Materials

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Methods
**Materials**

**Media**

- Tryptose Agar (HiMedia Laboratories, India)
- Todd Hewitt Broth (HiMedia Laboratories, India)
- Zobell Marine Broth (HiMedia Laboratories, India)
- Luria Bertani (LB) broth (g L\(^{-1}\))
  
<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptone</td>
<td>10 g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>5 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>10 g</td>
</tr>
</tbody>
</table>

For agar, 1.8% of bacteriological agar was added to broth and autoclaved.

- Blood Agar (g L\(^{-1}\))

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptose agar</td>
<td>41 g</td>
</tr>
<tr>
<td>Fresh Sheep Blood</td>
<td>50 mL</td>
</tr>
</tbody>
</table>

Fresh sheep blood was added to the medium after sterilization.

**Bacterial strains**

- *Escherichia coli* EPI300 (Epicentre, Madison, USA)
- *Escherichia coli* DH5α (Laboratory collection)
- *Streptococcus pyogenes* SF370 (M1 serotype; ATCC 700294D-5)
- *S. pyogenes* clinical isolates - SP5 (M56; EU636227), SP7 (st38; EU636229), SP11 (M65; EU660377), SP22 (M89; FJ798733), SP30 (M100; EU660379) and SP31 (M74; EU660380) (Thenmozhi et al., 2011)

**Vectors**

- Plasmid – pUC18 (Laboratory collection) (Figure 16)
- Fosmid – pCC1FOS (Epicentre, Madison, USA) (Figure 17)
Figure 16. Vector map of pCC1FOS used in the present study.

Figure 17. Vector map of pUC18 used in the present study.
**Antibiotic stocks**

**Chloramphenicol** *(12.5 mg mL⁻¹)*

125 mg of chloramphenicol (HiMedia Laboratories, India) was dissolved in 10 mL of ethanol and stored at 4 °C.

**Ampicillin** *(100 mg mL⁻¹)*

100 mg of ampicillin sodium salt (HiMedia Laboratories, India) was dissolved in 1 mL of sterile distilled water and stored at 4 °C.

**Erythromycin** *(10 mg mL⁻¹)*

10 mg of erythromycin (HiMedia Laboratories, India) was dissolved in 1 mL of ethanol and stored at 4 °C.

**Tetracycline hydrochloride** *(10 mg mL⁻¹)*

10 mg of tetracycline hydrochloride (HiMedia Laboratories, India) was dissolved in 1 mL of sterile distilled water and stored at 4 °C.

**Enzyme stocks**

**Lysozyme** *(10 mg mL⁻¹)*

10 mg of lysozyme (Sigma Aldrich, Switzerland) was dissolved in 1 mL of 10 mM Tris-HCl (pH 8.0). The stock solution was stored at -20 °C.

**Proteinase K** *(20 mg mL⁻¹)*

20 mg of proteinase K (Sigma Aldrich, Switzerland) was dissolved in 1 mL of solution containing 50 mM Tris-HCl (pH 8.0) and 1.5 mM calcium acetate. The stock solution was stored at -20 °C.

**RNase** *(10 mg mL⁻¹)*

10 mg of RNase (Sigma Aldrich, Switzerland) was dissolved in 1 mL of sterile distilled water. The stock solution was stored at -20 °C.
**Materials and Methods**

**Restriction and ligation enzymes**

*Not*I, *BamHI, Sau*3A*I, *Hind*III, *Kpn*I and T4 DNA ligase were purchased from New England Biolabs (NEB), USA.

**DNA Ladders**

1 kb ladder, 100 bp ladder and Lambda *Hind*III marker were purchased from Thermo Scientific, USA.

**Reagents and buffers**

**1 M Tris-HCl (pH 8.0)**

12.11 g of Tris was dissolved in 80 mL of distilled water, pH was adjusted to 8.0 with concentrated HCl and the volume was made up to 100 mL using distilled water. The solution was autoclaved and stored at 4 °C.

**0.5 M EDTA (pH 8.0)**

18.61 g of Na₂EDTA.2H₂O was added to 80 mL of distilled water and stirred vigorously. pH of the solution was adjusted to 8.0 with NaOH pellets and the final volume was made up to 100 mL with distilled water. The solution was autoclaved and stored at 4 °C.

