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Characterization and Identification of Biofilm Forming Bacterial Isolate *Shewanella* sp. DDR4

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Abstract: Bacteria can adhere to natural or artificial surfaces and form sessile multicellular communities known as biofilms. The natural and artificial surfaces covered by biofilms include cells and tissues of organisms, soils, sediments, pore in glaciers, thermal vent, pipelines, heat exchangers, separation membranes, and filters. In the marine environment, biofilms cover most subtidal and intertidal solid surfaces such as rocks, ships, loops, marine animals, and algae. Totally 10 bacterial isolates were obtained from three different ships anchored at the Royapuram harbour, Chennai, Tamil Nadu, India and screened for biofilm forming activity. The bacterial isolate DDR4 showed biofilm forming activity in the microtiter plate assay with a significant optical density of 0.632. Also an attempt was made to characterize the morphological, biochemical and molecular properties. Partial sequences of the 16S rRNA genes of the marine bacterial isolate was determined following the amplification of 16S rRNA genes, these sequences were aligned with the sequences of representative species of the genus *Shewanell*a sp. and phylogenetic characters of the isolate *Shewanell*a sp EF559251 with other closely related bacterial isolates were analyzed.

Kew words: Biofilm, marine bacteria, *Shewanella* sp. 16S rRNA genes analysis, and phylogenetic tree.

Introduction

A biofilm is a structured community of microorganisms encapsulated within a self-developed polymeric matrix and adherent to a living or inert surface. Biofilms are also often characterized by surface attachment, structural heterogeneity, genetic diversity, complex community interactions, and an extracellular matrix of polymeric substances. Formation of a biofilm begins with the attachment of free-floating microorganisms to a surface. These first colonists adhere to the surface initially through weak, reversible van der Waals forces. If the colonists are not immediately separated from the surface, they can anchor themselves more permanently using cell adhesion structures such as pili [11,15,16].

Extracellular polysaccharicides are adhesions bacterial appendages such as pili and flagella have been found to play important roles in both the initial stages of attachment to the surface as well as biofilm formation. The genus *Shewanalla is* one of the typical deep sea bacterial genera. One of the best examples of a microorganism causing biofouling is *Shewanalla oneidensis,* an

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extensively studied, gram negative, facultatively anaerobic bacterium, formerly classified as *Shewanella putrefaciens*.

The biocorrosion of steel in the presence of *Shewanella oneidensis* has been documented by Lee and Newman, [14]. Laboratory model systems were developed for studying *Shewanella putrefaciens* adhesion and biofilm formation under batch and flow conditions. *S. putrefaciens* plays a major role in food spoilage and may cause microbially induced corrosion on steel surfaces. *S. putrefaciens* bacteria suspended in buffer adhered readily to stainless steel surfaces *S. putrefaciens* also formed biofilms on stainless steel in a flow system [7]. *S. oneidensis* plays an important role in microbially induced corrosion of metal surfaces due to its capability to reduce solid ferric iron. Since this requires cellular adhesion to the metal surface, biofilm growth is an essential feature of this process [13].

S. oneidensis and related *Shewanella* species have been isolated from corroding steel pipelines a mechanistic understanding of the role of *Shewanella* biofilm formation and metabolic activity may help to facilitate corrosion control [14]. In soil and sediment environments, *S. oneidensis* biofilms form on mineral surfaces and are critical for mediating the metabolic interaction between this microbe and insoluble metal oxide phases. In order to develop an understanding of the molecular basis of biofilm formation, Kai *et al,* [12] investigated *S. oneidensis* biofilms developing on glass surfaces in a hydrodynamic flow chamber system.

Shewanella algae is a mesophilic marine bacterium and is a recently defined species closely related to the more psychrotolerant *Shewanella putrefaciens* [9]. Strains of *S. algae* probably play an important role in the environment, e.g., in the turnover of inorganic material, since the organism is capable of reducing Fe (III) in anaerobic respiration [19]. The detailed study on molecular identification of biofilm forming bacteria from Royapuram harbour, Chennai, Tamil Nadu are very scanty. Keeping these points in view, the present study is aimed to characterize and identify the biofilm forming bacteria in biofilm samples.

Materials and methods

Collection of biofilm sample

The biofilm samples were collected from three different ships anchored at the Royapuram harbour, Chennai, Tamil Nadu during. The samples were brought to the laboratory in ice box for further processing. Isolation of bacteria was carried out immediately upon retrieval of the samples to the laboratory.

