Environmental Science & Technology

pubs.acs.org/est

Article

Small Interfering RNAs (siRNAs) Negatively Impact Growth and Gene Expression of Environmentally Relevant Bacteria in *In Vitro* Conditions

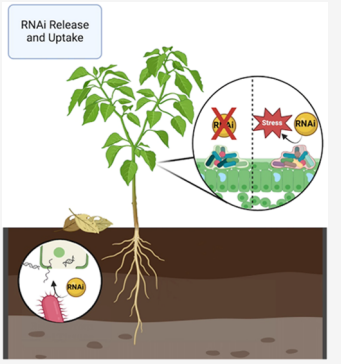
S. Un Jan Contreras, L. K. Redfern, L. W. Maguire, S. I. Promi, and C. M. Gardner*

Cite This: *Environ. Sci. Technol.* 2024, 58, 13856−13865

Read Online

ACCESS | Metrics & More | Article Recommendations | Supporting Information

ABSTRACT: Rising global populations have amplified food scarcity and ushered in the development of genetically modified (GM) crops containing small interference RNAs (siRNAs) that control gene expression to overcome these challenges. The use of RNA interference (RNAi) in agriculture remains controversial due to uncertainty regarding the unintended release of genetic material and downstream nontarget effects, which have not been assessed in environmental bacteria to date. To evaluate the impacts of siRNAs used in agriculture on environmental bacteria, this study assessed microbial growth and viability as well as transcription activity with and without the presence of environmental stressors. Results showed a statistically significant reduction in growth capacity and maximum biomass achieved when bacteria are exposed to siRNAs alone and with additional external stress ($p < 0.05$). Further transcriptomic analysis demonstrated that nutrient cycling gene activities were found to be consistently and significantly altered following siRNA exposure, particularly among carbon (*xylA, FBPase, limEH, Chitinase, rgl, rgh, rgaE, mannanase, ara*) and nitrogen (*ureC, nasA, narB, narG, nirK*) cycling genes ($p < 0.05$). Decreases in carbon cycling gene transcription profiles were generally significantly enhanced when siRNA exposure was coupled with nutrient or antimicrobial stress. Collectively, findings suggest that certain conditions facilitate the uptake of siRNAs from their surrounding environments that can negatively affect bacterial growth and gene expression activity, with uncertain downstream impacts on ecosystem homeostasis.



KEYWORDS: *RNA interference, transcriptomics, soil bacteria, small interfering RNA, genetic biotechnology, nutrient cycling*

INTRODUCTION

New strategies using RNA-based genetic biotechnology tools to engineer genetically modified (GM) crops have been developed to meet the growing food demands of the global population and to mitigate the controversy surrounding the use of transgenes in crops cultivated for human consumption. This has culminated in the development of more than 500 GM crops that possess phenotypes altered by conventional DNA-based, RNA-based, or combined approaches.[1] Many modern GM crops rely on RNA interference (RNAi) tools such as small interfering RNAs (siRNAs) that mimic natural gene-silencing mechanisms to alter gene expression and improve crop yield and quality.[2−5] GM crop phenotypes can be persistently altered by the exogenous introduction of double-stranded RNA (dsRNA)-derived siRNAs[6] ranging from cosmetic alterations (e.g., nonbrowning apples), nutritional improvements (e.g., increased starch content), reduced allergen content, and reduced susceptibility to pest predation.[2,3,7−12] While the small size of siRNAs enables high binding specificity in the target organism,[13] it may also increase the risk of nontarget binding in other organisms including soil bacteria that contain complementary regions within their genomes.[5,14,15] Risks to beneficial bacteria posed by nontarget gene silencing include reduced microbial growth, altered metabolism, and diminished nutrient cycling activity, which could lead to bottom-up impacts on larger ecosystems if sufficient numbers of keystone species are impacted.[5,16] No research to date has identified siRNA-mediated nontarget transcriptomic impacts in environmental bacteria, leaving the genetic changes caused by siRNA exposure and their downstream consequences undefined.[5]

Concerns persist regarding risks of RNA release from GM crops and potential downstream impacts on the surrounding ecosystem. Studies have consistently demonstrated that genetic material including engineered transgenes is released intact from GM crop debris, root exudates, and from human waste into wastewater treatment plants.[17−19] This supports the potential for siRNA release in the environment where it may come into contact with environmental bacteria associated with soils, surface water, and groundwater.[3] Recent research has also

Received: February 16, 2024
Revised: July 22, 2024
Accepted: July 23, 2024
Published: July 27, 2024

shown that single-stranded RNA (ssRNA) and dsRNA derived from agricultural biotechnology are much more persistent in soils than previously thought. This indicates that environmental matrices can act as siRNA reservoirs and increase their potential to remain bioavailable for uptake to native bacteria containing Argonaute and DICER-like proteins, with unknown consequences or estimations of potential gene targets.[3,6,19−26] Parker et al. determined that RNA fate in soil environments is dominated by adsorption to soil particles and microbial uptake,[20] with dsRNA persisting for months while ssRNA degrades in minutes under the same conditions.[21] RNA sorption was also found to increase in soils with high clay content, including a corresponding positive impact on persistence.[3] However, most available studies regarding modern RNA-based genetic contaminants have focused on the physicochemical fate of larger dsRNA fragments (i.e., >500 bp) and left the intracellular fate and consequences of smaller siRNA constructs unexplored.[5,20,21]

Due to the paucity of data regarding potential nontarget gene silencing risks posed by bacterial exposure to siRNAs used in agriculture, the objective of this study was to evaluate the influence of siRNA exposure on the viability and metabolic activity of model bacteria ubiquitously found across environmental matrices. It was hypothesized that siRNAs would alter growth patterns and transcriptomic activity of environmental bacteria following exposure due to uptake and nontarget gene silencing. This was achieved by (i) analyzing microbial growth patterns following exposure to model siRNAs with and without additional stressors in microenvironments and (ii) identifying and quantifying alterations to gene expression in upscaled batch reactors using the GeoChip 5S microarray.

MATERIALS AND METHODS

Model siRNA Identification and Sourcing. PVY, AC2, and AC4 siRNAs used in commercially available GM crops[4,8,27,28] were selected as model siRNAs in this experiment based on their potential for nontarget alignments determined by BLAST analysis, differing GC content (40−70%), and sequence dissimilarity across model siRNAs (Table S1). BLAST analyses were conducted in the BLASTN program by using the relevant cDNA sequence for each siRNA as the input sequence to identify potential nontarget alignments. BLASTN searches utilized the nucleotide collection standard database, were optimized for highly similar alignments, and search parameters were adjusted for a short input sequence. Resulting data were filtered to obtain alignments relevant only to prokaryotes (filtering *via* taxid: 2, taxid: 2157; taxid: 2323, taxid: 81490, taxid: 198431). PVY, AC4, and AV2 siRNAs were sourced from Integrated DNA Technologies (Coralville, Iowa) and were stored at −20 °C in 200 μL aliquots to avoid degradation caused by freeze−thaw cycles.

Evaluation of siRNA Impacts on Bacterial Growth in Microenvironments. To study the changes in growth patterns and viability resulting from siRNA exposure, model bacteria *Bacillus subtilis* (GC: 43.6%),[29] *Pseudomonas putida* G7 (GC: 62.13%),[30] and *Paenibacillus* sp. 300A (GC: 40.5%)[31] were cultured in R2A media with and without siRNAs and additional external stresses. Strains were chosen for their differences in cell wall structures and motility characteristics, allowing for a comparative analysis of how siRNAs influence microbial growth. Growth assessments were carried out in 96-well plates. Conditions included: (i) Abiotic controls: 100% R2A without bacteria; (ii) bacteria only controls: 100% R2A and model bacteria; (iii) SiRNA only controls: 100% R2A, bacteria, and siRNAs; (iv) stress only controls: R2A, bacteria, and an external stressor, and (v) SiRNA + stress: R2A, bacteria, siRNAs, and external stressors. Each condition was assessed in triplicate. External stressors selected for these experiments were nutrient stress (i.e., 10−100% R2A),[32] envelope stress (i.e., Kanamycin: 5×10^{-2}−5 μg/mL or Ampicillin: 1×10^{-2}−1×10^{2} μg/mL),[33,34] and osmotic stress (i.e., 1.25−10 μg/mL tryptophan)[35] (Table S2). Appropriate low, medium, and high concentrations for each stressor were determined for each strain prior to growth assessments. Concentrations were chosen based on environmentally relevant concentrations to ensure the presence of a stress-based response without causing populations to die out. The stability of siRNA in R2A and sorption to microplate well surfaces were also assessed (Figure S1).