**50X Tris Acetate EDTA (pH 7.7)**

242.2 g (2 M) of Tris and 37.2 g of (0.1 M) Na₂EDTA.2H₂O were dissolved in 600 mL of distilled water and the pH of the solution was adjusted to 7.7 with glacial acetic acid (~ 57 mL). The final volume was made up to 1 litre with distilled water, autoclaved and stored at room temperature.

**Sucrose TE buffer**

10.3 g of sucrose was dissolved in 80 mL of distilled water containing 2.5 mL of 1 M Tris-HCl (pH 8.0) and 5 mL of EDTA (0.5 M). The final volume was made up to 100 mL with distilled water, autoclaved and stored at 4 °C until use.
**DNA extraction buffer**

1.21 g (100 mM) of Tris-HCl, 3.72 g (100 mM) of sodium EDTA (pH 8.0), 1.6394 g (100 mM) of sodium phosphate, 8.766 g (1.5 M) of NaCl and 1 g (1 %) of CTAB were dissolved in 90 mL of distilled water and the volume was made up to 100 mL and autoclaved.

**Ethidium bromide (10 mg mL$^{-3}$)**

100 mg of ethidium bromide (EtBr) was dissolved in 10 mL of sterile distilled water by stirring overnight. The solution was stored at 4 ºC in amber coloured screw capped tube.

**6X DNA loading dye**

4 g (40 % w/v) of sucrose, 25 mg (0.25 %) of bromophenol blue and 25 mg (0.25 %) of xylene cyanol were added to 8 mL of distilled water and dissolved by mixing well. The volume was made up to 10 mL with distilled water and stored at 4 ºC.

**10 % SDS**

1 g of SDS was added to 8 mL of sterile distilled water, heated to 65 ºC to dissolve and the final volume was made up to 10 mL.

**0.4 N NaOH**

1.6 g of NaOH was dissolved in 90 mL of sterile distilled water and the final volume was made up to 100 mL.

**5 M potassium acetate**

49.075 g of potassium acetate was dissolved in 100 mL of distilled water and autoclaved.
Solution I

2.5 mL of 1 M Tris-HCl (pH 8.0), 2 mL of 0.5 M Na₂EDTA.2H₂O and 0.9 g of dextrose (glucose) constituting 25 mM, 10 mM and 50 mM respectively were dissolved in 90 mL of distilled water, and the final volume was made up to 100 mL.

Solution II

Equal volume of 0.4 N NaOH and 2 % SDS were mixed just prior use.

Solution III

60 mL of 5 M potassium acetate, 11.5 mL of glacial acetic acid and 28.5 mL of distilled water were mixed and autoclaved. The resulting solution is 3 M with respect to potassium and 5 M with respect to acetate.

3 M sodium acetate (pH 5.2)

24.61 g of sodium acetate was dissolved in 80 mL of distilled water. pH was adjusted to 5.2 and the volume was made up to 100 mL with distilled water.

Equilibration of phenol

The saturated phenol was equilibrated using 0.5 M Tris-HCl (pH 8.0). Then 0.1 M Tris-HCl (pH 8.0) was added and equilibrated. The equilibration step with 0.1 M Tris-HCl was repeated until the pH of phenol reaches 7.8. To the equilibrated phenol, 0.1 volume of 0.1 M Tris-HCl (pH 8.0) containing 0.2 % β-mercaptoethanol was added and stored at 4 °C.

Phenol: chloroform: isoamyl alcohol

Equilibrated phenol, chloroform and isoamyl alcohol were mixed just before use in the ratio of 25:24:1 (v/v).

Chloroform: isoamyl alcohol

Chloroform and isoamyl alcohol were mixed just before use in the ratio of 24:1 (v/v).
X-Gal (2 %)

20 mg of X-Gal was dissolved in 1 mL of DMSO and stored in amber coloured screw capped tube at -20 °C.

1 M IPTG

238 mg of IPTG was dissolved in 1 mL of sterile distilled water and stored at -20 °C.