Isolation of marine bacteria

The Zobell agar [23] medium was used for the isolation of marine bacteria. The collected biofilm samples were diluted upto 10^{-6} and 0.1 ml of the each diluted sample were inoculated on the medium. Plates were incubated at 28 ± 2°C for 24-48 hours. Three replicates were maintained for each dilution. After incubation, the bacterial colonies were purified and maintained in Zobell agar medium for further investigation [20].

Biofilm formation in polystyrene microtiter plate wells

The bacterial isolates were grown over night in nutrient broth at 37°C. Aliquots of 100 µl were inoculated in six parallel wells of a 96 well microtiter plate and incubated at 37°C for 24 hours. After the incubation period the wells were rinsed with physiological saline to remove the detached cells and fixed with 2ìl of 99.99 % ethanol for 10 minutes. The attached bacterial material was then stained by adding 2 µl of crystal violet (1 %) for 20 minutes. The amounts of attached cells were measured using an ELISA reader at 570 nm [1,18].

Characterization and identification of biofilm forming bacteria

Morphological and biochemical characterization

The isolate which showed maximum biofilm forming activity was then characterized using various morphological, biochemical and molecular characters. Gram staining and motility tests were performed for preliminary identification of the isolate [2]. Morphological parameters studied include colony color, size, shape and margin. Biochemical tests such as catalase, sugar

fermentation and nitrate reduction were carried out by Dalton, *et al*., [5].

Molecular phylogenetic characterization of biofilm forming bacteria

Isolation of chromosomal DNA

Isolates were inoculated in Luria Bertani broth and incubated in a shaking incubator for overnight. 1.5 ml of culture broth was centrifuged at 10,000 rpm for 15 minutes to obtain the pellet. 250 µl of lysis buffer was added to the pellet to lyses the cells and followed by adding 25 µl of 25 % sodium dodecyl sulfate (SDS). Mixture was incubated at 60°C for 20 minutes at room temperature. About 75 µl of sodium per chlorate was added to the mixture and incubated at 27°C for 30 minutes. It was centrifuged at 10,000 rpm for 10 minutes and separated the supernatant. Phenol, chloroform and iso-amyl alcohol (25:24:1) was added to the supernatant and kept for 30 minutes. Mixture was centrifuged at 10,000 rpm for 10 min and separated the upper aqueous layer. Double volume chilled ethanol was added to the aqueous layer along the sides and stored in refrigerator to precipitate the DNA. Mixture was centrifuged at 10,000 rpm for 15 minutes and the supernatant was discarded. The pellet was dried and suspended in 100 µl TE buffer and stored in refrigerator [4].

Separation of chromosomal DNA by agarose gel electrophoresis

1 % Agarose gel was prepared in 1X TBE buffer. 3 µl Bromophenol blue (Tracking Dye) was added to 7 µl of DNA sample and mixed well. DNA samples were loaded into the gel and run the sample until the tracking dye reached three fourth of the gel. Ethidium bromide staining solution was prepared and the gel was stained for 5-10 minutes. The DNA bands were observed on UV-transilluminator.

PCR amplification of 16S rDNA

The bacterial 16S rDNA was amplified by PCR using universal primer pair of 1.0 µl forward primer (5'AGAGTTTGATCCTGGCTCAG3') and 1.0 µl reverse primer (5'ACGGCTACCTTGT TACGACT3'). The 50 µl reaction mixture contained the following components: 5.0 µl template DNA, 5.0 µl of 10X buffer, 2.0 µl of Taq DNA polymerase, 2.0 µl of dNTP mixture and sterile water 34.0 µl. The 50 µl reaction mixture was taken in 0.5 ml of microcentrifuge tube. The total 50µl mixture in the tube was gently spin for 10 seconds and allowed to settle the contents. The PCR program was carried out in the following manner of 30 cycles with an initial denaturation step at 94°C for 2 minutes, followed by denaturation step at 94°C for 45 seconds, annealing at 52°C for 30 seconds, extention at 72°C for 1 minute and final extention at 72°C for 2 minutes. 10 µl of PCR product with 2 µl of loading dye was mixed and loaded on a 1 % agarose gel and analyzed electrophoretically at 60V for 45 minutes. The gel was observed on UV-transilluminator [22].

DNA sequencing

The amplified 16S rDNA sample was sent to First Base, Singapore for partial 16S rDNA nucleotide sequencing. Specific sequencing primer (5'GTATTACCGCGGCGTGCTGG 3') was provided along with the samples for sequencing.

Phylogenetic analysis

The 16S rDNA nucleotide sequence was obtained from sequencing the PCR product. A BLAST of obtained sequence was performed with that of available EMBL database using the site http://www.ncbi.nlm.nih.gov/genebank and the 16S rDNA nucleotide sequence was subjected to Phylogenetic analysis using Bioinformatics tool available in online www.genebee.msu.su/services/html.