To construct culturing microenvironments, bacteria were precultured in R2A broth (HIMEDIA, Kenett Square, PA) containing 13 mM of ammonium acetate to facilitate competence.[36] *B. subtilis*, *P. putida*, and *Paenibacillus* 300A were incubated for 24 h at 30 °C and 100 rpm. Conditions were based on individual growth patterns to ensure that the bacterial strains were in the exponential growth phase prior to inoculating microplates. Microplate wells contained a total of 280 μL: 180 μL of R2A broth with or without an added stressor, 20 μL of preculture, and 80 μL of siRNA corresponding to a concentration of 50,000 ng/mL. This concentration represents a high-risk scenario within current known ranges of nucleic acids in soils.[37,38] Optical Density (OD) was measured hourly at 600 nm using a SpectraMax iD3 microplate reader with temperature maintained at 30 °C. The impact of siRNAs on growth was determined as mean ΔOD, where

$$\Delta OD = OD_{siRNA+stress} - OD_{stressonly}$$

Or,

$$\Delta OD = OD_{siRNAonly} - OD_{bacteriaonly}$$

and,

ΔOD < 0: bacteria experience a net diminished growth when siRNAs were present *in vitro*

ΔOD = 0: siRNAs had no additional effect on growth

ΔOD > 0: bacteria experience a net enhanced growth when siRNAs were present *in vitro*

Upscaling Microcosms for Transcriptomic Analysis. Upscaled culture flasks (3 mL) were constructed to recreate high-risk microplate growth conditions to identify the genes affected by nontarget gene silencing following siRNA exposure using GeoChip 5S (Glomics Inc., Norman, OK). Microplate conditions with the most significantly altered growth patterns were selected for this task: (i) *P. putida* and *B. subtilis* exposed to nutrient and AV2 siRNA stresses, (ii) *P. putida* and *B. subtilis* exposed to an envelope (ampicillin) and AV2 siRNA stresses, and (iii) relevant Control conditions. All conditions were prepared in triplicate.

To construct upscaled reactors, 100 μL of microbial preculture was used to inoculate 2.9 mL of R2A broth, with or without relevant stressors and siRNAs (Table S2). Reactors were incubated at 30 °C and 100 rpm. To ensure that RNA was extracted from similar cell numbers from each species and prevent concentration-based impacts on downstream gene expression data, RNA was extracted while cultures were

https://doi.org/10.1021/acs.est.4c01685
Environ. Sci. Technol. 2024, 58, 13856−13865

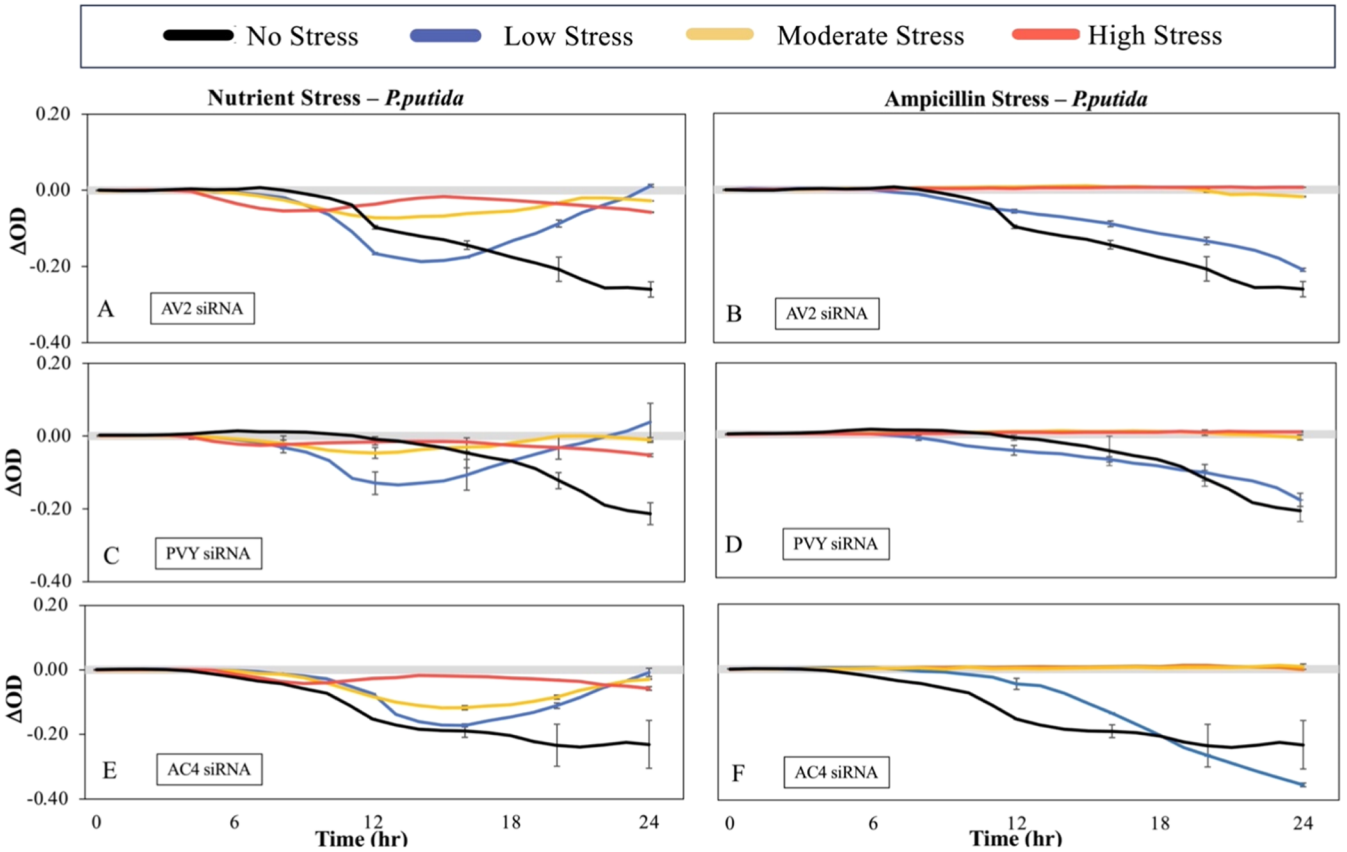


Figure 1. Differences in growth (ΔOD) for *P. putida* exposed to stressors and: (A, B) the AV2 siRNA; (C, D) PVY siRNA; and (E, F) AC4 siRNA ($\Delta OD = OD_{siRNA+stress} - OD_{stressonly}$ or $\Delta OD = OD_{siRNAonly} - OD_{bacteriaonly}$). Negative values indicate a net diminished growth when siRNAs were present *in vitro*. Positive values indicate a net enhanced growth when siRNAs are present *in vitro*. Differences in mean OD values for triplicate measurements are presented for hourly data. Error of mean difference is reported at 6 h intervals. Nutrient stress was assessed using 100% R2A (low stress), 50% R2A (moderate stress), or 10% R2A (high stress). Ampicillin-induced envelope stress was assessed at the following concentrations: 1.0 (low stress), 1×10^1 (Moderate stress), and 1×10^2 μg/mL (high stress). Evaluations of tryptophan-based osmotic stress and kanamycin-induced envelope stress are presented in Figure S2. Complete OD mean, standard deviation, and 95% CI data for individual conditions are presented in File S2 (Excel).

growing in the initial exponential phase. Total RNA was isolated using the Qiagen RNeasy Mini kit following manufacturer instructions and standard QA/QC protocols.[39−41] RNA was quantified using a Qubit 4.0 fluorometer and transformed to cDNA using the High-Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific, Waltham, Massachusetts). A mass of 1 μg high-quality cDNA (i.e., 260/280 = 1.8, 260/230 ≥ 1.7) was sent overnight to Glomics for GeoChip analysis. Mass was calculated using a Qubit 4.0 fluorometer, and quality measurements were determined using a Nanodrop spectrophotometer.

Statistical Analysis. Growth curve data were analyzed using Growthcurver (v. 0.3.1) in R (v. 4.4.0) to obtain the following parameters for each condition:[42,43] carrying capacity (k), growth rate (r), time when the population reaches half the carrying capacity (t_mid), doubling time (t_gen), calculated area under the curve (AUC) (auc_l), and empirical AUC (auc_e). Growth parameters in SiRNA + stress conditions were compared with respect to Control conditions to determine statistical significance using two-tailed Welch's t tests.

