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Method Article

Adaption of microarray primers for iron transport and homeostasis gene expression in *Pseudomonas fluorescens* exposed to nano iron



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A B S T R A C T

Modified protocols were adapted for PCR and culture based methods for the analysis of *Pseudomonas fluorescens* cells exposed to nanoscale zero-valent iron (NZVI) and iron (Fe) in bacterial growth nutrient media was determined by a modified atomic absorption spectrometric (AAS) analysis method. We adapted sets of microarray primers used to quantify gene expression of *pvdS* and a bacterioferritin-associated ferredoxin gene for use in real-time quantitative reverse transcription (qRT-PCR) analysis. *pvdS* is one of a cluster of genes regulating the synthesis of the siderophore pyoverdine that was also measured using chrome azurol S (CAS) plates.

- The current protocol provides a detailed qRT-PCR method for quantifying genes involved in the acquisition and utilization of Fe in *P. fluorescens* cells exposed to NZVI.
- The qRT-PCR results were independently corroborated with 2 culture based methods, growth curves and chrome azurol S (CAS) plate.
- The modified AAS method was used to measure Fe in Tryptic Soy Broth (TSB) medium where sodium (Na) causes inference in iron measurement.

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A R T I C L E I N F O

Method name: Real-time quantitative reverse transcription (qRT-PCR) analysis

Keywords: qRT-PCR, Azurol S (CAS), Atomic absorption, Pyoverdine, Ferredoxin

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Specifications Table

Subject Area:	Environmental Science
More specific subject area:	Adoption of microarray primers to qRT-PCR
Method name:	Real-time quantitative reverse transcription (qRT-PCR) analysis
Name and reference of original method:	Lim, C. K.; Hassan, K. A.; Tetu, S. G.; Loper, J. E.; Paulsen, I. T., The Effect of Iron Limitation on the Transcriptome and Proteome of <i>Pseudomonas fluorescens</i> Pf-5. <i>Plos One</i> 2012, 7 (6).
Resource availability:	Not applicable

Method details

In this method, we quantified expression of two functional genes partially controlling nutrient acquisition and utilization of Fe in *P. fluorescens* by developing a qRT-PCR protocol for primers previously included in a microarray analysis by Lim et al. [2]. In addition, we deployed a chrome azurol S method containing a desferal control that was modified from Shin et al. [2] as an independent verification of our qRT-PCR results. Gene expression is dependent upon the amount of Fe available to bacterial cells as was verified in our associated research paper published in *NanoImpact* [3]. The amounts of Fe released in the TSB media from nanoscale zero-valent iron (NZVI) and microscale zero-valent iron (MZVI) during a 48 h test period were measured. TSB is a nutrient rich media which was found to interfere in iron determination. So, we also validated our Fe determination methods using an atomic absorption spectrophotometry (AAS) with Tryptic Soy Broth (TSB) as the matrix.

Experimental procedure

In order to verify that decreases, increases or absence of mRNA activity were due to the interaction of the NZVI with *P. fluorescens* and not the result of the NZVI altering the biochemistry of the qRT-PCR reactions, we independently verified our qRT-PCR results using two separate culture techniques. A set of growth curves were obtained from *P. fluorescens* control cultures receiving no NZVI and cultures exposed to NZVI concentrations of 1, 2 and 5 g L⁻¹. The same bacterial culture stocks were grown on CAS agar plates without Fe and with Fe additions (MZVI or NZVI) at treatment rates of 1, 2 and 5 g L⁻¹ (see Sinha et al. [3] for details). Viable *P. fluorescens* cell numbers in cultures treated with 2 and 5 g L⁻¹ NZVI in the growth curve and blue agar CAS assay experiments were insufficient to determine the presence of siderophores or obtain viable cell counts. Therefore, messenger ribonucleic acid (mRNA) was extracted and converted to cDNA from *P. fluorescens* cultures not receiving Fe additions and from cultures grown for 24 h in the presence of 1 g L⁻¹ NZVI, 1 g L⁻¹ MZVI and 2 g L⁻¹ MZVI. “*P. fluorescens* RNA was isolated using the UltraClean[®] Microbial RNA Isolation Kit (MOBIO Laboratories Inc., Carlsbad, CA) and purified with Ambion[®] DNA-free[™] DNase Treatment and Removal Reagents (MOBIO Laboratories Inc., Carlsbad, CA). The SuperScript[®] VILO[™] cDNA Synthesis Kit was used to synthesize cDNA from mRNA (Invitrogen, Grand Island, NY)” (taken from Sinha et al. [3]).