Prediction of 16S rRNA restriction sites analysis

The restriction sites present on the bacterial 16S rRNA was analyzed using the NEB Cutter program [6].

Prediction of 16S rRNA secondary structure

The 16S rRNA of *Shewanella* sp.DR4 gene was analyzed for dot plot containing the base pair probabilities, minimum free energy (MFE) structure, centroid structure drawing encoding base-pair probabilities, mountain plot representation of the MFE structures using the RNAalifold webserver.

Results and discussion

Isolation

In the present study, the biofilm samples were collected from three different ships. The samples were plated on Zobell agar medium for bacterial isolation. Totally 10 isolates were obtained 3 isolates from ship I, 2 isolates from ship II and 5 isolates from ship III. The diversity of bacterial isolate was increased due to the nutritive status of water. The biofouling bacteria have been reported from different habitats. Nozue *et al.,* [17] has isolated *Shewanella* species associated with aquatic habitats. Thus it is obvious that *Shewanella* spp., are adopted to diverse habitats which vary widely in space and time and also diverse environmental conditions. The present investigation is different from the previous findings as the *Shewanella* spp., has been isolated from biofilm samples of ships hull.

Biofilm assay

Totally 10 isolates were obtained from three ships and named as RB1, RB1a, RB1b, RB2, RB21, RB22, DDR1, DDR2, DDR3 and DDR4. Among the 10 isolates only DDR4 showed maximum biofilm forming activity in the microtitre plate assay with a significant OD of 0.632. The other isolates did not showed significant biofilm forming activity. (Fig.1, Table1). The microtitre plate method was done by Abdi-Ali *et al.,* [1]. A

Table 1. Screening of Biofilm forming activity in bacterial isolates

Name of the Bacterial isolates	Biofilm forming activity (Optical density)
Control	0.000
RB1	0.053[b]
RB1a	0.051[c]
RB1b	0.045[d]
RB2	0.042[e]
RB21	0.043[e]
RB22	0.046[d]
DDR1	0.039[f]
DDR2	0.038[f]
DDR3	0.023[g]
DDR4	0.632[a]

A value in the each column represents the mean of three replications. Statistically the means of the three experiments were not significantly different ($P< 0.05$). Values in the same column with different letters are significantly different at $P< 0.05$ in accordance with Fisher's least significant difference test.

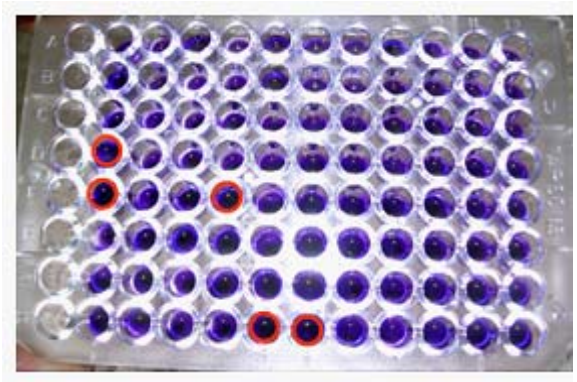

Fig. 1. Screening of biofilm forming activity by microtitre plate with bacterial growth stained with crystal violet

similar biofilm screening has been done by Bhosale *et al.,* [3] using floating glass cover slips on the sterilized filtered sea water to check the adherence of *Bacillus* spp*.,* and *Pseudomonas* spp., biofilms and then staining the cover slips with gentian violet.

Characterization and identification of biofilm forming bacteria

Since only one bacterial isolates were found to possess significant biofouling activity, they were justifiably chosen for the taxonomic characterization. The different parameters namely morphological, biochemical and molecular characters were used for characterization and identification of bacterial isolates.

Morphology

The biofouling bacteria show a notable array of distinguishing microscopic morphology**.** The bacterial isolate DDR4 was observed as orange color circular flat colonies on Zobell agar medium. The staining report showed Gram negative, non spore forming, non capsulated and motile cells.

Biochemical characteristics

Various biochemical characteristics of biofouling bacteria were used for their identification. In the present investigation it was found that the bacterial isolate DDR4 was negative for indole, voges-proskauer and lactose fermentation. In TSI agar, alkaline slant and heavy production of H_2S was observed and was found to be positive for methyl red, citrate utilization, mannitol motility, nitrate reduction, oxidase, catalase, sucrose, maltose and dextrose fermentations (Table 2). The present findings is similar to the findings of Ivanova *et al*. [10]. According to them Shewanella species are gram-negative, motile rods, with the ability to ferment glucose and *Shewanella* species produce H_2S from both organic and inorganic sources.