GeoChip analyses were used to identify genes with significantly increased or decreased probe intensities (as a proxy for up- or downregulation) following exposure to siRNAs, with observed probe intensity being directly proportional to transcription activity.[44] Preliminary QA/QC analysis of GeoChip data was conducted as described by Wu et al. and He et al.[44,45] Briefly, outlier reads and reads with a signal-to-noise ratio of <2.0 were removed, the intensity of surveyed genes across replicates was normalized to average transcript abundance (as % relative abundance) of each gene, and genes with sufficient intensities were binned by function (e.g., nutrient cycling). Initial analysis was performed using ANOSIM multivariate tests to compare data across gene categories (e.g., carbon cycling genes).[46] Differences among individual genes were analyzed using ANOVA and further assessed using t tests to identify significant changes in gene expression in individual treatments relative to Controls.[47−51] Statistical significance was assigned at $p < 0.05$ and moderate significance at $0.10 > p \geq 0.05$. Significance was confirmed through Benjamini-Hochberg correction analyses ($q < 0.05$).[48,52,53] Analysis was confined to genes present in *Pseudomonas* and *Bacillus* to avoid identification of false positive or negative correlations.

RESULTS AND DISCUSSION

Bacterial Growth Following siRNA Exposure. Bacterial growth patterns were strongly impacted by the presence of external stressors and siRNAs, both alone and in combination. Treatments containing siRNAs consistently had negative

https://doi.org/10.1021/acs.est.4c01685
Environ. Sci. Technol. 2024, 58, 13856−13865

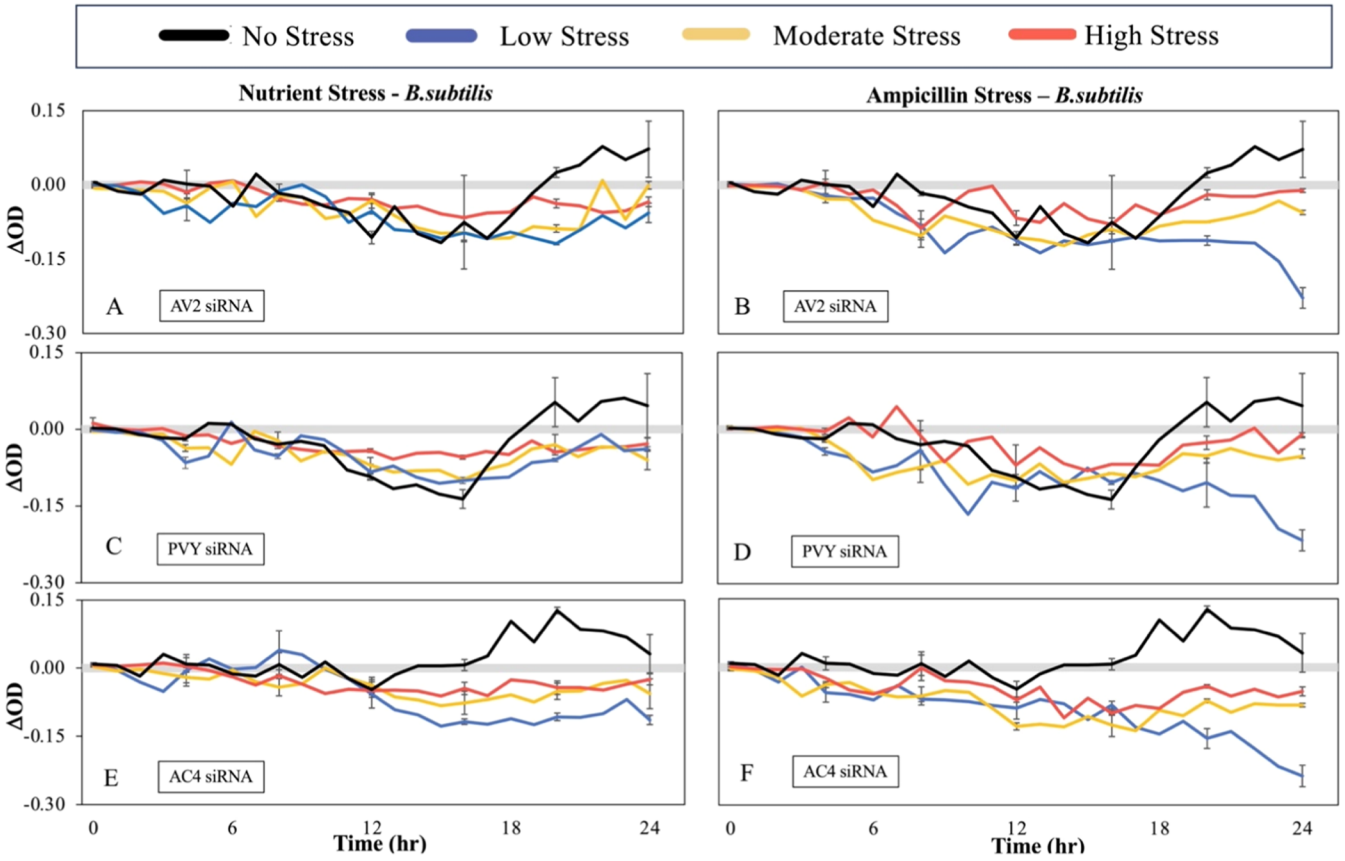


Figure 2. Differences in growth (ΔOD) for *B. subtilis* exposed to stressors and: (A, B) the AV2 siRNA; (C, D) the PVY siRNA; and (E, F) the AC4 siRNA ($\Delta OD = OD_{siRNA+stress} - OD_{stressonly}$ or $\Delta OD = OD_{siRNAonly} - OD_{bacteriaonly}$). Negative values indicate a net diminished growth when siRNAs were present *in vitro*. Positive values indicate a net enhanced growth when siRNAs were present *in vitro*. Difference in mean OD values for triplicate measurements are presented for hourly data. Error of mean difference is reported at 6 h intervals. Nutrient stress was assessed using 100% R2A (low stress), 50% R2A (moderate stress), or 20% R2A (high stress). Ampicillin-induced envelope stress was assessed at the following concentrations: 5×10^{-2} (low stress), 5×10^{-1} (Moderate stress), and 5.0 μg/mL (high stress). Evaluations of tryptophan-based osmotic stress and kanamycin-induced envelope stress are presented in Figure S3. Complete OD mean, standard deviation, and 95% CI data for individual treatments are presented in File S2 (Excel).

differences in OD (ΔOD) values, and bacteria exposed to siRNAs experienced diminished growth after correction for the influence of nutrient, envelope, or osmotic stress (Figures 1,2 and S2−S4). Often, growth was most negatively impacted (i.e., ΔOD_{max} was lowest) when bacteria were exposed solely to AV2, AC4, or PVY siRNA stress. This indicates that siRNA exposure alone can significantly negatively impact cell viability. For *P. putida* exposed to AV2 siRNAs, the SiRNA only control had an *auc_l* and maximum OD (OD_{max}, a proxy for maximum biomass achieved) 40 and 18% smaller, respectively, when compared to the bacteria only control (ΔOD = −0.26; p_{auc_l} = 0.0296, p_{ODmax} = 0.0733) (Figure 1). This is suggestive of a smaller *P. putida* population within the SiRNA only controls. These trends were also observed for SiRNA only conditions involving PVY (ΔOD_{max} = −0.21) and AC4 (ΔOD_{max} = −0.23) siRNAs.

Multiple growth parameters were also observed to decrease when external stress was added, especially within nutrient stress treatments (Figures 1,2 and S4). Across all conditions, *auc_l* and *auc_e* parameters were most consistently altered following exposure to siRNAs within SiRNA + stress and SiRNA only conditions when compared to the stress only controls ($p < 0.05$) (Tables S3−S5), followed by *k*, *t_mid*, *r*, and finally *t_gen*, respectively. As *auc_l* and *auc_e* parameters are proxy measurements of population size, these decreases imply a lower carrying capacity, a slower doubling time, or a combination following exposure to siRNAs.

