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# Isolation, Screening and Characterization of Exopolysaccharide Producing Bacteria

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***Authors' contributions***

*This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.*

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## ABSTRACT

Exopolysaccharides(EPSs) are essential metabolites synthesized and excreted by certain microorganisms in response to extreme condition of pH, temperature, salinity, osmotic stress and other contaminants for survival in such adverse environment. The present study focuses on isolation of exopolysaccharide producing bacteria from extreme environment of oil polluted soil of Ogoni land and marine water of Bonny Island. Screening of EPS producing abilities of the selected isolates were estimated using two approaches *viz* gravimetric analysis of EPS dry weight and quantification assay for total carbohydrate content by phenol sulphuric acid method. Result revealed that a total of forty (40) different colonies were suspected to produce exopolysaccharide after preliminary screening by selecting thick ropy-like colony formers on agar medium. Secondary screening indicated that twelve (12) isolates produced precipitates above 1000mg/l of total dry weight and eight (8) isolates produced highest EPS yield above 1000mg/l of culture media with carbohydrate content determination. Five isolates with the code WAS1, WAS11, SC6, SOS7 and SOS10 produced significantly higher EPS compared to other bacterial colonies isolated and were termed as most potent EPS producers. These isolates were identified based on 16S rDNA sequence as

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*Providencia stuarti, Escherichia coli, Shewanella chilikensis* and *Bacillus nealsonii*. The result of the present study indicated that these strains have the potentials of producing high exopolysaccharides and can be explored in biotechnological industries.

*Keywords: Bacteria; characterization; exopolysaccharide; marine water and polluted soil.*

## 1. INTRODUCTION

Microbial polysaccharides are macromolecules of glycosidic linkages of several monosaccharide secreted by microorganisms. These polymeric substances can be localized intracellularly as glycogen or extracellularly in the cell [1]. Based on their association with the cell, extracellular polymeric substance can be grouped as covalently bound capsular EPS and loosely bound slime EPS [2]. Loosely bound extracellular polymeric substances comprise about 90% of exopolysaccharide as their main component [3,4].

In the last few decades, there has been considerable rising interest in microbial production of extracellular polysaccharide due to their exceptional properties, and easier extraction and purification processes at higher extents from culture medium [5]. Microbial producers of extracellular polysaccharide can be isolated from different ecological niche with diverse environmental stress which includes marine water, fresh water and soil ecosystem as the most potential sources. Bacterial producing EPS can be generally isolated from environment containing high amount of organic substances such as contaminant soil like crude oil polluted soil [6]. The earth surfaces consist of about 70% marine environment with wide range of microbial diversity such as algae, bacteria, fungi and cyanobacteria [7]. Microbial habitats in such extreme ecological condition develop new adaptive strategies like synthesis of secondary metabolites for continued existence in the surrounding microenvironment. These metabolites like exopolysaccharides play essential physiological role in protecting cells from desiccation, predation, osmotic stress and antimicrobial effects [8]. Besides, they play the function of necessary ecological activities such as adhesion to solid surfaces and formation of biofilm [9].

Moreover, microbial polysaccharides represent an unexplored renewable resource for potential application in industrial biotechnology [10]. Recently, researches toward EPS production have extensively received broad attention due to their different unique complex chemical structures with novel functionalities [11]. To date, studies toward isolation, production and characterization of new exopolysaccharide is constantly in progress. Hence, crucial roles of these polysaccharides direct their emerging uses as biosorbent, binders, coagulants, emulsifiers, stabilizers, gelling agent, thickeners and viscosifiers [12,13].

In this study, exopolysaccharide producing bacteria were isolated from extreme environment of oil polluted soil and marine water and screened for high producing strains. The selected strains were identified using morphological, biochemical and molecular characterization.

## 2. MATERIALS AND METHODS

### 2.1 Sample Collection

Oil polluted soil sample from Ogoniland (Latitude 4.41º, Longitude 7.14º) and marine water sample from Bonny Island both in Rivers State were used for this study. The soil sample was collected 10 meters depth below the soil surface with hand trowel into a sterile plastic bag and transported to the laboratory for further study. The water sample was collected just below the water layer at the middle of the sea with a sterile container and transported immediately to the laboratory, stored at 4ºC until further use.

