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Quantitative detection of selenate-reducing bacteria by real-time PCR targeting the selenate reductase gene

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ABSTRACT

We designed a primer set to target selenate reductase (SerA) for detecting selenate reducing bacteria (SeRB). Our *serA* gene-based PCR primer set has high specificity in that it and positively amplified some SeRB, but not denitrifying bacteria (DB). Phylogenetic analysis of *serA* clone sequences of environmental samples from selenate-reducing membrane biofilm reactor (MBfR) biofilms showed that these sequences were closely grouped and had high similarity to selenate reductase gene sequences from SeRB *Thauera selenatis* and DB *Dechloromonas*; however, they were distant to other genes from dimethylsulfoxide (DMSO) enzyme family. Constructing a standard curve targeting the *serA* gene, we found that the good linearity for the qPCR assay when applied it to quantify SeRB in MBfR biofilms, and the gene copies of SeRB correlated well to the selenate removal percentages. Our results demonstrated the feasibility of using the *serA* gene-based PCR primer set to detect and quantify SeRB in environmental samples.

1. Introduction

A variety of natural, agricultural, and industrial processes can cause serious selenium- (Se) environmental contamination; among which the main toxic forms of Se are soluble selenate (SeO_4^{2-}) and selenite (SeO_3^{2-}) [18,31]. Soluble forms of Se impose serious health concerns because of their toxicities to organisms [12]. Hence, regulations in the United States have set up a maximum contaminant level (MCL) for drinking water of 50 μg-Se/L [46]). Bacterial strains capable of respiratory selenate reduction, a process that produces insoluble selenium (Se^0), have been isolated from different environmental sources [8,11,21,34,35,26]. Though these selenate-reducing bacteria (SeRB) have been studied for decades, their population dynamics in the environment remain unknown.

Estimating the abundance and growth of functional bacteria helps to assess accurately the potential of biological remediation and further optimize current treatment processes. Real-time qPCR was invented in 1993 [17] and has been applied to quantitatively detect functional bacterial groups by their specific genes (functional genes or conserved partial *16S rRNA* gene). For instance and after as first reported by Braker et al. [5], the *nirK* and *nirS* gene were intensively applied to target denitrifying bacteria (DB) in many different types of environmental samples [2,50]. Nozawa-Inoue et al. [30] developed a very accurate assay using *pcrA* gene to quantitatively analyze the abundance of perchlorate-reducing bacteria (PRB). The abundances of methanogens and *Dehalococcoides* have successfully been detected by qPCR as well [53,10]. Nevertheless and to our knowledge, scientific evidence is missing on the quantification of SeRB by qPCR.

Part of the problem is the frequent association of selenate reduction with denitrification process. Based on previous studies, selenate reduction has often been suggested to be a side reaction of the respiratory nitrate reductases. Both the membrane-bound (Nar) and periplasmic (Nap) nitrate reductases from *Paracoccus denitrificans* and *Paracoccus pantotrophus* were found to have selenate reductase activity [37,3]. However, researchers also found that nitrate reductases are poor reducers of selenate and may not contribute significantly to global selenate reduction [37,3,7]. Although common in practice [37], tracking denitrifying bacteria as surro-

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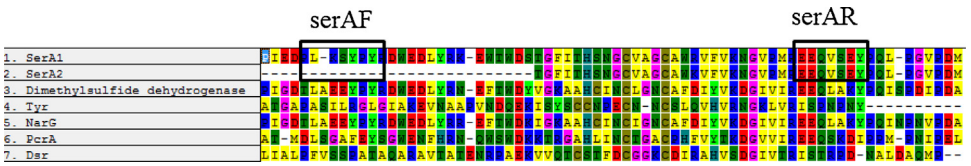

Fig. 1. Sequence alignment for selenate reductase and related molybdo-enzymes. The black-boxed parts represent positions with identical residues in all seven sequences, and the gray-shaded parts represent positions with identical residues in most of the sequences. serA, selenate reductase sequence 1 (SerA1) from *Thauera selenatis* [AX; AJ007744]; serA, selenate reductase sequence 2 (SerA2) from *Dechloromonas sp*. A34 [ACV70151]; Dimethylsulfide dehydrogenase (AF453479); *Tyr*, Trimethylamine-*N*-oxide reductase (cytochrome C, *Desulfitobacterium dichloroeliminans*); *narG*, nitrate reductase from *Sagittula stellate* E-37; *PcrA*, perchlorate reductase from *Dechloromonas agitate* [AAO49008]; *Dsr*, anaerobic dimethylsulfoxide reductase from *Salmonella enterica subsp. enterica serovar Montevideo str*. [IA2010008286, EHP19435].

gate for the abundance of selenate reducers is not accurate enough. Thus, designing specific primers to target SeRB is necessary.

In the process of biological reduction of SeO_4^{2-}, bacteria respire SeO_4^{2-} as a terminal electron acceptor to SeO_3^{2-} by selenate reductase (SerABC) [41] or nitrate reductase (Nar or Nap) [15,37]. SeO_3^{2-} is then reduced to Se^0 by one of the following pathways: (1) periplasmic nitrite reductase (Nir) [11], or (2) hydrogenase I [52], and (3) through non-enzymatic processes. This last pathway refers to gram-negative bacteria having glutathione, which can react with SeO_3^{2-} to form triselensulfide [13] to be further reduced to Se^0 [40].