Reduced growth was also consistently observed when *P. putida* was exposed to an siRNA combined with an external stressor, and growth impacts were most severe among low stress conditions (i.e., ΔOD_{max}: −0.38 to −0.17; Figures 1 and S2). Under the lowest tested concentration for nutrient stress (i.e., 10% R2A), *P. putida* exposed to SiRNA + stress had an *auc_l* 75% lower than the SiRNA only control (p_{auc_l} = 0.0249) and 46% lower than the stress only control (p_{auc_l} = 9.16 × 10^{-6}), indicating that changes to *P. putida* growth were not the result of an external stressor alone. Similarly, OD_{max} in the SiRNA + stress treatment was 74% lower than that of the SiRNA only control (p_{ODmax} = 0.0096) and 15% lower than that of the stress only control (p_{ODmax} = 0.0123). This further suggests that the combination of a stressful environment and siRNA exposure during microbial replication results in a reduction in the overall size of the population. Of the tested stress conditions, exposure to nutrient and envelope (antibiotic) stresses significantly altered the six studied growth curve parameters ($p < 0.05$) when compared to controls, while tryptophan stress did not (Table S3). This suggests that tryptophan stress is not a sufficient osmotic stress to induce a defense response. Increasing external stress also had a negative impact on *P. putida* growth, with reduced overall growth

https://doi.org/10.1021/acs.est.4c01685
Environ. Sci. Technol. 2024, 58, 13856−13865

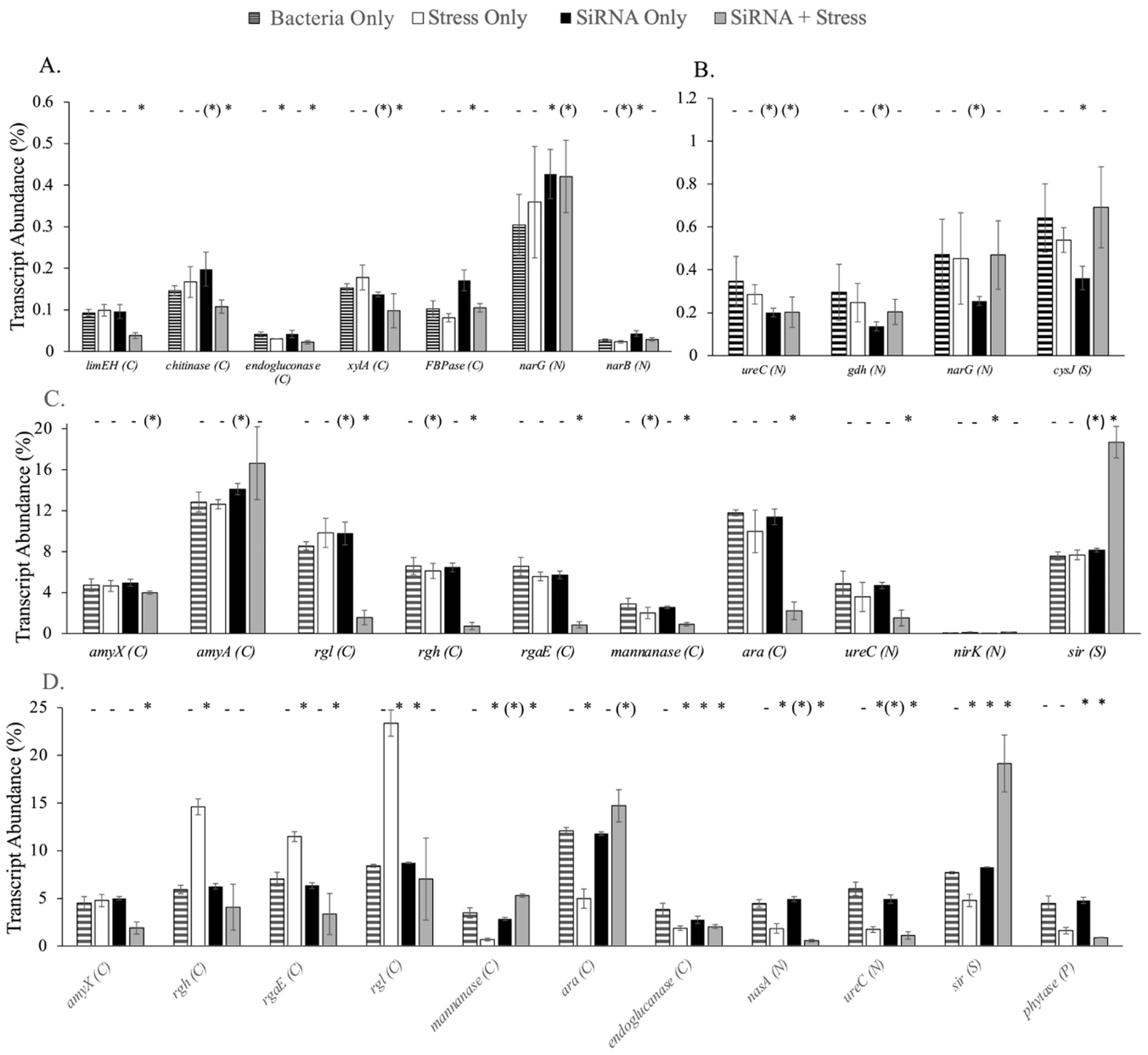


Figure 3. Gene transcripts for carbon cycling (*amyX, amyA, rgl, rgh, rgaE, mannanase, ara, limeEH, Chitinase, endogluconase, xylA, FBPase*), nitrogen cycling (*narG, narB, ureC, gdh, nasA, nirK*), sulfur cycling (*cysJ*), and phosphorus cycling (*phytase*) genes normalized to total transcripts detected for (A) *P. putida* exposed to ampicillin and AV2 siRNA stress; (B) *P. putida* exposed to nutrient and AV2 siRNA stress; (C) *B. subtilis* exposed to envelope (ampicillin) and AV2 siRNA stress; and (D) *B. subtilis* exposed to nutrient and AV2 siRNA stress. Statistical significance is denoted with asterisks, moderate significance is denoted with asterisks, and no significance is denoted with "-". Statistical significance was assigned at $p < 0.05$ and moderate significance at $0.10 > p \geq 0.05$.

frequently observed under moderate and high stress conditions. However, this impact on growth was largely due to external stress alone (i.e., $\Delta OD \sim 0$), with little or no additional siRNA-driven effects. This suggests that abiotic stresses can act as a more powerful driver of bacterial metabolic adaptations relative to siRNAs under certain conditions. We also observed several notable exceptions to growth trends in some stress + SiRNA treatments: both *P. putida* and *Paenibacillus* cultures experienced enhanced growth (i.e., $\Delta OD > 0$) with exposure to siRNAs in the presence of moderate and high levels of kanamycin stress (Figures S2 and S4). This suggests that bacterial gene expression may be altered by siRNA exposure and result in an increased resistance to antimicrobial stress. Further research is needed to elucidate mechanisms of potential compensatory gene regulation following siRNA exposure, especially considering the implications for increased antimicrobial resistance among environmental bacteria.

B. subtilis cultures (Figure 2) also displayed significantly reduced growth following exposure to siRNAs and stressors, alone and collectively ($\Delta OD_{max} \geq -0.26$). In the SiRNA only control (AV2), *auc_l* was reduced by 41% and OD_{max} was 26% lower than the bacteria only control ($\Delta OD_{max} = -0.12$; $p_{auc\_l} = 0.0052$, $p_{ODmax} = 0.1004$) (Figure 2). As with *P. putida*, the growth capacity most significantly reduced with the introduction of low-level nutrient or antimicrobial (kanamycin) stress: *auc_l* was reduced by 78% ($p_{auc\_l} = 0.0072$) and 49% ($p_{auc\_l} = 0.0002$) relative to SiRNA only and stress only

https://doi.org/10.1021/acs.est.4c01685
Environ. Sci. Technol. 2024, 58, 13856−13865

controls, respectively. Similar growth trends were observed for *Paenibacillus* 300A (Figure S4), although to a lesser extent. For example, *auc_l* and OD_{max} parameters in the *Paenibacillus* SiRNA only control were 29 and 26% lower than in the bacteria only control, respectively. SiRNA and stress combinations also had a lesser, but still statistically significant, effect on *Paenibacillus* growth when compared to *B. subtilis* and *P. putida* growth. Thus, *B. subtilis* and *P. putida* cultures were prioritized for upscale transcriptomic analyses. *B. subtilis* and *Paenibacillus* cultures also displayed unique growth behavior, with OD values stagnating and crashing around hour 20 (File S2). Small biofilm flocs were observed in these cultures upon visual inspection. We hypothesize that this led to greater variation in OD readings across 24 h and that >20 h OD values may underestimate cell numbers because of flocculation-sedimentation. We further hypothesize that this did not occur in other treatments, as added stressors may have decreased biofilm formation.

Collectively, growth data strengthen the hypothesis that siRNAs can negatively affect the growth patterns and community structures of environmental bacteria, with impacts increasing as external stress increases. The AV2 siRNA had the most significant impact on the tested growth parameters for *P. putida* and *B. subtilis* ($p < 0.05$), while AC4 had the largest impact on *Paenibacillus* 300A ($p < 0.05$) (Table S4). This strongly suggests that siRNA sequences have disparate effects on different bacteria, and that impact is tied to the sequence similarity between the siRNA and nontarget locations in the microbial genome. However, growth impacts and the magnitude of those impacts were not predicted by siRNA and genomic GC content alone but are likely influenced by the GC content of microlocations within vulnerable genes.