qRT-PCR method

qRT-PCR analyses were conducted to determine whether genes that regulate siderophore production and the utilization of Fe stores in *P. fluorescens* cells were actively producing mRNA in the presence of 1 g L⁻¹ NZVI and MZVI. Under Fe limiting conditions *P. fluorescens* obtains Fe from outside the cell and from stores of Fe rich proteins [1,4–6]. One of the cluster of genes that regulates the synthesis of the siderophore pyoverdine is *pvdS* (PFL 4190) [7]. The other gene bacterioferritin-associated ferredoxin (PFL 4858) is involved in mobilization and storage of Fe from the protein, bacterioferritin B) [1]. This gene is activated when the bacteria utilizes stored Fe in the protein.

All cDNA samples were diluted with DNA/RNase free water for qRT-PCR analysis and amplified with primers used to isolate genes that regulate Fe acquisition in *P. fluorescens* [1]. The two primer sets were originally included in a microarray experiment and designed to amplify a 145 bp fragment of the

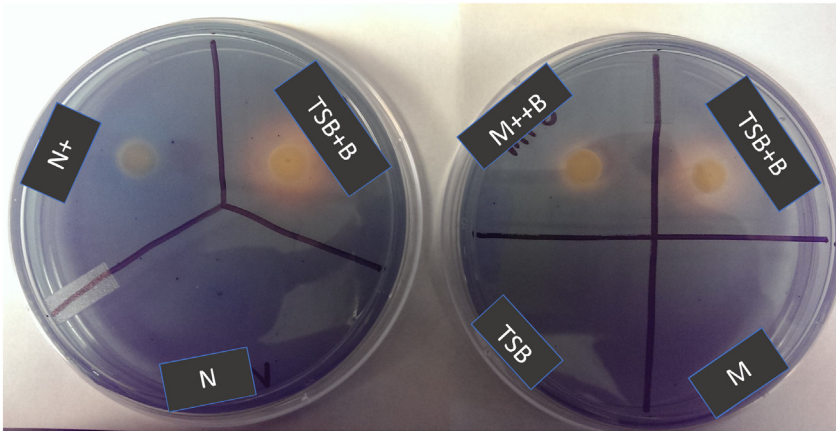


Fig. 1. Siderophore production in *Pseudomonas fluorescens* colonies grown on CAS agar plates. Siderophore production was assessed using chrome azurol S (CAS) plates. Presence of a halo on the CAS plates indicates that *P. Fluorescens* colonies are producing and excreting siderophores and absence of a halo indicates that no siderophores were produced. Legends: **N+B**: NZVI + Bacteria; **TSB+B**: Tryptic Soy Broth + Bacteria; **M+B**: MZVI+Bacteria; **N**: NZVI only; **TSB**: Tryptic Soy Broth only; and **M**: MZVI only.

pvdS gene, PFL_4190-qF *pvdS* CAGATCACCTCCTCGTTCAA and PFL_4190-qR *pvdS* CGCCATGAATAAC-CACATTC, and a 172 bp fragment of the bacterioferritin-associated ferredoxin gene, PFL_4858-qF ferredoxin CGACGGACAAATTCGCGAAGCG and PFL_4858-qR ferredoxin CACAGGGTAGGGGATTGCCGC (Invitrogen, Carlsbad, CA) [1]. In order to adapt and optimize the primers for use in qRT-PCR protocols, we varied template concentration (4, 40, 400 ng DNA μL^{-1}), primer concentrations (100–400 nM), annealing temperature (55–65 °C) and the timing of the thermocycler protocol. Lim et al. [1] did not use the above primers for quantitative analyses. Therefore, authors are not aware of any other published research using these primers for qRT-PCR.