Molecular characterization

The classification of bacteria using morphological and physiological characteristics does not necessarily lead to the identification of phylogenetically coherent taxa. However some of these characters were shown to be variable with changing media and environmental conditions. Several chemotaxonomic markers (isozyme patterns, fatty acid profile) were used for species and strain level differentiation with little success. To evaluate the bacteria, different PCR based molecular methods namely 16S rDNA sequencing, RAPD, STRR, etc., may be used at all taxonomic levels. The sequence analysis of genes encoding 16S rDNA is currently the most promising approach for phylogenetic classification of bacteria.

The comparison of rDNA sequence is a powerful tool for detecting phylogenetic and

Table 2. Biochemical characteristics of *Shewanella* sp.DDR4

No.	Name of the test	Result
1	Indole	Negative
2	Methyl red	Positive
3	Voges-Proskauer	Negative
4	Citrate	Positive
5	TSI	K/A, H_2S
6	Mannitol motility	Positive
7	Nitrate reduction	Positive
8	Dextrose fermentation	Positive
9	Sucrose fermentation	Positive
10	Maltose fermentation	Positive
11	Lactose fermentation	Negative
12	Catalase	Positive
13	Oxidase	Positive

evolutionary relationship among bacterial species. In the present study the DNA was isolated from the bacterial isolate DDR4 and was analyzed by agarose gel electrophoresis. A sharp band was obtained in the gel. The DNA was found to be approximately 500 bp in length.

The amplification 16S rDNA gene of the bacterial isolate DDR4 was performed by PCR technique using universal primers. PCR product was analyzed in 1% agarose gel. A sharp band was observed which confirmed the PCR product. The amplified 16S rDNA sample was sent to First base, Singapore for partial 16S rDNA nucleotide sequencing. The nucleotide sequence was obtained from sequencing the PCR product. A BLAST of the obtained nucleotide 16S rDNA sequence for the bacterial isolate DDR4 was performed and the sequence showed similarity with the *Shewanella* sp. The 16S rDNA sequences of *Shewanella* sp. DDR4 have been deposited in Gene Bank http://www.ncbi.nlm.nih.gov/genebank and the sequences accession numbers is NCBI - EF 559251. Similar kinds of results has been reported for *Shewanella* genus isolated from deep sea sediments of the west pacific was determined by their 16S rDNA sequence analysis [8].

Phylogenetic analysis is all about understanding the evolutionary relationship between the microorganisms. The easiest way to depict any evolutionary relationship between a group of organisms is building up a phylogenetic tree. A phylogenetic tree of the isolate DDR4 was constructed using its 16S rDNA sequence with that of the other *Shewanella* sp. from the NCBI data base. The resultant phylogenetic tree showed similarity of the isolate DDR4 only at the genus level, where as the isolate did not show any similarity at the species level. (Fig.2). A similar work has been reported as 16S rDNA sequences of *Shewanella* sp. is closely related bacteria such as *Pseudoalteromonas haloplanktis* and *Marinospirillum minutulum* [21].

The restriction sites present on the 16S rRNA of *Shewanella* sp. DDR4 was analyzed using the NEB cutter program and it showed the site for various commercial and NEB (New England Biolabs) restriction enzymes which is around 35 in number. Also it showed the GC and AC contents to be 44% and 55% respectively (Fig. 3).