Gene Activity Affected by siRNA Exposure. Surveyed genes responsible for nutrient (i.e., C/N/P/S) cycling (Figure 3), organic remediation, and metal homeostasis (Figures S5 and S6) showed significant differences in transcription activity following exposures to AV2 siRNAs, with impacts dependent on the presence and type of external stressors.

Following siRNA exposure, carbon cycling gene activity was most significantly altered in *B. subtilis* (ANOSIM $P < 0.05$) and *P. putida* (ANOSIM $P < 0.05$). In total, transcription of nine carbon cycling genes (i.e., *xylA, FBPase, limEH, Chitinase, rgl, rgh, rgaE, mannanase, ara*) was significantly affected relative to bacteria only controls following siRNA exposure ($p < 0.05$). For eight of nine genes, altered expression was intensified under SiRNA + stress conditions when compared to stress only and SiRNA only conditions. This indicates that the combination of siRNA stress and environmental stress results in a stronger impact on transcription patterns rather than individual exposure to the stressor or siRNA (*B. subtilis*: ANOSIM $P = 0.019$; *P. putida*: ANOSIM $P = 0.023$). The activity of *P. putida limEH* ($p = 0.003$), endoglucanase ($p = 0.012$), Chitinase ($p = 0.044$), and *xylA* ($p = 0.021$) genes was strongly reduced by the presence of exogenous AV2 siRNAs and ampicillin stress (Figure 3A). The *limEH* gene (41.1% GC) shows no significant change in expression when exposed to siRNAs or external stress alone (bacteria only transcript abundance: 0.093 ± 0.008%; stress only: 0.099 ± 0.014% and $p = 0.260$; SiRNA only: 0.096 ± 0.017% and $p = 0.407$; SiRNA + stress condition: 0.038 ± 0.007%). This trend was also observed for Chitinase, endoglucanase, and *xylA* activity in *P. putida* when cultured under the same environmental conditions. Carbon cycling activity was more severely diminished in *B. subtilis* when exposed to AV2 siRNAs and ampicillin-induced envelope stress; transcription declined sharply for *amyX* (47.7% GC), *rgl* (48.7% GC), *rgaE* (51.8% GC), *rgh* (45.8%), and *mannanase* (59.9% GC) genes (Figure 3C). Among these, *rgl* and *rgaE* genes had the most significant reductions in transcript activity when exposed to siRNAs and envelope stress, decreasing from 8.554 ± 0.403 to 1.559 ± 0.708% ($p = 0.0002$) and from 6.545 ± 0.891 to 0.848 ± 0.300% ($p = 0.002$), respectively. *RgaE* ($p = 0.019$), *mannanase* ($p = 0.033$), and *ara* ($p = 0.006$) gene transcription also followed these trends. These carbon cycling genes play critical roles in environmental carbon degradation, the breakdown of organic compounds, release to bioavailable N/P/S, and net CO_2 production.[54,55] Among carbon cycling genes, only *Chitinase* (*P. putida*), *FBPase* (*P. putida*), and *amyA* (*B. subilits*) appeared upregulated following siRNA exposure, though only changes to *FBPase* transcription in SiRNA only treatments were significantly different from bacteria only controls ($p = 0.020$).

Impacts on *P. putida* and *B. subtilis* carbon cycling activity were less consistent when cultures were exposed to AV2 siRNAs and reduced nutrient availability, though significant differences were still observed (*B. subtilis*: ANOSIM $P = 0.020$; Figure 3B,3D). In *B. subtilis*, *amyX, rgaE*, and endoglucanase activity decreased significantly in SiRNA + stress conditions relative to bacteria only controls ($p = 0.0003$, 0.018, 0.005, respectively). While only *rgl* and endoglucanase transcription was negatively impacted by siRNA exposure alone ($p = 0.036$, 0.021), nutrient stress alone resulted in significant upregulations to *rgh, rgaE*, and *rgl* genes ($p = 0.002$, 0.003, 0.012, respectively). As these genes are involved in nutrient assimilation from more complex carbon substrates (*rgh*: lignin biodegradation; *rgaE*: pectin biodegradation; *rgl*: rhamnogalacturonan biodegradation), this may be indicative of efforts to increase relevant protein production to counteract increased nutrient scarcity.[56] However, similar impacts to *rgh, rgl, rgaE*, and endogluaonase transcription were not observed when nutrient stress was coupled to siRNA exposure. The presence of additional external stressors may induce bacteria to assimilate siRNAs differently into their cell compartments, reducing the predictability of gene silencing events. This suggests that siRNAs can alter cellular metabolic responses to external stressors, with downstream implications for fitness and viability.

Alterations in nitrogen and sulfur cycling gene expression were also observed for *P. putida* and *B. subtilis* exposed to siRNAs and ampicillin stresses. For *P. putida* (Figure 3A), differences were frequently moderately significant and were observed primarily when bacteria were exposed to siRNAs alone rather than in conjunction with additional stressors (ANOSIM $P = 0.046$). Nitrogen cycling mediated by *narG* (40.8% GC) and *narB* (50.0% GC) genes was significantly upregulated when exposed to siRNAs alone ($p = 0.046$ and $p = 0.032$, respectively), although not when siRNA and ampicillin stresses were combined. Under siRNA stress alone (Figure 3B), effects on *P. putida* expression of *ureC* (65.8% GC, $p = 0.078$), *gdh* (49.4% GC, $p = 0.084$), and *narG* ($p = 0.073$) were moderate, while expression of sulfur cycling gene *cysJ* was significantly decreased when nutrient stress was added (56.1% GC, $p = 0.039$). SiRNA + stress treatments were associated with large variations in gene expression, further demonstrating that siRNAs exert disparate impacts across variations in microbial strains and environmental conditions.

https://doi.org/10.1021/acs.est.4c01685
Environ. Sci. Technol. 2024, 58, 13856−13865

In contrast, nitrogen gene transcription in *B. subtilis* was largely downregulated following AV2 siRNA exposure (ANOSIM $P = 0.022$). SiRNA exposure significantly reduced the capacity for *B. subtilis* nitrogen cycling *via ureC* and *nasA* genes (*ureC* $p = 0.035$; *nasA*: 68.7% GC, $p = 0.003$), responsible for ammonification and assimilatory nitrogen reduction and are essential for converting inorganic nitrogen into organics like proteins and amino acids.[57−59] *UreC* was strongly downregulated under SiRNA + stress conditions compared to stress and SiRNA only controls when exposed to envelope stress (*ureC* transcript abundance: 4.882 ± 1.209% bacteria only control; 1.516 ± 0.772% SiRNA + stress condition) ($p = 0.010$) (Figure 3C). *UreC* and *nasA* responded similarly when under siRNA and envelope stresses (Figure 3D, *ureC* transcript abundance: 6.011 ± 0.702% bacteria only control, 1.112 ± 0.389% SiRNA + stress condition $p = 0.0008$; *nasA* transcript abundance: 4.486 ± 0.405% bacteria only control, 0.569 ± 0.132% SiRNA + stress condition $p = 0.0008$). Alterations in transcription behavior imply that free RNA constructs can be translocated across the cellular membrane, affect transcription patterns, and ultimately alter bacterial dynamics with their environments. As a result, it is hypothesized that the silencing or downregulation of these genes can subsequently have negative impacts on major steps of cycling of these nutrients in the environment and ecosystem. Large-scale disruptions to microbial metabolisms may have downstream impacts on the decomposition of organic matter, nutrient availability, soil fertility, and ecosystem stability.[55]

However, not all interactions resulted in a decreased transcription activity. When *P. putida* was exposed to envelope stress and the AV2 siRNA, we observed several increases in gene expression relevant to organic compound biodegradation (Figure S5, *bphF1* gene, $p = 0.065$) and denitrification (*narG*, $p = 0.077$). While not statistically significant, these genes may be more highly expressed because of their role in substrate breakdown, which may be advantageous when cells respond to unknown compounds in their internal or external environment (e.g., siRNA, ampicillin). *B. subtilis* under siRNA and envelope stress had significantly decreased carbon cycling activity *via* the *ara* gene (45.3% GC, transcript abundance: 2.217 ± 0.863%, SiRNA + stress; 11.781 ± 0.295%, bacteria only control; 9.972 ± 2.080%, stress only control; 11.387 ± 0.769% SiRNA only control) ($p = 0.0006$) (Figure 3C). This was also observed for the sulfur cycling gene *sir* (57.5% GC, transcript abundance: 18.677 ± 1.537%, siRNA + stress; 7.562 ± 0.403%, bacteria only control; 7.674 ± 0.473%, stress only control; 8.137 ± 0.188%, SiRNA only control) ($p = 0.001$). Similar patterns were observed for *B. subtilis ara*, *sir*, and *mannanase* genes under nutrient stress (Figure 3D), *terC* expression (55.9% GC, metal homeostasis), and *amiE* expression (64.2% GC, organic remediation) (*terC*, $p = 0.001$; *amiE*, $p = 0.0002$) (Figure S6).

