d8 (2 μL) was added to each 5 mL sample as an internal standard from a methanol stock by the autosampler. Extracted samples were analyzed by GC-MS/MS (Agilent Intuvo 9000 GC with an Agilent 7000C MS Triple Quad). The method detection limit (MDL) for dioxane was 0.047 $\mu\text{g/L}$. Complete purge and trap GC-MS/MS settings MDL calculations are provided in the Supporting Information.

Statistical Analyses. Statistical significance between treatments was assessed using a Student's *t*-test (paired, two-tailed, $\alpha = 0.05$). Analyses were completed using GraphPad Prism 8.3.0 software (San Diego, CA).

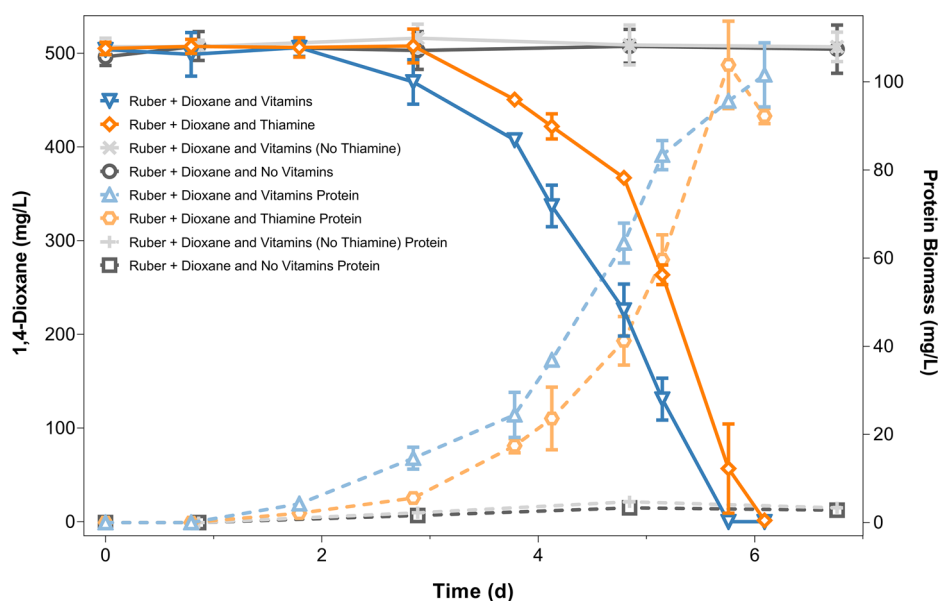


Figure 1. *R. ruber* 219 growth and dioxane depletion curve conducted in AMS minimal media with and without the addition of vitamin mixtures. Both growth and dioxane consumption in the treatments amended with thiamine-only closely mirrored the treatments amended with all vitamins. No growth was observed in treatments lacking thiamine. Error bars represent ± 1 standard deviation among triplicate reactors.

Table 1. Kinetic Parameters of Select Metabolic Dioxane-Degrading Bacteria^a

Strain	q_{\max} (mg dioxane/ mg protein/day)	K_s (mg/L dioxane)	Yield (mg protein/ mg dioxane)	S_{\min} (μ g/L dioxane)	Reference
<i>Pseudonocardia dioxanivorans</i> CB1190	1.65 ± 0.05	6.32 ± 0.22	0.45 ± 0.09	487.14 ± 173.45^b	Barajas-Rodriguez <i>et al.</i> (2018) ²⁶
<i>Pseudonocardia dioxanivorans</i> CB1190	26.40 ± 0.19	160.00 ± 44.00	0.09 ± 0.002	3389.35 ± 1200.15^b	Mahendra <i>et al.</i> (2006) ⁴⁶
<i>Mycobacterium dioxanotrophicus</i> PH-06	Not Reported	78.00 ± 10.00	0.16	Not Reported	He <i>et al.</i> (2017) ⁴⁷
<i>Rhodococcus ruber</i> 219	5.00 ± 0.24^c	0.015 ± 0.03^c	0.24 ± 0.02^c	0.49 ± 1.16^b	This Work

^aParameters calculated using Aquasim 2.1. Final Monod model χ^2 values averaged 0.01 ± 0.006 for triplicate reactors. ^b S_{\min} uncertainty estimated using Monte Carlo analysis ($n = 500$). ^cError-values for K_s , q_{\max} , and yield values calculated for *R. ruber* 219 in this work are the standard deviations for triplicate reactors.

RESULTS AND DISCUSSION

Thiamine Facilitates the Rapid Metabolism of Dioxane by *R. ruber* 219. Previous work reports *R. ruber* 219 as either a slow metabolizer⁸ or cometabolizer³² of 1,4-dioxane. However, neither growth nor dioxane removal was observed in cultures without vitamins, or in cultures amended with vitamins but lacking thiamine after 7 days (Figure 1) and 34 days (Figure S1). In contrast, the strain rapidly grew and metabolized dioxane when supplemented with vitamins. Cultures containing only thiamine mirrored both the full MD-VS vitamin mixture's growth and depletion rates. We observed a statistically significant improvement in the growth rate with the full vitamin mixture (p -value = 0.035). Yield values averaged 0.24 ± 0.02 mg-protein per mg-dioxane for the full vitamin mixture and 0.18 ± 0.03 mg-protein per mg-dioxane for thiamine-only reactors.