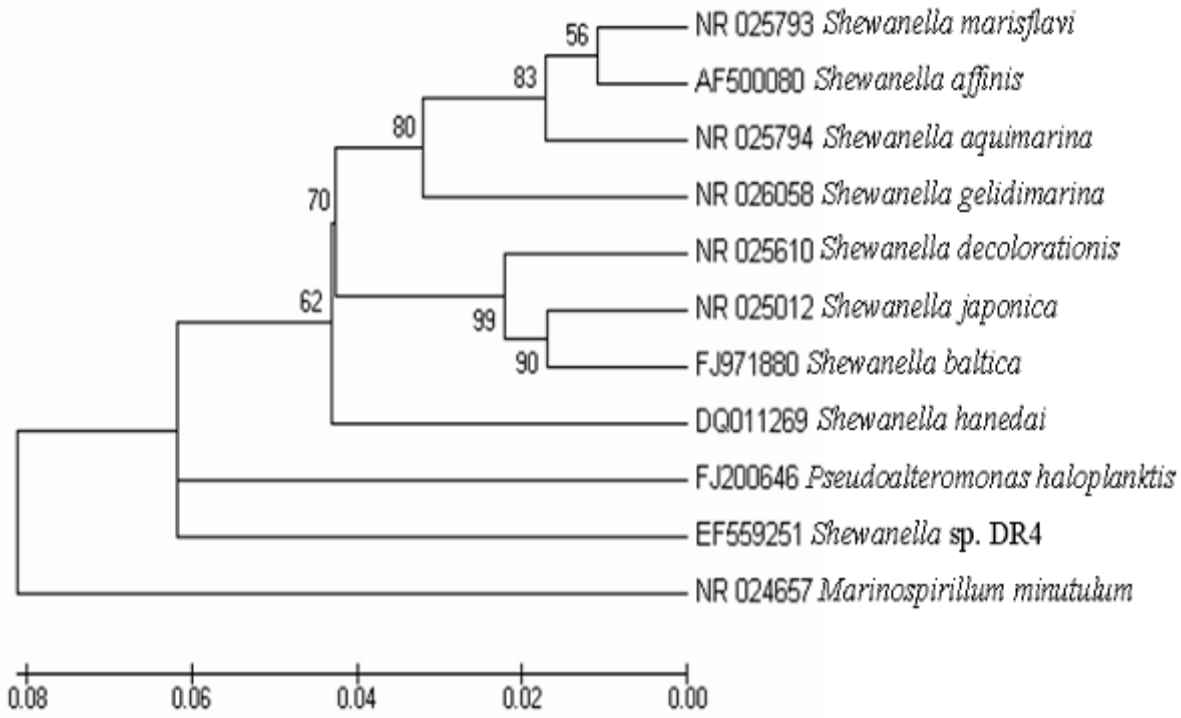

Fig. 2. 16S rDNA-based Phylogenetic tree of *Shewanella* sp. DDR4 with other *Shawanella* spp obtained from NCBI using Neighbor Joining method

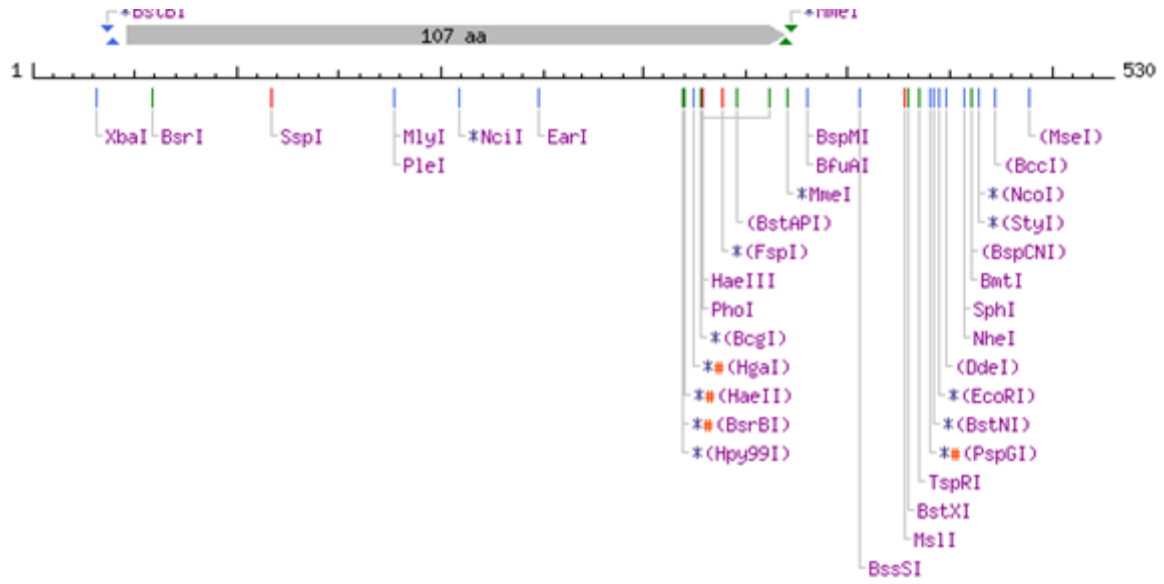

Fig. 3. Restriction site analysis of 16S rDNA of *Shewanella* sp. DDR4 using NEB cutter program

The optimal secondary structure of 16S rRNA gene of *Shewanella* sp DDR4 in dot-bracket notation with a minimum free energy

The optimal secondary structure of 16S rRNA gene of *Shewanella* sp DDR4 in dot-bracket notation with a minimum free energy of -186.30 kcal/mol is given in Fig. 4. If partition function folding was selected, an ensemble structure depicting the base pair probabilities summarized by pseudo bracket notation with the additional symbols ‘,’, ‘|’, ‘{‘, ‘}’ is shown on the results page too. Here, the usual ‘(‘, ‘)’, ‘.’, represent bases with a strong preference (more than 2/3) to pair upstream (with a partner further 3'), pair down-stream, or not pair, respectively. ‘{‘, ‘}’, and ‘,’ are just weaker version of the above and ‘|’ represents a base that is mostly paired but has pairing partners both upstream and downstream. In this case open and closed brackets need not match up. This pseudo bracket notation is followed