The consistency of down-regulation trends is strongly suggestive of nontarget gene silencing, though additional experiments are needed to verify whether siRNAs are directly or indirectly affecting genes surveyed in this study. It is necessary to differentiate the mechanistic contributions of external stressors to observed downregulations, as the creation of environmentally relevant stress conditions may drive cells to decrease production of proteins not essential for survival in an effort to conserve energy.[60−62] Exposure to antimicrobial compounds like ampicillin upregulate genes directly and indirectly relates for neutralizing those toxins (e.g., *terC*, *amiE*).[63,64] Nutrient limitations may be counteracted by the upregulation of genes involved in nutrient scavenging to improve enzyme−substrate complex formation to increase the rate or efficiency of energy generation.[65] Gene upregulation may also occur if unaffected genes are expressed to compensate for the loss or decreased activity of siRNA-affected genes. Gene expression patterns under the SiRNA + stress conditions were not always correlated to the SiRNA only controls, indicating other genetic factors influence metabolic activity. In several cases, gene expression in SiRNA only controls increased under the tested conditions, while the expression of the same gene in SiRNA + stress conditions decreased (e.g., *Chitinase*, *FBPase*, *amyA*, *nasA*). This may be explained by cell efforts to increase relevant protein production to counteract the silencing or toxicity effects associated with siRNA exposure.[63,64] This is significant for bacteria in environmental matrices and human microbiomes that might have elevated exposure to GM crop tissues or other materials containing RNAi genetic biotechnology.[66]

This work demonstrates that siRNA exposure can impact bacterial growth and gene expression, with potential implications for ecological homeostasis in environments that frequently encounter RNAi including agricultural and human microbiomes. Data demonstrate statistically significant differences in *auc_l* (as a proxy for growth capacity) and OD_{max} growth parameters of bacteria cultured in the presence of model siRNAs with and without the addition of external stress. Differences in strain growth suggest that siRNA exposure has a direct and negative influence on population dynamics, increasing the time required for microbial populations to multiply and ultimately reducing overall population biomass and viability. If sufficient fractions of a microbial population are reduced, the efficiency of microbial ecosystem services may also be impacted including organic matter degradation,[67] increasing nutrient bioavailability,[68] and carbon sequestration.[69] Findings also demonstrate that siRNA exposure can lead to the altered expression of individual genes, thereby affecting protein production and cell functions. Critically, exposure to siRNA and external stressors appear to have effects on transcription activity and are not necessarily predicted by the presence of a single stressor alone, microbial phylogeny, or the GC content of target genomes and genes. Findings are also suggestive of an inverse relationship between increasing external stress and siRNA-driven impacts. However, statistical analyses failed to identify gene expression trends that can be easily predicted by siRNA sequence or external stress type, requiring further research incorporating additional model siRNAs and more comprehensive transcriptomic approaches.

While results suggest siRNAs can pass through bacterial cell membranes to exert a gene silencing effect and present evidence of genetic dysregulation, study limitations necessitate further research. Observed transcriptomic alterations may be due to multiple mechanisms, including nontarget alignments between the siRNA and surveyed nutrient cycling genes or upstream genes that control their expression (e.g., genes located within the same operon), the precise nature of which was not resolved here. Additional controls (e.g., siRNAs currently used in agriculture that do not exert silencing effects once they can be identified), varying siRNA concentrations to determine no-effect exposure levels, and more complex environments (e.g., soils) would provide further insights into exposure response mechanisms and more accurately predict up- or down-regulation events. Due to the rapid implementation of siRNAs as a tool in modern agriculture, environmental

https://doi.org/10.1021/acs.est.4c01685
Environ. Sci. Technol. 2024, 58, 13856−13865

assessments of GM crops are needed to avoid these potential risks. Additional research is needed to understand siRNA transport and intracellular fate, as their potential to influence lateral gene flow across generations remains unknown. Research needs also include assessing siRNA activity ranges and influence in food webs, as well as leveraging these findings to establish more complete risk assessment profiles.[70]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.4c01685.

Stability of siRNAs in R2A media; growth curves for *P. putida* exposed to siRNAs and tryptophan or kanamycin stress; growth curves for *B. subtilis* exposed to siRNAs and tryptophan or kanamycin stress; growth curves for *Paenibacillus* 300A exposed to siRNAs and nutrient, ampicillin, tryptophan, or kanamycin stress; metal homeostasis and organic remediation gene transcription data for *P. putida;* nutrient cycling, metal homeostasis, and organic remediation gene transcription data for *B. subtilis*; summary of model siRNAs used in this study; summary of stressor conditions used in this study; selected statistical significance values for *P. putida* growth assays; selected statistical significance values for growth assays containing kanamycin or ampicillin (PDF)

Average, standard deviation, and 95% CI statistics for growth curve experiments (XLSX)

■ AUTHOR INFORMATION

Corresponding Author

C. M. Gardner − *Department of Civil and Environmental Engineering, Washington State University, Pullman, Washington 99164, United States; Maseeh Department of Civil, Architectural, and Environmental Engineering, University of Texas at Austin, Austin, Texas 78712, United States;* orcid.org/0000-0002-1292-2083; Email: courtney.gardner@austin.utexas.edu

Authors

S. Un Jan Contreras − *Department of Civil and Environmental Engineering, Washington State University, Pullman, Washington 99164, United States*

L. K. Redfern − *Department of Bioengineering, Civil Engineering, and Environmental Engineering, Florida Gulf Coast University, Fort Myers, Florida 33965, United States;* orcid.org/0000-0002-9839-7469

L. W. Maguire − *Maseeh Department of Civil, Architectural, and Environmental Engineering, University of Texas at Austin, Austin, Texas 78712, United States*

S. I. Promi − *Maseeh Department of Civil, Architectural, and Environmental Engineering, University of Texas at Austin, Austin, Texas 78712, United States*

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.est.4c01685

Author Contributions

The manuscript was written through the contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This research was supported by funding provided by the USDA (BRAG Award#2019-33522-30203) and NSF (CBET award#2129531). The authors would also like to acknowledge Haluk Beyenal at Washington State University for providing *Paenibacillus* sp. 300A cultures for this experiment. TOC art was created with Biorender.com.

■ REFERENCES

(1) Kamle, M.; Kumar, P.; Patra, J. K.; Bajpai, V. K. Current perspectives on genetically modified crops and detection methods. *3 Biotech* **2017**, *7* (3), No. 219.

(2) Ali, N.; Datta, S. K.; Datta, K. RNA interference in designing transgenic crops. *GM Crops* **2010**, *1* (4), 207−213.

(3) Zhang, K.; Wei, J.; Hartz, K. E. H.; Lydy, M. J.; Moon, T. S.; Sander, M.; Parker, K. M. Analysis of RNA Interference (RNAi) Biopesticides: Double-Stranded RNA (dsRNA) Extraction from Agricultural Soils and Quantification by RT-qPCR. *Environ. Sci. Technol.* **2020**, *54* (8), 4893−4902.

(4) Un Jan Contreras, S.; Gardner, C. M. Environmental fate and behaviour of antibiotic resistance genes and small interference RNAs released from genetically modified crops. *J. Appl. Microbiol.* **2022**, *133* (5), 2877−2892.

(5) Arpaia, S.; Christiaens, O.; Giddings, K.; Jones; Mezzetti, B.; Moronta-Barrios, F.; Perry, J. N.; Sweet, J. B.; Taning, C. N.; Smagghe, G.; Dietz-Pfeilstetter, A. Biosafety of GM Crop Plants Expressing dsRNA: Data Requirements and EU Regulatory Considerations. *Front. Plant Sci.* **2020**, *11*, No. 940.

(6) Li, H.; Guan, R.; Guo, H.; Miao, X. New insights into an RNAi approach for plant defence against piercing-sucking and stem-borer insect pests. *Plant, Cell Environ.* **2015**, *38* (11), 2277−2285.