Two types of selenate reductase have been reported: periplasmic selenate reductase and membrane-bound selenate reductase [48,29]. While the membrane-bound selenate reductase has a broad substrate specificity [43], the periplasmic selenate reductase shows a restricted substrate specificity and does not reduce nitrate, chlorate, or sulphate [41]. The selenate reductases belong to the molybdenum dependent reductase (molybdoreductase) family [19]. The alignment of the periplasmic selenate reductase (SerA) with the periplasmic (NapA) and membrane-bound (NarG) nitrate reductases suggests that SerA is more closely related to NarG than to NapA.

Based on the limited information of the *serA* gene, the objective of this study was to design a real-time quantitative PCR (qPCR) assay to quantitatively detect periplasmic serA enzyme-containing selenate-reducing bacteria (SeRB) in environmental samples. To date, this is the first report of qPCR assay developed to detect SeRB. We analyzed the partial *serA* sequences for SeRB and DB isolates, as well for selenate-, and nitrate-reducing membrane biofilm reactor (MBfR) biofilms. Developing such an assay improves the specificity and accuracy to detect SeRB instead of using nitrate reductases as surrogates.

2. Materials and methods

2.1. Primer design

To identify conserved regions, deduced SerA protein sequences from *Dechloromonas* sp. A34 (GeneBank accession ACV70151) and *Thauera selenatis* (Q9S1H0) were aligned using Clustal W [44]. Although the serA from *Dechloromonas* is a putative selenate reductase, this strain is reported capable to reduce selenate. To identify unique SerA sequence regions, Fig. 1 shows SerA protein sequences from the strains above mentioned along with several molybdoenzyme sequences from the dimethyl sulfoxide (DMSO) reductase family. All selected proteins play key functions in anaerobic respiratory reduction of oxyanions such as nitrate, perchlorate, trimethylamine-*N*-oxide, and dimethylsulfoxide [27], in addition to selenate.

We designed the primer set, serAF (5′-CCGCTCAAGTCCTATCCCTAC-3′), and serAR (5′-ATACTCGCTCACCTGCTCCTC-3′), by using primer premier 5.0 [14] and by referring the sequences and the degrees of genetic code degeneracy. The primer sequences correspond to nucleotide positions 136–156 and 283–303 of the *serA* gene from *T. selenatis*.

2.2. Comparison of serA with functional genes in the DMSO family

We tested the serA primers with DNA from three pure cultures of known SeRB strains (*T. selenatis*, ATCC 55363; *Thauera*. sp., lab isolated; and *Pseudomonas stutzeri*, lab isolated) and two DB strains (*Dechloromonas agitate*, ATCC 700666 and *Pseudomonas sp*., lab isolated). All DB strains were proved with fast denitrification capability and harbor the *narG* and *nirS* genes (unpublished data). We also amplified the *narG* and *nirS* gene from the tested strains to compare the specificity of the designed primers. We then amplified the *serA* and *nirS* genes from five samples of a selenate-, and nitrate-reducing MBfR, cloned the amplicons by using a TOPO TA cloning kit (Invitrogen, China). Positive clones were sequenced following screening with M13 universal primers [16]. We constructed a phylogenetic tree of the deduced *serA* gene sequences of selected 20 positive clones, and corresponding sequences of enzyme genes in the DMSO redutase family (i.e., respiratory nitrate reductase gene (*narG*), formate dehydrogenase gene (*fdhF*), arsenate respiration reductase (*arrA*) and perchlorate reductase (*pcrA*)), by using the neighbor-joining method [38].

2.3. Plasmid standard curve for quantification

For plasmid construction, we amplified the MBfR samples with designed *serA* primers, cloned the amplicon, and picked one of the positive clones for sequencing. We then constructed the plasmid containing the *serA* gene to generate a standard curve based on serial dilutions containing between 10^7 and 10^1 ng/μl target gene copies, which were calculated directly from the concentration of extracted plasmid measured at 260 nm.

2.4. qPCR analysis of serA/nirS/narG in selenate-, and nitrate-reducing MBfR samples

We set up the selenate-reducing MBfR in our previous work and kept the influent concentration of selenate at 1 ppm through stages 1–5 [22]. Each stage lasted at least 24 days. However, we varied the nitrate concentration with 0, 10, 0, 1, and 5 ppm of N/L and then reported its effect on selenate reduction. The reduction rate of selenate and the microbial ecology of the MBfR were greatly influenced by addition of nitrate: 40%, 10% and 60% selenate reduction was achieved when 0, 10, 0 ppm nitrate-*N* was added to the MBfR through stages 1–3. Furthermore, selenate reduction remained steady at ~60% when 1 or 5 ppm of nitrate-*N* was reintroduced in Stage 4 and 5, respectively. During those time periods, we took routine samples for ion analysis to validate steady-state conditions and to quantify the acceptor fluxes prior to take a biofilm sample. Biofilm sampling and DNA Extraction were performed as described previously [22]. We amplified the selenate-, and nitrate-reducing MBfR samples with the designed *serA* primer set to target the *serA* gene. In addition, nirS and narG primer sets [23,45] were