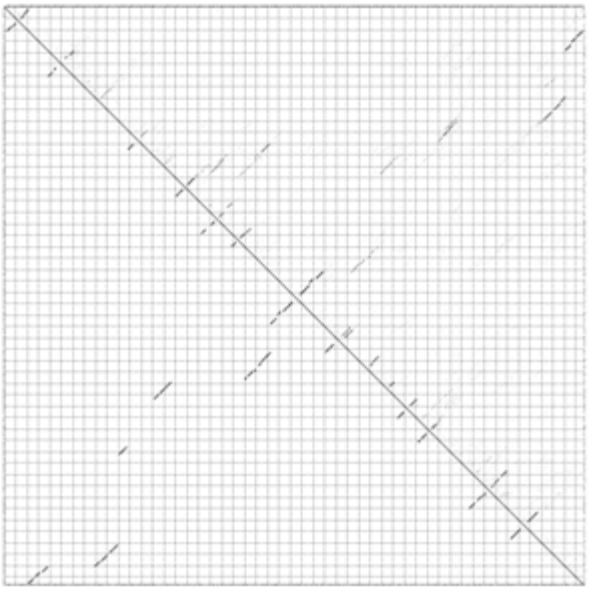

Fig. 4. Dot Plot Containing the Base Pair Probabilities of 16S rRNA gene of *Shewanella* sp DDR4

by the ensemble free energy $F = -kT \ln Q$ in kcal/mol. Considering alignments of RNA sequences, these energies include the covariance term (they're not physical energies).

Thermodynamic ensemble prediction of 16S rRNA gene of *Shewanella* sp DDR4

The free energy of the thermodynamic of 16S rRNA gene of *Shewanella* sp DDR4 ensemble is -192.47 kcal/mol. The frequency of the MFE structure of 16S rRNA gene of *Shewanella* sp DR4 in the ensemble is 0.00 %. The ensemble diversity is 118.73.

This dot plot consists of an upper and a lower triangle of a quadratic matrix. In both dimensions, each letter of the primary structure is assigned to a matrix index *i* and *j*, respectively. Matrix entries at position *i,j* are filled by black boxes indicating a base pair *(i,j)*. In the upper triangle, the size of the boxes is proportional to the base pairing probability where small boxes indicate low and large boxes high probability to form a base pair (i, j). The lower triangle is filled by boxes of equal size, depicting the secondary structure with minimal free energy.

Minimum free energy (MFE) structure

The MFE structure of an RNA sequence is the secondary structure that contributes a minimum of free energy. As an RNA secondary structure can be uniquely decomposed into loops and external bases the loop-based energy model treats the free energy *F(s)* of an RNA secondary structure *s* as the sum of the contributing free energies F_L of the loops *L* contained in *s*. According to the chosen energy parameter set and a given temperature (defaults to 37°C) the secondary structure *s* that minimizes *F(s)* is computed. MFE structure drawing encoding base-pair probabilities of 16S rRNA gene of *Shewanella* sp DDR4 (Fig. 5). The centroid structure of an RNA sequence is the secondary structure with minimal base pair distance to all other secondary structures in the Boltzmann ensemble. Centroid structure drawing encoding base-pair probabilities of 16s rRNA gene of *Shewanella* sp DDR4 (Fig. 6). A mountain plot represents a secondary structure in a plot of height versus position, where the height m(k) is given by the number of base pairs enclosing the base at position k. I.e. loops correspond to plateaus (hairpin loops are peaks), helices to slopes. Mountain plot representation of the MFE structure of 16s rRNA gene of *Shewanella* sp DDR4 (Fig. 7). This study clearly reveals the biofilm forming bacteria *Shewanella* sp. DDR4 in the biofilm sample. The bacterial isolate was characterized and identified by different parameters such as morphological, biochemical and molecular characters. Identifying the mechanism of biofilm formation in ship hull by *Shewanella* sp. DDR4 will be the further course of action.