(7) Davuluri, G. R.; Van Tuinen, A.; Fraser, P. D.; Manfredonia, A.; Newman, R.; Burgess, D.; Brummell, D. A.; King, S. R.; Palys, J.; Uhlig, J.; Bramley, P. M.; et al. Fruit-specific RNAi-mediated suppression of DET1 enhances carotenoid and flavonoid content in tomatoes. *Nat. Biotechnol.* **2005**, *23* (7), 890−895.

(8) Andersson, M.; Melander, M.; Pojmark, P.; Larsson, H.; Bülow, L.; Hofvander, P. Targeted gene suppression by RNA interference: An efficient method for production of high-amylose potato lines. *J. Biotechnol.* **2006**, *123* (2), 137−148.

(9) Bachman, P. M.; Huizinga, K. M.; Jensen, P. D.; Mueller, G.; Tan, J.; Uffman, J. P.; Levine, S. L. Ecological risk assessment for DvSnf7 RNA: A plant-incorporated protectant with targeted activity against western corn rootworm. *Regul. Toxicol. Pharmacol.* **2016**, *81*, 77−88.

(10) Dubrovina, A. S.; Kiselev, K. V. Exogenous RNAs for gene regulation and plant resistance. *Int. J. Mol. Sci.* **2019**, *20* (9), No. 2282.

(11) Lilley, C. J.; Kyndt, T.; Gheysen, G.*Genomics and Molecular Genetics of Plant-Nematode Interactions*; Springer, 2011; pp 517−541.

(12) Ryffel, G. U. Making the most of GM potatoes. *Nat. Biotechnol.* **2010**, *28* (4), No. 318.

(13) Qiu, S.; Adema, C. M.; Lane, T. A computational study of off-target effects of RNA interference. *Nucleic Acids Res.* **2005**, *33* (6), 1834−1847.

(14) Yu, A. M.; Choi, Y. H.; Tu, M. J. RNA drugs and RNA targets for small molecules: Principles, progress, and challenges. *Pharmacol. Rev.* **2020**, *72* (4), 862−898.

(15) Davidson, B. L.; McCray, P. B. Current prospects for RNA interference-based therapies. *Nat. Rev. Genet.* **2011**, *12* (5), 329−340.

(16) Lundgren, J. G.; Duan, J. J. RNAi-based insecticidal crops: Potential effects on nontarget species. *Bioscience* **2013**, *63* (8), 657−665.

(17) Baetz, U.; Martinoia, E. Root exudates: The hidden part of plant defense. *Trends Plant Sci.* **2014**, *19* (2), 90−98.

(18) Sasse, J.; Martinoia, E.; Northen, T. Feed Your Friends: Do Plant Exudates Shape the Root Microbiome? *Trends Plant Sci.* **2018**, *23* (1), 25−41.
(19) Chatterjee, A.; Zhang, K.; Rao, Y.; Sharma, N.; Giammar, D. E.; Parker, K. M. Metal-Catalyzed Hydrolysis of RNA in Aqueous Environments. *Environ. Sci. Technol.* **2022**, *56* (6), 3564−3574.
(20) Parker, K. M.; Borrero, V. B.; Van Leeuwen, D. M.; Lever, M. A.; Mateescu, B.; Sander, M. Environmental Fate of RNA Interference Pesticides: Adsorption and Degradation of Double-Stranded RNA Molecules in Agricultural Soils. *Environ. Sci. Technol.* **2019**, *53* (6), 3027−3036.
(21) Zhang, K.; Hodge, J.; Chatterjee, A.; Moon, T. S.; Parker, K. M. Duplex structure of double-stranded RNA provides stability against hydrolysis relative to single-stranded RNA. *Environ. Sci. Technol.* **2021**, *55* (12), 8045−8053.
(22) San Miguel, K.; Scott, J. G. The next generation of insecticides: DsRNA is stable as a foliar-applied insecticide. *Pest Manage. Sci.* **2016**, *72* (4), 801−809.
(23) Mitter, N.; Worrall, E. A.; Robinson, K. E.; Li, P.; Jain, R. G.; Taochy, C.; Fletcher, S. J.; Carroll, B. J.; Lu, G. Q.; Xu, Z. P. Clay nanosheets for topical delivery of RNAi for sustained protection against plant viruses. *Nat. Plants* **2017**, *3*, No. 16207, DOI: 10.1038/nplants.2016.207.
(24) Domingues, S.; Nielsen, K. M. Membrane vesicles and horizontal gene transfer in prokaryotes. *Curr. Opin. Microbiol.* **2017**, *38*, 16−21.
(25) Lee, H. C.; Li, L.; Gu, W.; Xue, Z.; Crosthwaite, S. K.; Pertsemlidis, A.; Lewis, Z. A.; Freitag, M.; Selker, E. U.; Mello, C. C.; Liu, Y. Diverse pathways generate microRNA-like RNAs and Dicer-independent small interfering RNAs in fungi. *Mol. Cell* **2010**, *38* (6), 803−814.
(26) Olina, A. V.; Kulbachinskiy, A. V.; Aravin, A. A.; Esyunina, D. M. Argonaute proteins and mechanisms of RNA interference in eukaryotes and prokaryotes. *Biochemistry* **2018**, *83* (5), 483−497.
(27) McCue, K. F.; Ponciano, G.; Rockhold, D. R.; Whitworth, J. L.; Gray, S. M.; Fofanov, Y.; Belknap, W. R. Generation of PVY Coat Protein siRNAs in Transgenic Potatoes Resistant to PVY. *Am. J. Potato Res.* **2012**, *89* (5), 374−383.
(28) Saxena, S.; Kesharwani, R. K.; Singh, V.; Singh, S. Designing of putative siRNA against geminiviral suppressors of RNAi to develop geminivirus-resistant papaya crop. *Int. J. Bioinf. Res. Appl.* **2013**, *9* (1), 3−12.
(29) Moszer, I. The complete genome of Bacillus subtilis: From sequence annotation to data management and analysis. *FEBS Lett.* **1998**, *430* (1−2), 28−36.
(30) Varner, P. M.; Gunsch, C. K. Complete Genome Sequence of NAH7-Harboring Pseudomonas putida Strain G7. *Microbiol. Resoure. Announce.* **2022**, *11* (10), No. e00394-22.
(31) Kiehl, P. M.; Cagle, S. M.; Kinkel, T. L.; Stenglein, M. D. Whole-genome sequence of Paenibacillus sp., isolated from soil, Fort Collins, Colorado, USA. *Microbiol. Resour. Announce.* **2023**, *12* (10), No. e0054223.
(32) Poole, K. Bacterial stress responses as determinants of antimicrobial resistance. *J. Antimicrob. Chemother.* **2012**, *67* (9), 2069−2089.
(33) Guest, R. L.; Wang, J.; Wong, J. L.; Raivio, T. L. A bacterial stress response regulates respiratory protein complexes to control envelope stress adaptation. *J. Bacteriol.* **2017**, *199* (20), 10−1128.
(34) Mitchell, A. M.; Silhavy, T. J. Envelope stress responses: balancing damage repair and toxicity. *Nat. Rev. Microbiol.* **2019**, *17* (7), 417−428.
(35) Weber, A.; Kögl, S. A.; Jung, K. Time-dependent proteome alterations under osmotic stress during aerobic and anaerobic growth in *Escherichia coli*. *J. Bacteriol.* **2006**, *188* (20), 7165−7175.
(36) Lu, N.; Massoudieh, A.; Liang, X.; Kamai, T.; Zilles, J. L.; Nguyen, T. H.; Ginn, T. R. A kinetic model of gene transfer via natural transformation of Azotobacter vinelandii. *Environ. Sci. Water Res. Technol.* **2015**, *1* (3), 363−374.
(37) Sodnikar, K.; Kaegi, R.; Christl, I.; Schroth, M. H.; Sander, M. Transport of double-stranded ribonucleic acids (dsRNA) and deoxyribonucleic acids (DNA) in sand and iron oxide-coated sand columns under varying solution chemistries. *Environ. Sci.: Process Impacts* **2023**, *25* (12), 2067−2080.
(38) Ogram, A.; Sayler, G. S.; Gustin, D.; Lewis, R. J. DNA adsorption to soils and sediments. *Environ. Sci. Technol.* **1988**, *22* (8), 982−984.
(39) Tang, W.; Hu, Z.; Muallem, H.; Gulley, M. L. Quality assurance of RNA expression profiling in clinical laboratories. *J. Mol. Diagn.* **2012**, *14* (1), 1−11.
(40) Azzarà, A.; Chiaramonte, A.; Filomeni, E.; Pinto, B.; Mazzoni, S.; Piaggi, S.; Guzzardi, M. A.; Bruschi, F.; Iozzo, P.; Scarpato, R. Increased Level of DNADamage in SomeOrgans of Obese Zucker Rats by c-H2AX Analysis Alessia. *Environ. Mol. Mutagen.* **2010**, *405*, 391−405.
(41) Shi, L.; Tong, W.; Goodsaid, F.; Frueh, F. W.; Fang, H.; Han, T.; Fuscoe, J. C.; Casciano, D. A. QA/QC: Challenges and pitfalls facing the microarray community and regulatory agencies. *Expert Rev. Mol. Diagn.* **2004**, *4* (6), 761−777.
(42) Sprouffske, K.; Wagner, A. Growthcurver: An R package for obtaining interpretable metrics from microbial growth curves. *BMC Bioinf.* **2016**, *17* (1), No. 172.
(43) R Core Team, R.. *R: A Language and Environment for Statistical Computing*, 2013.
(44) He, Z.; Gentry, T. J.; Schadt, C. W.; Wu, L.; Liebich, J.; Chong, S. C.; Huang, Z.; Wu, W.; Gu, B.; Jardine, P.; Criddle, C.; Zhou, J. GeoChip: a comprehensive microarray for investigating biogeochemical, ecological and environmental processes. *ISME J.* **2007**, *1* (1), 67−77.
(45) Wu, L.; Liu, X.; Schadt, C. W.; Zhou, J. Microarray-based analysis of subnanogram quantities of microbial community DNAs by using whole-community genome amplification. *Appl. Environ. Microbiol.* **2006**, *72*, 4931−4941.
(46) Gao, Y.; Ding, J.; Yuan, M.; Chiariello, N.; Docherty, K.; Field, C.; Gao, Q.; Gu, B.; Gutknecht, J.; Hungate, B. A.; Le Roux, X.; et al. Long-term warming in a Mediterranean-type grassland affects soil bacterial functional potential but not bacterial taxonomic composition. *npj Biofilms Microbiomes* **2021**, *7* (1), No. 17.
(47) Xue, K.; Xie, J.; Zhou, A.; Liu, F.; Li, D.; Wu, L.; Deng, Y.; He, Z.; Van Nostrand, J. D.; Luo, Y.; Zhou, J. Warming alters expressions of microbial functional genes important to ecosystem functioning. *Front. Microbiol.* **2016**, *7*, No. 668.
(48) Liu, F.; Ryu, T.; Ravasi, T.; Wang, X.; Wang, G.; Li, Z. Niche−dependent sponge hologenome expression profiles and the host-microbes interplay: a case of the hawaiian demosponge Mycale Grandis. *Environ. Microbiome* **2024**, *19* (1), No. 22.
(49) Zhou, J.; Li, P.; Wang, Y.; Liu, H.; Wei, W.; Yuan, C.; Wang, H. Arsenic mobilization in a high arsenic groundwater revealed by metagenomic and Geochip analyses. *Sci. Rep.* **2019**, *9* (1), No. 12972.
(50) Poirel, J.; Joulian, C.; Leyval, C.; Billard, P. Arsenite-induced changes in abundance and expression of arsenite transporter and arsenite oxidase genes of a soil microbial community. *Res. Microbiol.* **2013**, *164* (5), 457−465.
(51) Addis, S. D.; Formel, S. K.; Kim, Y. J.; Varner, P. B.; Raudabaugh, D. B.; Lefevre, E.; Bernik, B. M.; Elango, V.; et al. Alterations of endophytic microbial community function in Spartina alterniflora as a result of crude oil exposure. *Biodegradation* **2022**, *33* (1), 87−98.
(52) Benjamini, Y.; Hochberg, Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J. R. Stat Soc: Ser. B: Stat. Methodol.* **1995**, *57* (1), 289−300.
(53) Gardner, C. M.; Hoffman, K.; Stapleton, H. M.; Gunsch, C. K. Exposures to semivolatile organic compounds in indoor environments and associations with the gut microbiomes of children. *Environ. Sci. Technol. Lett.* **2021**, *8* (1), 73−79.
(54) Li, X.; Rui, J.; Xiong, J.; Li, J.; He, Z.; Zhou, J.; Yannarell, A. C.; Mackie, R. I. Functional potential of soil microbial communities in the maize rhizosphere. *PLoS One* **2014**, *9* (11), No. e112609.