Once optimized, concentrations in the master mix and the thermocycler program were held constant for the two primer pairs. cDNA was amplified using a PikoReal™ Real-Time PCR System (Thermo Scientific, Wilmington, DE). Each 20 μL reaction contained 10 μL Fast SYBR® Green Master Mix (Applied Biosystems Inc., Foster City, CA), 0.4 μL each of the forward and reverse primer at 10 μM , 8.2 μL RNA/DNA free water (H_2O), and 1 μL RNA/DNAase free H_2O that contained 4 ng of cDNA template. Environmental samples were run in triplicate. The qRT-PCR thermocycler protocol used was

Table 1
Siderophore production in *Pseudomonas fluorescens* colonies grown on CAS agar.

Treatment	Time of Sampling ^a Hours (h)					
	0	6	12	18	24	30
Bacterial Control (No NZVI or MZVI)	absent	absent	halo	halo	halo	halo
NZVI (1 g L ⁻¹)	absent	absent	absent	absent	absent	absent
NZVI (2 g L ⁻¹)	absent	absent	absent	absent	absent	absent
MZVI (1 g L ⁻¹)	absent	absent	absent	absent	absent	absent
MZVI (2 g L ⁻¹)	absent	absent	absent	absent	absent	absent
NZVI (1 g L ⁻¹) + Bacteria	absent	absent	absent	absent	absent	absent
NZVI (2 g L ⁻¹) + Bacteria	absent	absent	absent	absent	absent	absent
MZVI (1 g L ⁻¹) + Bacteria	absent	absent	absent	halo	halo	absent
MZVI (2 g L ⁻¹) + Bacteria	absent	absent	absent	halo	halo	absent

Presence of a halo on the CAS plates indicates that *P. Fluorescens* colonies are producing and excreting siderophores and absence of a halo indicates that no siderophores were produced.

^a Siderophore production was assessed using chrome azurol S (CAS) plates.

7Table 2
Details of AAS setup used in iron determination.

Element	Wavelength (nm)	Slit (mm)	Lamp Current (mA)	Air (L/min)	Acetylene (L/min)
Fe	248.33	0.2	30	10.0	3.16

min at 95 °C followed by 40 cycles of 95 °C for 10 s, and 60 °C for 30 s with a data acquisition step, followed by a melt curve analysis ($R^2 = 0.99$). Genomic DNA isolated from organismal controls and cDNA from TSB samples were run in triplicate. Master mix containing no cDNA was run in triplicate and served as a control. Standard curves were generated for each functional gene using 5 serial dilutions of genomic DNA isolated from *Pseudomonas fluorescens* Migula (ATCC[®] BAA-477D[™], Manassas, VA) ranged from 8.6 ng μL^{-1} to 8.6 fg μL^{-1} .

Chrome azurol S (CAS) plate preparation

This method is a common protocol used for screening various microorganisms for their ability to produce siderophores [8] but includes an additional internal control, desferal [2]. All glassware was acid washed and triple rinsed with double distilled water (ddH₂O) to remove any metals. The CAS media consisted of a blue dye, a nutrient mixture and a Casamino acid solution. The blue dye contained three separate solutions: 0.06 g CAS (Fluka Chemicals) in 50 mL of ddH₂O (Solution 1); 0.0027 g of FeCl₃·6H₂O in 10 mL of 10 mM HCl (Solution 2) and 0.073 g of HDTMA in 40 mL of ddH₂O (Solution 3). Specifically, 9 mL of Solution 2 was added to 50 mL of Solution 1 which was then added to 40 mL of Solution 3 and autoclaved to complete the blue dye production. The nutrient mixture contained Minimal Media 9 Salt Solution Stock (15 g of KH₂PO₄, 25 g of NaCl and 50 g of NH₄Cl in 500 mL of ddH₂O), 20% glucose (20 g of glucose in 100 mL of ddH₂O) and NaOH stock (25 g of NaOH in 150 mL ddH₂O, pH 12). The Casamino acid solution was made by dissolving 3 g of Casamino acid in 27 mL of ddiH₂O. Potential iron contaminants were removed with 3% 8-hydroxyquinoline in chloroform. The casamino solution (27 mL) and chloroform (3 mL) were mixed together (total of 30 mL) and allowed to stand at 4 °C until two separate layers formed after which the supernatant was put through a 0.2 μm PPT filter and stored in an air tight container.