### 2.2 Isolation and Purification

Serial dilution method was adopted for isolation of bacteria from the samples. A total of 1 g of the soil material and 1 ml of water samples were aseptically suspended in 10 ml sterile physiological saline separately, homogenized and labeled as stock solutions. Aliquots of 1 ml stock solutions were transferred to 9 ml of sterile physiological saline solutions and vortexed. They were diluted serially to sixth dilution. Spread plate method was adopted for enumeration of bacterial isolates by cultivating 0.1 ml of each diluent suspension on Bushnell Hass agar and

nutrient agar supplemented with 2% sucrose as carbon source in duplicate and incubated for 48h [2]. Isolates forming thick mucoid colonies were subsequently selected and purified by streaking on freshly prepared agar medium to obtain distinct colonies [14].

## 2.3 Screening and Selection of EPS Producing Bacteria

Screening of exopolysaccharide producing strength of isolates were assayed gravimetrically by setting the dry weight of exopolysaccharide produced by the isolates and quantitatively by phenol sulphuric acid method. A cell suspension of $1.5x10^8$ bacteria/ ml of overnight grown culture of each inoculum was prepared and inoculated in 50 ml nutrient medium supplemented with 2% sucrose, incubated in rotary shaker at 30ºC for 3 days at 120 rpm. Exopolysaccharides were extracted from cell free supernatant of the culture media with two volumes of cold ethanol and precipitated after refrigerating at 4ºC overnight. The precipitates were collected after centrifuging at 15,000 rpm for 20 mins and dried at 60ºC to constant weight. Pellets in form of EPSs were estimated by measuring total dry weight and assayed for total carbohydrate contents by phenol sulphuric acid test (Abdulrazack et al., 2017) [15]. Bacterial strains which produced most significant yields based on EPS dry weight and carbohydrate content values were selected for further study.

## 2.4 Physiological and Biochemical Identification

Selected bacterial isolates were identified by their morphological characterization based on size, shape and colony morphology on agar medium and examined by Grams staining reaction. Biochemical identification was based on Bergey's Manual of Determinative Bacteriology. Different biochemical tests performed include Indole, voges proskauer, citrate utilization, catalase, starch hydrolysis and carbohydrate fermentation of various sugars [16].

## 2.5 Molecular Characterization

**DNA extraction:** Genomic DNA extraction of strains WAS1, WAS11, SOS7, SOS10 and SC6 were carried out by boiling method. The selected bacterial strains were cultured in Luria Bertani (LB) broth overnight at 35ºC and the cells collected by centrifuging at 14000rpm for 3 minutes. The pellets were washed thrice with normal saline and resuspended in 500microlitres of elution buffer, heat-fixed at 95ºC for 20 minutes. The heated cells were ice-cooled immediately and spun at 14,000rpm for 3 minutes. The DNA holding supernatants were stored at -20ºC for further use.

**Amplification of 16S rRNA gene:** Polymerase chain reaction amplification of 16S rRNA region of extracted genomic DNA of the bacteria was performed using 27: 5$^{l}$-AGAGTTTGATCMTGGCTCAG-3$^{l}$ and 1492: 5'-CGGTTACCTTGTTACGACTT-3' as the forward and reverse primers [17]. The PCR was resolved in a reaction mixture of template DNA, forward and reverse primers, master mix and nuclease free water on an ABI 9700 Applied Biosystems thermal cycler at a final volume of 40 icrolitres. Amplification reaction conditions include initial denaturation at 95ºC for 5 minutes, denaturation at 95ºC for 30 seconds, annealing at 52ºC for 30 seconds and extension at 72ºC for 30 seconds for 35 cycles, final elongation at 72ºC for 5 minutes and hold at 4ºC.

**Gel electrophoresis:** 16S rRNA amplifications were estimated by resolving the amplicons on 1% agarose gel electrophoresis at 130V for 30 minutes and visualized on a blue light transilluminator.

**Sequencing:** The PCR products were sequenced using the BigDye Terminator kit at 96ºC for 10 seconds, 55ºC for 5 seconds and 60ºC for 4 minutes for 32 cycles on a 3510 ABI sequencer.