This experiment confirms that thiamine is the primary limiting cofactor for dioxane metabolism by *R. ruber* 219. Thiamine pyrophosphate, a thiamine derivative, is a known essential growth factor for the metabolism of amino acids and carbohydrates in microorganisms.⁴⁰ This finding also agrees with previous research that identified thiamine auxotrophy as a common trait among *Rhodococcus* spp.^{41,42}

***R. ruber* 219 Possesses an Incomplete Thiamine-Synthesis Pathway.** Annotation of the *R. ruber* 219 genome

assembly revealed the strain lacks the genes that encode molecular machinery to synthesize thiamine *de novo*. Specifically, *R. ruber* 219 lacks *thiC*, *thiL*, *thiF*, and *thiH* (Figure S2). However, *R. ruber* 219 possesses a complete thiamine salvaging pathway, including genes encoding for *thiE*, *thiD*, and *thiM* (Figure S3). The genomic context of these genes is detailed in Figure S4. Additionally, *R. ruber* 219 contains the *tenA* gene previously identified as required to salvage the 2-methyl-4-amino-5-hydroxymethylpyrimidine (HMP) component of thiamine from soil.⁴³ The ability to salvage HMP may allow *R. ruber* 219 to overcome the absence of *thiC*, a gene required to synthesize HMP *de novo*.⁴⁴ *R. ruber* 219 possesses the same thiamine salvaging genes as *Rhodococcus ruber* ZM07, a tetrahydrofuran-degrading strain and confirmed thiamine auxotroph.⁴⁵ Both strains also lack the genes required for thiamine *de novo* synthesis, suggesting that *R. ruber* 219 is also a probable thiamine auxotroph.⁴⁵

Biokinetic Parameters Suggest Thiamine-Grown *R. ruber* 219 Thrives at Low Dioxane Concentrations.

Resting cell dioxane-degradation experiments (Figure S4) were used to calculate Monod kinetic parameters (Table 1 and Figure S5) for thiamine-grown *R. ruber* 219. The half-saturation constant, K_s , for *R. ruber* 219 was calculated as 0.015 ± 0.03 mg/L dioxane (Table 1), the lowest reported K_s of any metabolic dioxane-degrader to date, at 2–4 orders of magnitude

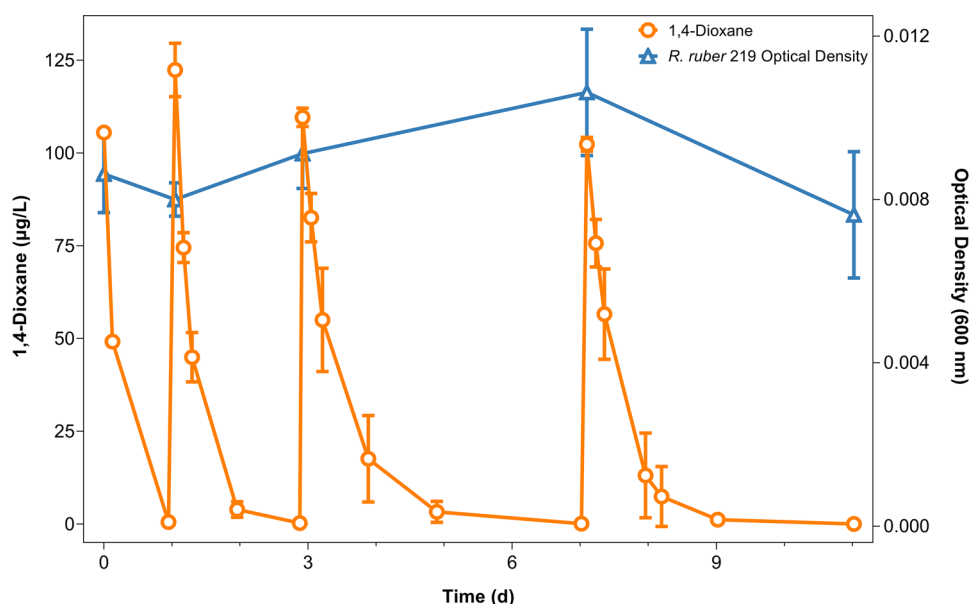


Figure 2. Degradation of dioxane by *R. ruber* 219 in semibatch experiments. Despite a low initial dioxane concentration ($\sim 100 \mu\text{g/L}$) and biomass ($\text{OD}_{600} = 0.0086 \pm 0.0008$), *R. ruber* 219 repeatedly degraded dioxane to $< 1 \mu\text{g/L}$ and below the MDL of $0.047 \mu\text{g/L}$ by day 11. The culture optical density remained stable ($0.0075\text{--}0.012$). Error bars represent standard deviations among quadruplicate reactors.