CAS Agar preparation consisted of the addition of 100 mL of MM9 solution to 375 mL of ddiH₂O after which 32.24 g of PIPES (piperazine-*N,N'*-bis (2-ethanesulfonic acid)) was added and dissolved in the solution. After addition of 15 g of Bacto Agar, the solution was autoclaved for 15 min and allowed to cool to 50 °C. Once the solution was cooled, 30 mL of sterile Casamino acid solution and 10 mL of sterile 20% glucose was added to the MM9/PIPES mixture. In the final step, 100 mL of blue dye solution was added while the solution was continuously stirred. The plates were poured using aseptic technic and the color of the plates were blue.

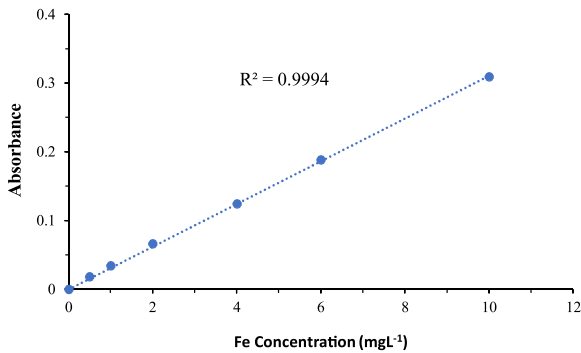


Fig. 2. Calibration Curve for Fe.

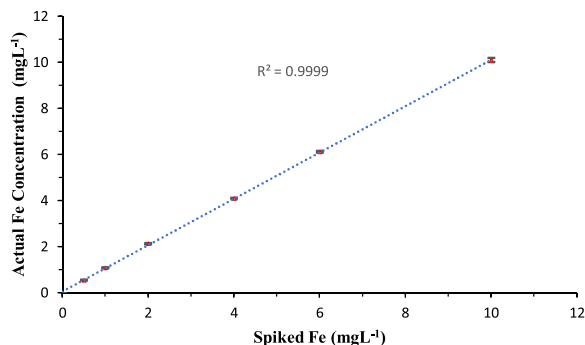


Fig. 3. Fe content in TSB media samples spiked with iron. Standard deviations ($n = 3$) were negligible but have been shown here as vertical error bars.

CAS agar plates from all treatments were replicated in triplicate. Plates were incubated with 100 μL of bacteria grown for 24 h in the presence of variable concentrations of NZVI and MZVI 1, 2 and 5 g L^{-1} . Controls included TSB broth with bacteria and TSB broth with NZVI concentrations of 1, 2 and 5 g L^{-1} without bacteria and MZVI 1, 2 and 5 g L^{-1} without bacteria. Standards were made using known concentrations of desferal a chelating reagent that was added to plates as an internal control [2] (Fig. 1 and Table 1).

AAS analysis of iron release from NZVI and MZVI in TSB media

Preparation of tryptic soy broth (TSB)

A measured amount (30 g) of TSB contains 17 g of pancreatic digest of casein, 3 g papaic digest of soybean, 5 g sodium chloride, 2.5 g dipotassium phosphate, and 2.5 g dextrose was dissolved in 1 L water and autoclaved. Experimental: Measured amount (20 mg) of NZVI or MZVI particles were added to 50 mL sterile centrifuge tubes (reactors) and 20 mL TSB medium was added to each reactor. The mixture was sonicated to evenly disperse the particles in the medium. The reactors were then placed onto a rotating incubator (180 rpm, 30 °C) shaker. A set of 3 sacrificial reactors (for both NZVI and MZVI) were withdrawn at different time interval (0, 6, 12, 18, 24, 30, 48 h) and aliquots were separated. A definite amount (14 mL) of aliquot sample was filtered with a 0.2 μm nylon housing syringe filter. The filtered sample was acidified with HNO_3 and retained for iron analysis. Iron was determined using a PerkinElmer Atomic Absorption Spectrometer (AAS, PinAAcle 900H) in Flame mode (Details of instrument Setup in Table 2).