## 2.6 DNA Data and Phylogenetic Analysis

The sequenced DNA of the strains obtained were edited using the bioinformatics algorithm Trace Edit and analyzed by comparing with closely related sequences retrieved from National Center of Biotechnology Information (NCBI) database using BLAST N. Significant alignment of the sequences were recognized with MAFFT. Using the neighbor-joining method, the evolutionary history was inferred in Molecular Evolutionary Gene Analysis (MEGA 6.0) [18]. The bootstrap consensus tree inferred from 500 replicates representing the evolutionary history of taxa was analyzed [19]. The evolutionary distances of closest identical bacterial species were computed using Jukes-Cantor method and phylogenetic tree constructed [20]. Data were submitted to GenBank database for accession numbers.

## 3. RESULTS

### 3.1 Isolation, Screening and Selection

Two sampling sites selected for this study the oil polluted soil and marine water were serially diluted, plated in nutrient agar medium supplemented with sucrose and incubated. Plates exhibited numerous bacterial counts, out of which a total of forty different colonies were selected and recorded as exopolysaccharide producing strains on the basis of ropy-like or mucoid-forming appearances on agar medium. These isolates were investigated for EPS producing potential using the criteria of gravimetric determination of EPS dry weight after cold ethanol precipitation of cell free supernatant of the cultured medium. Lower number of 20% of total isolates with production ability yield greater than 1.0 g/l was classified as strong EPS producers. Few numbers of the total isolates about 35% belongs to moderate EPS producers with producing ability yield at the range of 0.6-0.9 g/l while majority of the remaining isolates (45%) were grouped as weak producers with production yield less than 0.5 g/l as shown in Fig. 1.

A slight difference was observed from the quantification of carbohydrate content using phenol sulphuric acid test of precipitated EPS of different isolates when compared with result obtained from EPS dry weight. 30% of the total isolates secreted the highest carbohydrate value of >1000 mg/l, followed by another 30% of isolates with carbohydrate content between 700-1000 mg/l while the least carbohydrate value of <600 mg/l was observed with about 40% of the total isolates as shown in Fig. 2.