100 mM CaCl₂

1.475 g of CaCl₂.2H₂O was dissolved in 100 mL of distilled water and autoclaved.

0.2 % Hydrazine sulphate

0.2 g of hydrazine sulphate was dissolved in 100 mL of concentrated sulphuric acid.

5 % phenol

5 g of phenol was dissolved in 95 mL of sterile distilled water by heating and stirring at 60 °C and the final volume was made up to 100 mL.

0.4 % Crystal violet

400 mg of crystal violet was dissolved in 100 mL of distilled water and stored at room temperature.

20 % Glacial acetic acid

20 mL of glacial acetic acid was added to 80 mL of distilled water and stored at room temperature.

2 % Azocasein

100 mg of azocasein was dissolved in 5 mL of 0.1 M potassium phosphate buffer (pH 7.9) and stored at 4 °C.
0.1 % Acridine orange

10 mg of acridine orange was dissolved in 10 mL of distilled water and stored at room temperature in an amber coloured screw capped tube.

10 % Trichloro acetic acid

1 mL of trichloroacetic acid was dissolved in 9 mL of distilled water and stored at room temperature.

DEPC water

1 mL of diethyl pyrocarbonate was added to 999 mL of distilled water and mixed vigorously followed by incubation at 37 °C overnight and autoclaved.

30 % Acrylamide: bis-acrylamide solution (AB solution)

29 g of acrylamide and 1 g of bis-acrylamide were dissolved in 60 mL of distilled water by continuous stirring and the final volume was made up to 100 mL and stored in brown bottle at 4 °C.

20 % Ammonium persulfate (APS)

200 mg of ammonium persulfate was dissolved in 1 mL of sterile distilled water just prior use.

40 - 60 % linear denaturant gradient polyacrylamide gel for DGGE

Solution A (60 % denaturant): 9.2 mL of 30 % AB solution, 0.5 mL of 50X TAE, 6 mL of formamide, 6.3 g of urea, 50 µL of 20 % APS and 5 µL of TEMED were dissolved in 20 mL of distilled water and the final volume was made up to 25 mL.

Solution B (40 % denaturant): 9.2 mL of 30 % AB solution, 0.5 mL of 50X TAE, 4 mL of formamide, 4.2 g of urea, 50 µL of 20 % APS and 5 µL of TEMED were dissolved in 20 mL of distilled water and the final volume was made up to 25 mL.
Solution A and Solution B were mixed properly using a gradient mixer (Thermo scientific, USA) and poured into gel plate setup via automatic peristaltic pump (INGENY, The Netherlands).

**Stacking Gel (5 %) for DGGE**

2.53 mL of 30 % AB solution, 0.3 mL of 50X TAE, 50 µL of 20 % APS and 5 µL of TEMED were dissolved in 10 mL of distilled water and the final volume was made up to 15 mL.

**2,4-Di-tert-butyl-phenol (DTBP) (5 mg mL⁻¹)**

5 mg of DTBP (Catalogue no. 96-76-4, Sigma Aldrich, Switzerland) was dissolved in 1 mL of methanol and stored at 4 °C till further use.

**Betulin (5 mg mL⁻¹)**

5 mg of betulin (Catalogue no. 0210106.2, MP Biomedicals, USA) was dissolved in 1 mL of methanol and stored at 4 °C till further use.

**Lupeol (5 mg mL⁻¹)**

5 mg of lupeol (Catalogue no. 545-47-1, Sigma Aldrich, Switzerland) was dissolved in 1 mL of methanol and stored at 4 °C till further use.