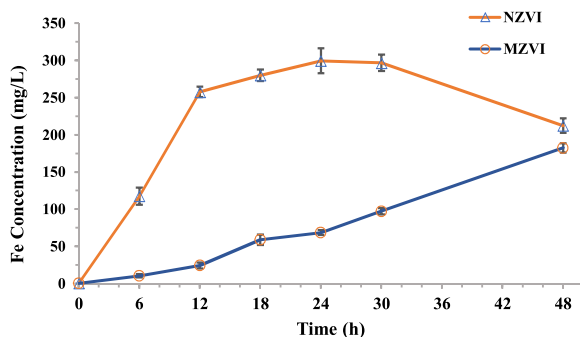


Fig. 4. Measurement of Fe-ion dissolution from nanoscale zero-valent iron (NZVI) and microscale zero-valent iron (MZVI) particles in TSB media using a method developed within this study. NZVI and MZVI dose 1 g L^{-1} . Error bar indicates the standard deviation value ($n = 3$). Independent method may have to be developed for different nanomaterials and/or media used.

Table 3
Iron dissolution (mg L^{-1}) in TSB media (NZVI and MZVI dose = 1 g L^{-1}) ($n = 3$).

Time (h)	Ionic Fe (mg L^{-1}) in TSB Media	
	NZVI	MZVI
0	0.52 ± 0.02	0.52 ± 0.02
6	117.95 ± 11.52	10.56 ± 2.29
12	257.79 ± 7.02	24.53 ± 4.12
18	280.27 ± 7.94	59.14 ± 7.21
24	299.80 ± 16.71	68.87 ± 3.41
30	297.05 ± 10.94	97.43 ± 4.02
48	212.59 ± 9.82	182.64 ± 6.08

Samples were diluted with 1% HNO_3 solution before Fe determination. A standard curve was prepared (Fig. 2) using a range of Fe concentrations ($0, 0.5, 1, 2, 4, 6, 10 \text{ mg L}^{-1}$) derived from an iron (Fe) standard containing $1000 \text{ mg Fe L}^{-1}$ Fe stock solution (Alfa Aesar). The limit of detection (LOD) of the AAS in Flame mode was $5 \mu\text{g L}^{-1}$. In our work the calculated LOD was $8.7 \mu\text{g L}^{-1}$ which was calculated as $\text{LOD} = 3 s m^{-1}$ where m is the calibration curve slope and s is the standard deviation of the blank solution signal in AAS [9]. The standard protocol was modified to take into account the high sodium (Na) content in the TSB medium. Although the wavelength for Fe (248.33 nm) determination in the instrument does not overlap with that of Na (589 nm), Sodium (Na) is an emissive element and interfered with our analysis. To determine the effects of the TSB media matrium on Fe determinations, we diluted the media with HNO_3 to achieve the predetermined concentrations of Fe. It was evident from our results (Fig. 3) that the Fe spiked TSB samples give satisfactory results ($R^2 = 0.9997$). The Fe content in blank standard (in the TSB matrix) was $0.14 \pm 0.01 \text{ mg L}$. Fe-ion dissolution patterns from NZVI and MZVI in TSB media were successfully studied using this method (Fig. 4 and Table 3). The Fe dissolution pattern from NZVI in this experiment is comparable with literature values of $350\text{--}360 \text{ mg L}^{-1}$ [10] and 198 mg L^{-1} [11] when 1 g L^{-1} NZVI was used.

Conclusion

Nanomaterials such as NZVI have different chemical properties and react differently than microscale (bulk) materials. Therefore, there is a need to develop and adapt current molecular, biochemical and chemical protocols for use with nanomaterials in order to account for the unique properties that nanomaterials exhibit. The authors have adapted protocols that are designed for non-nanomaterials and modified them for nano-iron and independently verified the qRT-PCR application results using two separate culture techniques that are currently deemed formal microbial protocols. Specifically, each independent protocol corroborated that variations in or the absence of mRNA activity obtained from the qRT-PCR protocol in this paper were due to the interaction of the NZVI with *P. fluorescens* and not the result of the NZVI altering the biochemistry of the qRT-PCR reactions. This work has validated that the existing methods can be for nanomaterials with careful modification if needed. The current methods were developed to address the needed modifications to existing protocols for use to evaluate NZVI. Specific methods may have to be developed for each nanomaterial tested.

Acknowledgements

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