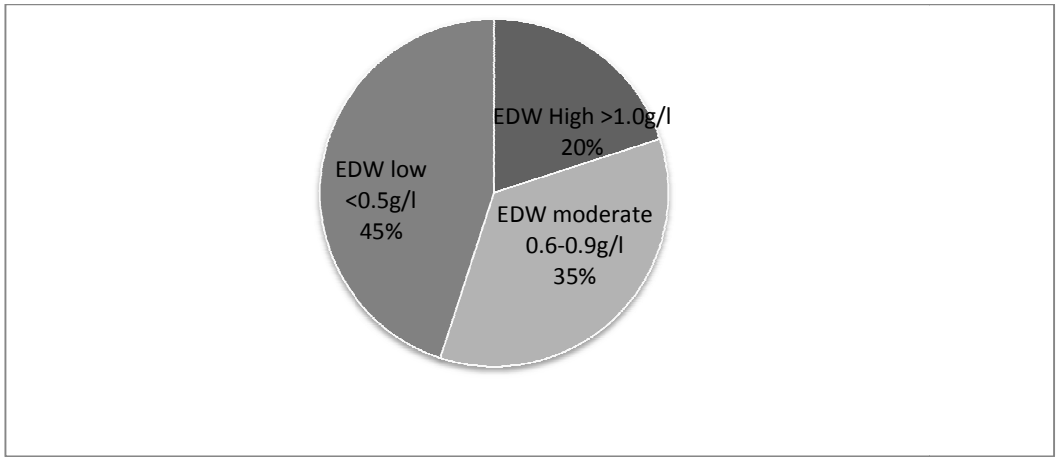


**Fig. 1. EPS dry weight of selected isolates**

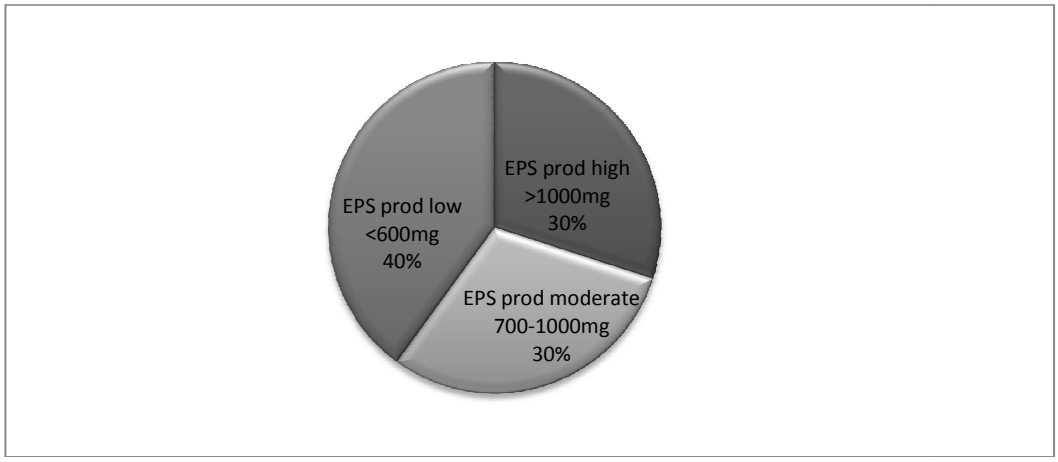


**Fig. 2. EPS production of selected isolates**

Five strains classified as strong EPS producers were selected as potential EPS producers based on quantitative screening and gravimetric analysis. These isolates were designated as WAS1, WAS11, SC6, SOS7 and SOS10.

### 3.2 Physiological, Morphological and Biochemical Identification

The morphological features of selected strains grown on nutrient agar medium were described in Table 1. Their biochemical characteristics are presented in Table 2.

The gel electrophoresis of the PCR products showed clear band of the marker (Lane 1) and base pairs of the five selected bacterial strains as represented in Fig. 3.

The identified bacterial strains were confirmed by sequencing and phylogenetic analysis of 16S rRNA gene. Blast analysis of the 16S rDNA sequences of the strains WAS1, WAS11, SOS7 and SC6 revealed that selected isolates showed close resemblance of 99% with *Escherichia coli, Shewanella chilikensis, Providencia stuarti and Bacillus nealsonii* respectively at nucleotide level. The organisms were confirmed as *Escherichia coli* strain WAS1 (accession number: MN511699), *Shewanella chilikensis* strain WAS11 (accession number: MN511701), *Providencia stuarti* strain SC6 (accession number: MN511702) *and Bacillus nealsonii* strain SOS7 (accession number: MN511700).

The most similarly matched results of 16S rRNA sequences were used for phylogenetic representation (Fig. 4).

## 4. DISCUSSION

Microbial exopolysaccharides are biopolymers secreted by microbial cells as loosely bound slimes in association with the cell surface [21]. Unlike plant polysaccharides, they can be synthesized at higher yield through controlled fermentation conditions. Previous studies showed that the quantities and composition of microbial EPS produced depends on species of organism isolated and its cultural conditions [22]. The successful isolation of bacteria producing EPS can pave way to realizing their biotechnology activities such as antimicrobial, antiviral, oil recovery enhancer, thickener, viscosifiers, suspenders and gelling agents [23]. These strains are described by their phenotypic appearances mostly as glistering, slimy-like or ropy-like colonies on agar medium [24].

**Table 1. Morphological characterization of EPS producing bacteria**

| Characteristics | Observations | | | | |
|---|---|---|---|---|---|
| | WAS1 | WAS11 | SC6 | SOS7 | SOS10 |
| Size | small | medium | large | large | medium |
| Shape | circular | oval | irregular | irregular | circular |
| Consistency | smooth | smooth | mucoid | mucoid | smooth |
| Opacity | opaque | opaque | translucent | translucent | opaque |
| Pigmentation | cream | white | gray | gray | red |
| Grams staining | -ve rod | -ve rod | -ve rod | +ve rod | -ve rod |