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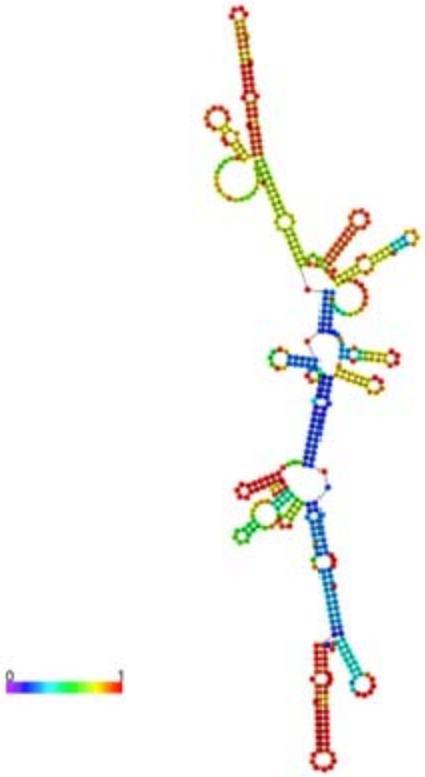

Fig. 5. MFE structure drawing encoding base-pair probabilities of 16S rRNA gene of *Shewanella* sp DR4

Fig. 6. Centroid structure drawing encoding base-pair probabilities of 16S rRNA gene of *Shewanella* sp DDR4

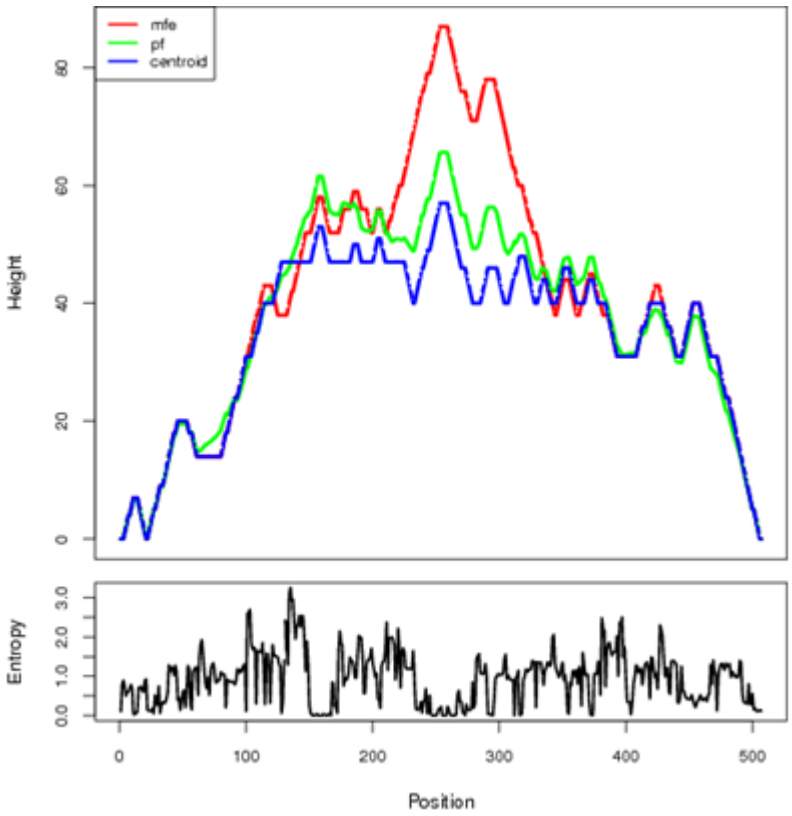

Fig. 7. Mountain plot representation of the MFE structure of 16S rRNA gene of *Shewanella* sp DDR4