**Methods for studying bacterial diversity using DGGE**

**Sample collection**

Seaweed (Gracilaria sp., Padina sp., Enteromorpha sp., Sargassum sp., and Turbinaria sp.), seagrass (Cymodaceae sp.) and seawater samples were collected from Karankadu coastal region (8° 28’N lat. and 77° 41’E long.), Palk Bay, Tamil Nadu, India in sterile plastic bags and transported immediately to the laboratory in cold condition. Seaweed and seagrass samples showing good physiological state alone were collected and were washed individually with sterile seawater to remove loosely attached bacteria and stored at -80 °C for further experiments.
Isolation of culturable epiphytic bacteria from seaweed and seagrass

One gram of samples were weighed and taken into sterile 50 mL centrifuge tubes containing 9 mL of PBS and vortexed vigorously to isolate the surface associated bacteria. The samples were serially diluted and $10^{-1}$ to $10^{-5}$ dilutions were plated on ZMA plates and incubated at room temperature for 48 h. After incubation, colonies with different morphology were selected and streaked on ZMA plates to get pure cultures. Glycerol stocks were prepared with 20 % glycerol and stored at -80 °C for future use.

Metagenomic DNA extraction from seaweed and seagrass

Total bacterial DNA from seaweed and seagrass samples were isolated using the protocol described by Burke et al. (2011a) with slight modifications. Five grams of seaweed and seagrass samples were placed individually into 13.5 mL of DNA extraction buffer. Samples were constantly agitated at 80 rpm for 1 h in room temperature. The samples were filtered to remove plant material and the remaining liquid was centrifuged at 3000 rpm for 15 min to remove any residual material. The supernatants were transferred to new tubes, and lysozyme was added to a final concentration of 150 µg mL$^{-1}$. The samples were then incubated in 37 °C water bath for 1 h with constant agitation. DNA was extracted using phenol, chloroform, and isoamyl alcohol (25:24:1 ratio, respectively) mixture. DNA was subsequently precipitated with 1/10 volume of 3 M sodium acetate (pH 5.2) and 2.5 volume of chilled ethanol. Pelleted DNA was washed once with 70 % ethanol, air dried, and resuspended in 0.5 mL of MilliQ water. Quantity and quality of the extracted DNAs were evaluated by submarine agarose gel (0.8 %) electrophoresis using 1X TAE buffer. After electrophoresis, the gel was stained for 20 min in EtBr (0.5 µg mL$^{-1}$),
rinsed for 10 min in distilled water and the bands were visualized using Gel documentation system (Bio-Rad, Laboratories, USA).

**Metagenomic DNA extraction from seawater**

DNA extraction from seawater was done using standard procedure as described by Venter *et al.* (2004) with slight modifications. Seawater (2 L) was filtered using Whatmann no.1 filter paper to remove debris and subsequently filtered again using Millipore 2 μm filter paper to separate bacterial cells. The filter paper was suspended in 5 mL of sucrose TE buffer and kept at -80 °C for 1 h. Subsequently lysozyme was added to a final concentration of 150 μg mL⁻¹ and incubated at 37 °C for 2 h. SDS was added to a concentration of 0.1 % and subjected to three freeze/thaw cycles. The sample was then treated with 100 μg mL⁻¹ of proteinase K at 55 °C for 3 h. Subsequent steps involving phenol: chloroform: isooamyl alcohol mixture extraction and ethanol precipitation were performed as described above. Finally, the quantity and quality of the extracted DNAs were evaluated by submarine agarose gel (0.8 %) electrophoresis using 1X TAE buffer.

**Amplification of V3 region of 16S rDNA for DGGE**

The variable V3 region of 16S rDNA was enzymatically amplified by nested PCR system, based on the initial amplification of >1300 bp of the 16S rRNA gene with P63f (5’-CAGGCCTAACACATGCAAGTC-3’) and R1378r (5’-CGGTGTGTACAAGGCCGGAAC G-3’) primers and a subsequent amplification with GC-clamped forward primer P338fG (5’CGCCCGCCCGCCCGGCGGGGGCGGGGGGGCACGGGGGGACTCCTACGGGAGGCAGCAG-3’) and reverse primer P518r (5’-ATTACCAGCGCTGCTGG-3’). PCR conditions used for the first cycle were an initial denaturation of 95 °C for 5 min; 25 cycles of 95 °C for 1 min, 55 °C for 1 min, 72 °C for 1.5 min and ended with a final extension of 72 °C for 10 min.
Similar cycle conditions were used for the second cycle of PCR except that the annealing temperature and extension time were reduced to 53 °C and 30 sec respectively. Only 25 cycles were allowed for both of the PCR cycles to avoid excess amplification of the dominant species. The size of the amplified products was verified by submarine agarose gel (1.5 %) electrophoresis using 1X TAE buffer.