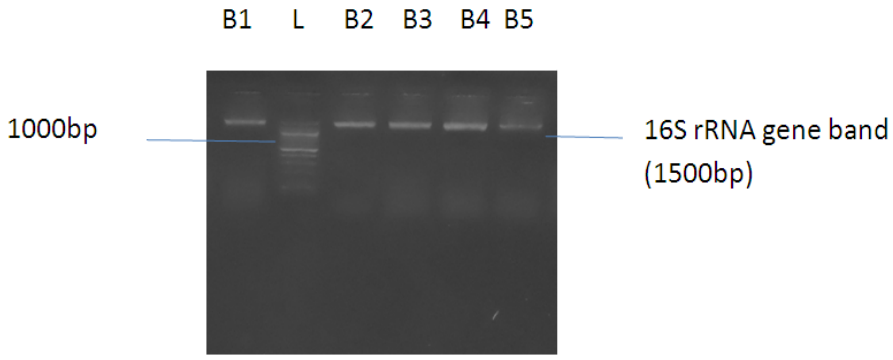


**Fig. 3. Agarose gel electrophoresis of the 16S rRNA gene of some selected bacterial isolates. Lanes B1-B5 represent the 16SrRNA gene bands (1500 bp), lane L represents the 1000bp molecular ladder**

**Table 2. Biochemical characterization of the isolates**

| Biochemical reaction | WAS1 | WAS11 | SC6 | SOS7 | SOS10 |
|---|---|---|---|---|---|
| Catalase | + | + | - | + | + |
| Oxidase | - | + | + | + | - |
| Citrate | + | + | - | - | + |
| Indole | - | + | - | - | + |
| VP | - | - | - | + | + |
| $H_2S$ | - | - | - | + | - |
| Starch hydrolysis | + | - | - | - | - |
| Motility | - | + | - | + | - |
| **Carbohydrate fermentation** | | | | | |
| Glucose | + | + | - | + | + |
| Fructose | + | + | - | + | + |
| Galactose | - | + | + | - | + |
| Maltose | + | + | + | - | + |
| Mannitol | - | + | + | - | + |
| Lactose | - | - | - | - | - |
| Sucrose | + | + | - | - | + |

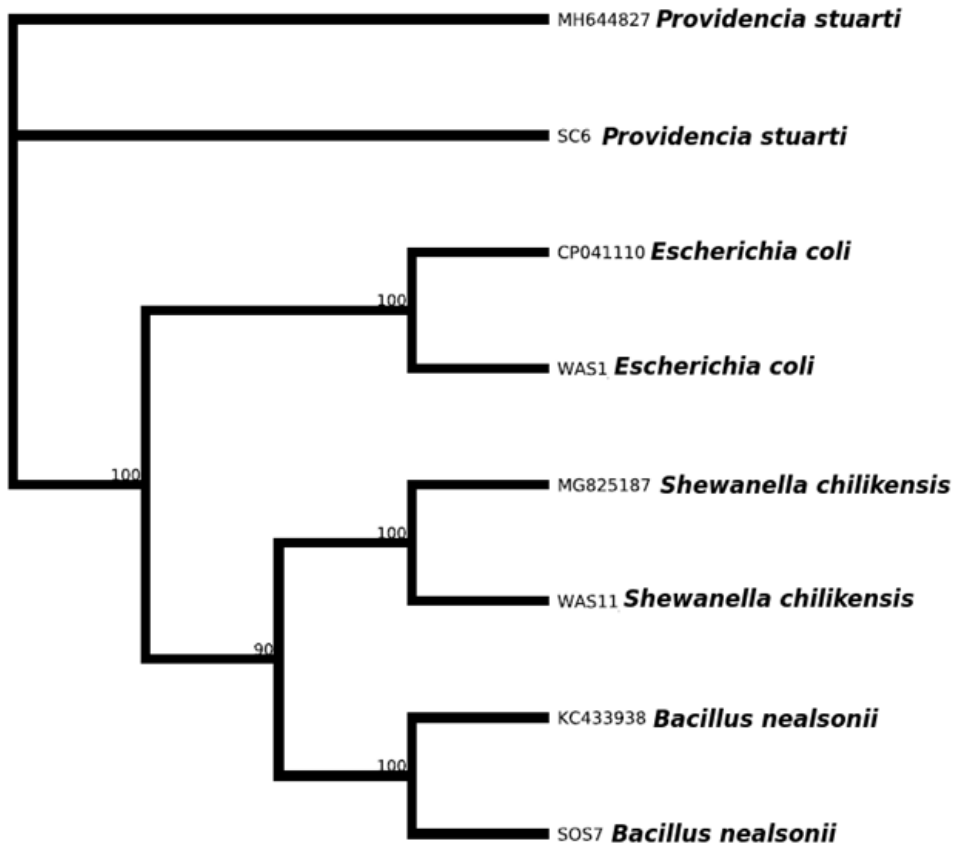


**Fig. 4. Phylogenetic tree showing the evolutionary distance between the bacterial isolates**

Based on these characteristics, forty different bacteria producing exopolysaccharide were selected out of all isolates from two samples in this study. Fang and Catchmark [25] worked on exopolysaccharide production from unexplored microbial diversity with this characteristic. Development of mucoid and slimy colonies of bacterial cultured on solid medium were utilized