References

1. **Abdi-Ali, A., Mohammadi-Mehr, M., Agha, A. (2006)**. Bactericidal activity of various antibiotics against biofilm producing *Pseudomonas aeruginosa*. Int. J. Antimicrob. Agents.. 27: 196-200.
2. **Allegrucci, M., Sauer, K. (2007).** Characterization of colony morphology variants isolated from *Streptococcus pneumoniae* biofilms. J. Bacteriol., 189: 2030-2038.
3. **Bhosale, S.H., Nagle, V.L., Jagtap, T.G. (2002).** Antifouling potential of some marine organisms from India against species of *Bacillus* and *Pseudomonas*. Mar. Biotechnol., 4: 111-118.
4. **Bollet, C., Gevaudan, M.J., Lamballerie, X., Zandotti, C., Micco, P. (1955).** A simple method for the isolation of chromosomal DNA from Gram positive or acid-fast bacteria. Nucleic Acids Research., 19: 4101-4102.
5. **Dalton, H.M., Poulsen, L.K., Halasz, P., Angles, M.L., Goodman, A.E., Marshall, K.C. (1994).** Substratum-induced morphological changes in a marine bacterium and their relevance to biofilm structure. J. Bacteriol., 176: 6900-6906.
6. **Dhanasekaran, D., Thajuddin, N., Rahhmi, Deepika,T.L and Gunasekaran, M. (2009).** Screening of Biofouling activity in marine bacterial isolate from ship hull. Int. J. Environ. Sci. Tech., 6(2), 197-202.
7. **Dorthe, B., Mette, H., Chartolte, J., Ingrid, H., Lone, G. (2001).** Shewanella putrefaciens adhesion and biofilm formation on food processing surfaces. Appl. Environ. Microbiol., 67: 2319-2325.
8. **Fengping, W., Peng, W., Mingxia, C., Xiang, X. (2004).** Isolation of extremophiles with the detection and retrieval of *Shewanella* strains in deep-sea sediments from the west pacific. Extremophiles., 8: 165-168.
9. **Fonnsenbech Vogel, B., Jorgensen, K., Christensen, H., Olsen, J.E., Gram, L. (1997).** Differentiation of *Shewanella algae* using ribotyping whole cell protein profiles, phenotypic characterization and 16S DNA sequence analysis. Appl. Environ. Microbio., 63: 2189-2199.
10. **Ivanova, E.P., Sawabe, T., Gorshkova, N.M., Svetashev, V.I., Miknailov, V.V,. Nicolau, D.V., Christen, R. (2000).** *Shewanella japonica* sp.,nov. Int. J. Syst. Evol. Microbiol., 51: 1027-1033.
11. **Kaplan, J.B., Ragunath, C., Ramasubbu, N. and Fine, D.H. (2003).** Detachment of *Actinobacillus actinomycetemcomitans* Biofilm Cells by an Endogenous ß-Hexosaminidase Activity. J. of Bacteriol., 185: 4693-4698.
12. **Kai, M., Renee, Saville, M., Soni, S., Dale, A.P., Alfred, M.S. (2004).** Initial phases of biofilm formation in *Shewanella oneidensis* MR-1. J. Bacteriol., 186: 8096-8104.
13. **Kris De Vriendt, Sofie, T., Wesley, C., Lina De Smet, Bart. D,, Jozef Van Beeumen. (2005).** Proteomics of *Shewanella oneidensis* MR-1 biofilm reveals differentially expressed proteins, including Agg A and Rib B. Proteomics, 5: 1308-1615.
14. **Lee, A.K., Newman, D.K. (2003).** Microbial iron respiration: impacts on corrosion processes. Appl. Microbiol Biotechnol., 62: 134-139.
15. **Marshall, K.C. (1985).** Mechanisms of bacterial adhesion at solid-liquid interfaces. In: Savage DC, Fletcher M, editors. Bacterial adhesion: mechanisms and physiological significance New York: Plenum Press. 133-161.
16. **Mayer, C., Moritz, R., Kirschner, C., Borchard, W., Maibaum, R., Wingender, J., Flemming, H.C. (1999).** The role of intermolecular interactions: studies on model systems for bacterial biofilms. Int. J. Biol. Macromol., 26: 3-16.
17. **Nozue, H., Hayashi, T., Hashimoto, Y., Ezaki, T., Hamasaki, K., Ohwada, K. (1992).** Isolation and characterization of *Shewanella alga* from human clinical specimens and emendation of the description of *S. alga.* Int. J. Syst. Bacteriol., 42: 628–34.

18. **Peeters, E., Nelis, H.J., Coeny, T. (2008).** Comparison of multiple methods for quantification of microbial biofilms grown in microtiter plates. J. Microbiol. Methods., 72:157-165.
19. **Rosello-Mora, R., Caccavo, F., Springer, N., Spring, S., Osterlehne,r K., Shuler, D., Ludwig, W., Amann, R., Schleifer KH. (1994).** Isolation and taxonomic characterization of a halotolerant facultatively iron-reducing bacterium. Syat. Appl. Microbiol., 17: 569-573.
20. **Saravanan, P., Prabagaran, S.R., Venkata Nancharaiah, Y., Krishnaveni, M., Venugopalan, V.P., Jayachandran, S. (2008).** Isolation and characterization of *Pseudoalteromonas ruthenica* (SBT033), an EPS-producing biofilm bacterium from the seawater intake point of a tropical power station. World J. Microbiol. Biotechnol., 24: 509-515.
21. **Vogel, B.F., Kasthuri, V., Masataka, S., Lone, G. (2005).** Idebntification of *Shewanella* baltica as the most important H_2S producing species during iced storage of Danish marine fish. Appl. Environ. Microbiol., 71: 6689-6697.
22. **Weisburg, W.G., Barns, S.M., Pelletier, D.A., Lane, D.J. (1991).** 16S ribosomal DNA amplification for phylogenetic study. J of Bacteriol. 173:697-703.
23. **Zobell, C.E. (1946).** Marine Microbiology, Chronica Botanica Co.,Waltham Mass.,USA.