lower than archetype dioxane-degrader CB1190. Furthermore, the maximum substrate utilization rate (q_{max}) for *R. ruber* 219 was within the same order of magnitude as CB1190, per Barajas-Rodriguez *et al.* (2018).²⁶ However, estimation of biokinetic parameters is sensitive to the experimental conditions and modeling methodology, which can result in significant discrepancies in the reported values for a single organism, as seen with the q_{max} and K_s estimates reported for CB1190 (Table 1).²⁶ The kinetic experiments and parameter estimations for *R. ruber* 219 closely mirror those used by Barajas-Rodriguez and Freedman (2018) for CB1190.²⁸ Additionally, we estimate that the minimum dioxane concentration required for growth (S_{min}) for *R. ruber* 219 is exceedingly low ($0.49 \pm 1.16 \mu\text{g/L}$), multiple orders magnitude lower than CB1190 (Table 1). These findings suggest that *R. ruber* 219 continually grows and degrades dioxane despite low dioxane concentrations in groundwater plumes ($< 100 \mu\text{g/L}$), unlike CB1190 and PH-06, which may stall at low dioxane concentrations.

Thiamine-Grown *R. ruber* 219 Repeatedly Degrades Dioxane to below Health Advisory Levels. A semibatch experiment was conducted to evaluate *R. ruber* 219's ability to degrade low initial dioxane concentrations ($\sim 100 \mu\text{g/L}$) to $< 0.35 \mu\text{g/L}$ (Figure 2). Thiamine-grown *R. ruber* 219 repeatedly degraded $\sim 100 \mu\text{g/L}$ dioxane to below $1 \mu\text{g/L}$ over 11 days. Furthermore, *R. ruber* 219 degraded dioxane to 0.099 ± 0.12 on day 7 and below the MDL of $0.047 \mu\text{g/L}$ on day 11. This was despite a low initial biomass concentration of $1.94 \pm 0.28 \text{ mg/L}$ protein ($\text{OD}_{600} = 0.0086 \pm 0.0008$). The culture biomass remained stable, ranging from an optical density of 0.0075 to 0.012. Apparent growth was observed at day 7 ($\text{OD}_{600} = 0.011 \pm 0.001$), but this measurement was not significantly higher than at day 0 (p -value = 0.053). The final culture biomass was measured at $0.84 \pm 0.14 \text{ mg/L}$ protein, indicating decay occurred, likely because of starvation after day 9. However, no significant change in optical density was observed between day 0 and day 11 ($\text{OD}_{600} = 0.008 \pm 0.001$, p -value = 0.463). Nevertheless, this experiment confirmed that thiamine-grown *R. ruber* 219 can

sustain degradation in dilute dioxane concentrations ($\sim 100 \mu\text{g/L}$) and degrade dioxane to $< 0.35 \mu\text{g/L}$.

Technical Implications. This study is the first to report sustained metabolic dioxane biodegradation to $< 0.35 \mu\text{g/L}$. Achieving this low concentration is critical for treating dilute dioxane plumes. Thiamine-grown *R. ruber* 219's Monod kinetics ($K_s = 0.015 \pm 0.03 \mu\text{g/L}$), exceeding low S_{min} ($0.49 \pm 1.16 \mu\text{g/L}$), and sustained degradation of low dioxane concentrations ($< 100 \mu\text{g/L}$) to $< 0.35 \mu\text{g/L}$ make the strain a promising candidate for bioaugmentation at dioxane contaminated sites. While promising, further field testing is needed to examine *R. ruber* 219's performance under *in situ* conditions (*i.e.*, extended time frames with low dioxane, oxygen, and temperature). Finally, further testing is needed to adjust thiamine dosage to field-scale applications. Potential losses of thiamine due to adsorption or uptake by nontargeted organisms will be important considerations. However, because *R. ruber* 219 possesses a complete thiamine salvaging pathway (Figure S4), thiamine supplementation may not be necessary in the field.

Identifying *R. ruber* 219 as a probable thiamine auxotroph underscores the need to explore growth supplements when isolating and characterizing contaminant-degrading strains. The reliance on minimal media likely caused *R. ruber* 219 to be overlooked, as may be the case with other potentially valuable organisms. Furthermore, our findings raise the possibility that *in situ* biostimulation with growth supplements might facilitate the biodegradation of dioxane as well as other contaminants to health advisory levels.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.estlett.1c00714>.

Experimental procedures and results, including the vitamin supplement formula (Section S1 and Table S1), *R. ruber* 219 genome sequencing and assembly (Section S2), kinetic model development (Sections S3 and S4 and Table S2), analytical methods (Section S5 and Tables

S3–S5), *R. ruber* 219 growth and depletion curve after 34 days (Section S6 and Figure S1), *R. ruber* 219 KEGG metabolic pathways (Sections S7 and Figures S2–S4), and Monod kinetic models (Section S8 and Figure S5 and S6). (PDF)

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Notes

The authors declare no competing financial interest.

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