DGGE

DGGE was performed for the PCR products obtained as above using a parallel gel containing 11 % (w/v) polyacrylamide. Denaturants used were urea and formamide. Each gel contained a 40 - 60 % linear gradient (top-bottom) of denaturants urea and formamide (100 % denaturant concentration corresponds to 7 M urea and 40 % (v/v) deionised formamide). Electrophoresis was carried out in INGENY PHOR U (The Netherlands) gel system at 100 V for 17 h at a constant temperature of 60 °C. After electrophoresis, the gel was stained for 45 min in SYBR Gold® (Invitrogen), rinsed for 10 min in 1X TAE buffer and the DGGE profiles were visualized using gel documentation system (Bio-Rad Laboratories, USA).

Diversity indices calculation

Each DGGE band was assumed as an operational taxonomic unit and the following diversity indices were calculated. (i) species diversity (S), which corresponds to the number of bands in a DGGE profile; (ii) simple index (Ii), which is calculated using the formula: \( I_i = \frac{n}{nM} \). Where, \( I_i \) is an index number for each band present in a DGGE profile; \( n \) is the number of DGGE bands in a given DGGE profile; \( nM \) is the number of bands in the DGGE profile with the highest number of bands.

Statistical analysis of DGGE fingerprint

The bacterial community structures of the samples were analyzed using Primer software v.6.1.9 (Primer-E). Initially a presence-absence matrix was generated.
based on the band pattern observed. All visible bands in every gel lane were taken into account for calculation. Only samples from one gel were compared with each other in order to ensure the comparability of band patterns. Bray-Curtis values without transformation were calculated. Sample similarities were depicted using cluster analysis and non-metric multidimensional scaling (NMDS).

**DGGE - band elution and sequencing**

In order to elucidate the common OTUs from different samples, selected prominent DGGE bands were sequenced. Using a sterile scalpel, bands were excised from the gel and the DNA was extracted using QIAGEN gel extraction kit. The extracted DNA was reamplified using P338f-P518r primer set and checked in denaturant gradient gel for the presence of single band and sequenced (Macrogen Inc., S. Korea). The sequences were manually assessed for errors and the ‘N’s observed in the sequences were edited with bases corresponding to the peaks using the program Chromas Lite (http://technelysium.com.au/?page_id=13). Processed sequences were assembled using Contig Assembly Program version 3 (CAP3) (http://doua.prabi.fr/software/cap3) (Huang and Madan, 1999) and the contigs were searched for hits in public database NCBI (http://www.ncbi.nlm.nih.gov/) using nucleotide Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990). The sequences were further submitted in European Nucleotide Archive (ENA) and accession numbers were obtained.

**Methods for studying bacterial diversity using illumina sequencing**

**Amplicon library construction for illumina sequencing**

Library construction for the seaweed and seagrass epiphytic metagenomes involved two PCR reactions (Kapa HiFi Hot start, Kapa Biosystems, Boston, US). The first reaction targeted the V3 region using primers 341F (5’-
CCTACGGGAGGCAGCAG-3’) and 518R (5’-ATTACCGCGGCTGCTGG-3’) with the starting amount of 20 ng of DNA. Cycle conditions were an initial denaturation at 98 °C for 3 min, followed by 20 cycles of 98 °C for 30 sec, 60 °C for 30 sec, 72 °C for 30 sec, and ended with a final extension step at 72 °C for 3 min. The amplified products of correct size were recovered by gel elution using minelute columns (Qiagen, Hilden, Germany).

For the next cycle of PCR, 150 ng of the amplified sample was used as template and modified primers (which include V3 specific sequences and adapter sequences) were used as suggested by Bartram et al., 2011. These modified primers had four degenerate bases in the forward primer to enhance base calling accuracy and cluster density and aids in identifying unique clusters. The reverse primer contained a 6-bp index (barcode) sequence for multiplexing (Table 6).