(55) Trivedi, P.; Delgado-Baquerizo, M.; Trivedi, C.; Hu, H.; Anderson, I. C.; Jeffries, T. C.; Zhou, J.; Singh, B. K. Microbial regulation of the soil carbon cycle: Evidence from gene-enzyme relationships. *ISME J.* **2016**, *10* (11), 2593−2604.
(56) Liao, L.; Wang, J.; Dijkstra, F. A.; Lei, S.; Zhang, L.; Wang, X.; Liu, G.; Zhang, C. Nitrogen enrichment stimulates rhizosphere multi-element cycling genes via mediating plant biomass and root exudates. *Soil Biol. Biochem.* **2024**, *190*, No. 109306.
(57) Kuypers, M. M. M.; Marchant, H. K.; Kartal, B. The microbial nitrogen-cycling network. *Nat. Rev. Microbiol.* **2018**, *16* (5), 263−276.
(58) Islam, F.; Yasmeen, T.; Arif, M. S.; Ali, S.; Ali, B.; Hameed, S.; Zhou, W. Plant growth promoting bacteria confer salt tolerance in Vigna radiata by up-regulating antioxidant defense and biological soil fertility. *Plant Growth Regul.* **2016**, *80* (1), 23−36.
(59) Wolińska, A.; Szafranek-Nakonieczna, A.; Banach, A.; Błaszczyk, M.; Stępniewska, Z. The impact of agricultural soil usage on activity and abundance of ammonifying bacteria in selected soils from Poland. *Springerplus* **2016**, *5* (1), No. 565, DOI: 10.1186/s40064-016-2264-8.
(60) Shimizu, K. Regulation Systems of Bacteria such as *Escherichia coli* in Response to Nutrient Limitation and Environmental Stresses. *Metabolites* **2014**, *4* (1), 1−35.
(61) Morano, K. A.; Grant, C. M.; Moye-Rowley, W. S. The response to heat shock and oxidative stress in saccharomyces cerevisiae. *Genetics* **2012**, *190* (4), 1157−1195.
(62) Elsholz, A. K. W.; Gerth, U.; Hecker, M. Regulation of CtsR Activity in Low GC, Gram+ Bacteria. In *Advances in Microbial Physiology*; Elsevier, 2010; Vol. *57*, pp 119−114.
(63) Coyne, S.; Rosenfeld, N.; Lambert, T.; Courvalin, P. and Périchon, B. Overexpression of resistance-nodulation-cell division pump AdeFGH confers multidrug resistance in Acinetobacter baumannii. *Antimicrob. Agents Chemother.* **2010**, *54* (10), 4389−4393.
(64) Wu, G.; Kang, H.; Zhang, X.; Shao, H.; Chu, L.; Ruan, C. A critical review on the bio-removal of hazardous heavy metals from contaminated soils: Issues, progress, eco-environmental concerns and opportunities. *J. Hazard. Mater.* **2010**, *174* (1−3), 1−8.
(65) Baral, B.; Akhgari, A.; Metsä-Ketelä, M. Activation of microbial secondary metabolic pathways: Avenues and challenges. *Synth. Syst. Biotechnol.* **2018**, *3* (3), 163−178.
(66) Lund, P.; Tramonti, A.; De Biase, D. Coping with low pH: Molecular strategies in neutralophilic bacteria. *FEMS Microbiol. Rev.* **2014**, *38* (6), 1091−1125.
(67) Hunting, E. R.; Barmentlo, S. H.; Schrama, M.; van Bodegom, P. M.; Zhai, Y.; Vijver, M. G. Agricultural constraints on microbial resource use and niche breadth in drainage ditches. *PeerJ* **2017**, *5* (12), No. e4175.
(68) Mueller, E. A.; Wisnoski, N. I.; Peralta, A. L.; Lennon, J. T. Microbial rescue effects: How microbiomes can save hosts from extinction. *Funct. Ecol.* **2020**, *34* (10), 2055−2064.
(69) Schmidt, R.; Gravuer, K.; Bossange, A. V.; Mitchell, J.; Scow, K. Long-term use of cover crops and no-till shift soil microbial community life strategies in agricultural soil. *PLoS One* **2018**, *13* (2), No. e0192953.
(70) Lundgren, J. G.; Duan, J. J. RNAi-Based Insecticidal Crops: Potential Effects on Nontarget Species. *Bioscience* **2013**, *63* (8), 657−665.

https://doi.org/10.1021/acs.est.4c01685
Environ. Sci. Technol. 2024, 58, 13856−13865