as morphological characteristics for screening and selection of EPS producing bacteria by Evans [26] and Patil and Shirsath [27]. Similarly, the study of Shukla and Dave screened 99 marine bacterial isolates based on formation of mucoid colonies as phenotypic characteristics on agar solid medium [7].

An essential nutritional requirement for growth and cell development of bacterial cells is carbohydrate which is a main component of their cytoskeleton [28]. Hence, carbon source containing growth medium function as cell development component and energy source for production and excretion of exopolysaccharides [29]. Therefore, medium containing a carbon source that is easily oxidized will enhance the growth of EPS producing bacteria [30]. In this study, nutrient medium containing 2% sucrose was used as carbon source for isolation and production of exopolysaccharide by the isolates. Pawar and others optimized EPS production of bacterial isolates with different concentration of sucrose and reported that maximum production was obtained from nutrient broth containing 2% sucrose [31]. Shukla and others discovered sucrose supplemented nutrient medium as suitable culture medium for exopolysaccharide production [32]. When different sugars as carbon sources in YE broth were compared for the production of EPS by different isolates, it was discovered that irrespective of selected isolate, 3% sucrose containing medium produced more than at least two fold higher EPS than other sugar [28].

Liu and others explained that highest EPS producing capability of bacteria is a criterion for selection of isolates [33]. In this study, two approaches, quantification of total carbohydrate content present in precipitated EPS by phenol sulphuric acid assay and gravimetric measurement of EPS were employed for selection of highest EPS producers from a battery of forty isolates. From both approaches, result revealed that five isolates designated as WAS1, WAS11, SC6, SOS7 and SOS10 produced copious amount of EPS of above 1.0 g/l and confirmed them to be the most potent producers of EPSs. There is similarity of this work to that achieved by Shukla and Dave where isolate PS-47 produced highest EPS of 1.052 mg/ml among others [7]. The purpose of the secondary screening was to obtain the efficient strain from the selected isolates which produces highest amount of EPS.

The development of a procedure for selection of most potential producers of exopolysaccharide from groups of selected bacterial isolates provide the opportunity to determine the interaction between precipitates cell dry weight and their carbohydrate contents. *Bacillus subtilis* produced EPS containing total carbohydrate of 0.91 mg/100 ml and 0.43 mg/100 ml when cultured on basal and malt media respectively [34]. Correspondingly, highest carbohydrate content of 12.76 g/l and 10.95 g/l was recorded by isolate J47 and J1 among other isolates when cultured on MRS medium [35]. Shukla and others reported highest EPS producing bacteria as *Bacillus* sp which produced EPS up to 12 g/l among others by gravimetric analysis [32]. Also, maximum exopolysaccharide production of 3.10 g/l was observed from rhizobal strain *Mesorhizobium loti* LMG 6125 among others selected [11]. The study of Tudor and others, *L. mesenteroides* 109 produced largest EPS of 19 g/l among other produced EPS when measured gravimetrically (2016).

Four different bacterial isolates were selected from 34 mucoid colony formers as potential better EPS producers after measurements of dry weight of precipitate produced at range of 1.75 to 2.24mg/ml of the medium (Mu'minah et al. 2015).

## 5. CONCLUSION

This work aimed at selecting most efficient strains that produces high amount of EPS from isolates of extreme environment and their identification to species of the isolates. The selected bacterial strains were identified based on reference to key of Bergey's Manual of Determinative Bacteriology on morphological, microscopic and biochemical characteristics. They were further confirmed by the sequencing and phylogenetic analysis of the 16S rRNA genes as *Escherichia coli* strain WAS1, *Shewanella chilikensis* strain WAS11, *Providencia stuarti* strain SC6, *Bacillus nealsonii* strain SOS7 and *Serratia* species strain SOS10.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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