Table 6. Details of the modified primers used in library construction

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence*</th>
</tr>
</thead>
<tbody>
<tr>
<td>V3F modified</td>
<td>5’-atgatacgggacccctacttacagactctactttctacctacgaatcttccgatct</td>
</tr>
<tr>
<td>V3R seaweed</td>
<td>5’-caagcagaagacggggtagatgtgactggagttcagacgtgtgc</td>
</tr>
<tr>
<td>V3R seagrass</td>
<td>5’-caagcagaagacggggtagatgtgactggagttcagacgtgtgc</td>
</tr>
</tbody>
</table>

* The lower case letters are the adapter sequences necessary for binding to the flow cell. Underlined lower case sequences are the binding sites for illumina sequencing primers and the uppercase sequences are the V3 region primers. The six base upper case red coloured fonts are the index sequences (Barcode).

The PCR conditions were same as the first cycle except that the number of cycles was reduced to six. The amplified products were cleaned up using Agencourt Ampure XP SPRI beads (Beckman Coulter, California, US) as per the manufacturer’s
instructions. The quality and size of the prepared library was validated by running an aliquot through High Sensitivity Bioanalyzer Chip (Agilent technologies, California, US) and quantified using Nano-spectrometer.

**Illumina sequencing**

PCR products with unique indices from each library were taken in equal nanogram quantities and subjected to 100-nucleotide paired-end multiplex sequencing using illumina GAIIX sequencer at Genotypic Technology Pvt. Ltd (Bangalore, India). Both the samples were pooled in a single lane. Image analysis and base calling were done using illumina analysis pipeline (Version 2.2).

**Initial processing of raw illumina reads**

Demultiplexing of raw illumina reads was done using Consensus Assessment of Sequence and Variation (CASAVA). Only high quality reads with more than 70% of bases with phred score greater than 20 were considered significant and taken for subsequent analysis. Reads with adapter contaminated sequences were removed. Using an automated Perl code, primer sequences, barcode and degenerate bases were removed generating processed reads.

**Illumina sequencing- Data analysis**

Duplicates and chimeras were removed from the processed reads using CD-HIT DUP with the minimum length of common sequence shared between a chimeric read and each of its parents set as 20 bases (Li W et al., 2012). Abundance ratio between a parent read and a chimeric read was set to 1. The resulting dataset was pre-screened using uclust for a minimum of 70% identity to ribosomal sequences and then clustered at 97% identity against RNA databases implemented in MG-RAST (namely RDP, Greengenes, SILVA LSU and SSU) (Glass and Meyer, 2011). Taxonomic assignment from phylum level to strain level was assigned based on the
hits. Abundance graphs were plotted based on the number of hits. Rarefraction curves were plotted using MG-RAST. The venn diagrams were made with venn diagram plotter jquery.venny, a tool developed by genotoul bioinfo (http://bioinfor.genotoul.fr). Diversity index and richness estimates were calculated using SPADE software (Hong et al., 2006). Krona graphs were plotted using Krona (Ondov et al., 2011).

**Methods for molecular identification of bacteria**

**Genomic DNA isolation**

Genomic DNA from positive isolate (R60) was isolated by CTAB-NaCl method. Overnight culture of R60, grown in ZMB at 28 °C with shaking (120 rpm) was taken in sterile 1.5 mL micro centrifuge tube and the cells were pelleted out by centrifugation at 8000 rpm for 10 min at 4 °C. To the pellet, 567 µl of sucrose TE buffer, 30 µl of 10 % SDS and 3 µl of proteinase K (20 mg mL⁻¹) were added and incubated at 37 °C for 1 h. After incubation, 100 µl of 5 M NaCl and 80 µl of 1 % CTAB were added and mixed gently by inverting the tube. The tube was again incubated at 65 °C for 10 min. Equal volume of phenol: chloroform: isoamyl alcohol (25: 24: 1) mixture was added to the tube and centrifuged at 10,000 rpm for 10 min at 4 °C. The aqueous phase from the tube was transferred to fresh 1.5 mL micro centrifuge tube and 2 µl of RNase (10 mg mL⁻¹) was added and incubated at 37 °C for 1 h. Equal volume of chloroform: isoamyl alcohol (24: 1) mixture was added to the tube, gently mixed and centrifuged at 10,000 rpm for 10 min at 4 °C. After centrifugation, the aqueous phase of the tube was again transferred into fresh 1.5 mL micro centrifuge tube and 0.6 volume of isopropanol was added and spun at 10,000 rpm for 10 min at 4 °C. The supernatant was discarded and the pellet was washed with 400 µl of 70 % ethanol. The pellet was air dried and dissolved in 30 µl Milli-Q water. The quality of genomic DNA isolated by the above mentioned method was checked.
by submarine agarose gel (0.8 %) electrophoresis using 1X TAE as running buffer and stained with EtBr (0.5 µg mL⁻¹). The isolated DNA was visualized using gel documentation system (Bio-Rad Laboratories, USA).

**PCR for amplification of 16S rRNA gene**

16S rRNA gene was amplified using primers 27f (5' - AGAGTTTGATCCTGGCTCAG -3') and 1492r (5' - ACGGCTACCTTGTTACGACTT -3') using the thermal cycler (Mastercycler® proS, Eppendorf AG, Germany). Cycle conditions were an initial denaturation at 95 °C for 10 min, followed by 35 cycles of 95 °C for 1 min, 55 °C for 1 min, 72 °C for 1.5 min, and ended with a final extension step at 72 °C for 10 min. The amplification was confirmed by running the PCR products in submarine agarose gel (0.8 %) electrophoresis using 1X TAE as tank buffer. The amplified products were cleaned and sequenced (Macrogen Inc., S. Korea). The sequences were manually assessed for errors, assembled using CAP3 and BLAST searched as described above. The hits with more than 99 % identity were considered same species and the sequence was further submitted in GenBank and accession number was obtained.

**Methods for subcloning using shotgun approach**

**Isolation of fosmid metaclone MC-81 and plasmid DNA**

The fosmid metaclone MC-81 and plasmid DNA from the subclones were isolated using alkaline lysis method as detailed below. For fosmid metaclone MC-81 isolation, the *E. coli* EPI300 cells containing metagenomic fosmid clone MC-81 was grown in LB medium supplemented with 12.5 µg mL⁻¹ of chloramphenicol and 13.5 mM concentration of L-arabinose (induction solution). This was incubated overnight with shaking at 37 °C. For plasmid isolation, the subclone pAB79 was grown in LB medium supplemented with 100 µg mL⁻¹ of ampicillin with shaking at 37 °C for 12 h.
The cultures (2 mL) were spun at 7000 rpm for 5 min and the pellets were resuspended in 150 µL of Solution I. Then 200 µL of Solution II was added, gently mixed and incubated in ice for 5 min. Solution III (150 µL) was added and incubated in ice for 10 min. The tubes were centrifuged and the supernatants were carefully transferred to a new microfuge tube. Equal volume of phenol: chloroform: isoamylalcohol mixture was added and centrifuged (10000 rpm; 10 min). The supernatants were transferred to a sterile tube and equal volume of chloroform: isoamylalcohol (24:1) mixture was added and centrifuged. The DNA was precipitated using 2.5 volume of ice cold ethanol and centrifuged (10000 rpm; 10 min). The obtained pellets were washed with 70 % ethanol, air dried and DNA was resuspended in sterile MilliQ water and stored at -20 °C.

**Partial digestion of fosmid metaclone MC-81**

Fosmid metaclone MC-81 (300 ng) was digested with varying concentrations of *Sau3AI* (5U, 2U, 1U, 0.1U, 0.05U, 0.025U and 0.01U) in the recommended buffer for 20 min at 37 °C. The enzyme was immediately inactivated by incubating at 70 °C for 15 min and the digested products were separated in submarine agarose gel (1 %) electrophoresis using 1X TAE at 2 V cm⁻¹ for 1 h, stained and visualized.

**Linearization of pUC18**

pUC18 vector has a single *BamHI* site at its MCS region. The vector was linearized by digesting with *BamHI* in the recommended buffer for 3 h at 37 °C. Enzyme inactivation was done by incubating the tube at 70 °C for 15 min. The digested products were separated in submarine agarose gel (1 %) electrophoresis using 1X TAE at 2V cm⁻¹ for 1 h, stained and visualized.
Ligation

For ligation, partially digested fosmid metaclone MC-81 insert and linearized pUC18 vector were taken in the ratio of 3:1. Ligation was carried out using 1U of T4 DNA ligase by incubating at 4 °C overnight.

Competent cell preparation

*E. coli* strain DH5α was grown to OD 0.6 and the culture was immediately kept in ice for chilling. The culture (20 mL) was spun at 3000 rpm for 15 min. The supernatant was drained and 10 mL of ice cold 100 mM CaCl₂ was added to resuspend the pellet fully. The suspension was kept in ice for 10 min and spun at 3000 rpm for 15 min. The supernatant was drained and the pellet was resuspended in 1 mL of 100 mM CaCl₂. Glycerol was added to the suspension and stored at -80 °C until needed.

Transformation and Blue-White selection

Competent *E. coli* DH5α cells (100 μl) were thawed in ice for 15 min. Ligation mixture was added to the cells and incubated in ice for 1h. Heat shock was given by incubating the cells at 42 °C for 90 sec and immediately plunging the tube into ice. The cells were incubated in ice for 30 min. LB broth (1.5 mL) was added to the cells and incubated at 37 °C for 1 h with shaking. The cells were centrifuged at 8000 rpm for 2 min and the excess medium was discarded. The cells were plated on LB agar plate supplemented with 100 μg mL⁻¹ ampicillin, 80 μg mL⁻¹ X-Gal and 20 mM IPTG. The plates were incubated at 37 °C overnight and observed for blue and white colonies.

Restriction analysis and sequencing of pAB79

Restriction analysis was carried out with 10 units of restriction endonucleases *viz.* KpnI and HindIII and incubated overnight at 37 °C. The digested products were
separated in submarine agarose gel (1 %) electrophoresis using 1X TAE at 2V cm\(^{-1}\) for 1 h, stained and visualized. The insert DNA present in pAB79 was sequenced (Macrogen Inc., S. Korea) and the sequence was further submitted in GenBank and accession number was obtained.

**Methods for biofilm and virulence assays against GAS**

**Screening of culturable bacteria, metaclones and subclones for antibiofilm activity**

Marine bacterial isolates were grown in ZMB at 28 °C for 48 h in shaker (120 rpm). Metaclones were grown in LB medium supplemented with 12.5 µg mL\(^{-1}\) of chloramphenicol for 48 h at 37 °C. The subclones were grown in LB medium supplemented with 100 µg mL\(^{-1}\) of ampicillin for 48 h at 37 °C. After incubation, the cultures were centrifuged and cell free culture supernatants (CFCS) were collected and individually assessed for antibiofilm activity against reference strain *S. pyogenes* SF370 in 24 well polystyrene plates. Briefly, \(~1 \times 10^5\) cells from an overnight culture of *S. pyogenes* SF370 were used to inoculate Todd Hewitt broth supplemented with 0.5 % yeast extract and 1 % glucose (THYG) containing 15 % CFCS (for subclones 20 % CFCS was used). The plates were incubated for 24 h at 37 °C in static condition. Subsequently, spent media and planktonic cells were discarded and the wells were washed with sterile distilled water to remove loosely adhered cells. To quantify biofilm formation, wells were stained with 0.4 % crystal violet for 10 min. Excess stain was removed by washing the wells twice with sterile distilled water. The cell bound crystal violet was extracted using 20 % glacial acetic acid and its absorbance was measured at 570 nm (Spectramax M3, Molecular Devices, USA). The amount of stain bound to the wells is directly proportional to the biofilm formation. Hence, a decrease in absorbance at 570 nm compared to control suggests biofilm inhibition.
Percentage of biofilm inhibition was calculated using the formula: Percentage of inhibition = \[\frac{(\text{Control OD value} - \text{Treated OD value})}{\text{Control OD value}} \times 100\] (Thenmozhi et al., 2009).

**Heat inactivation assay**

The heat stability of the active lead present in MC-81 CFCS was assessed by incubating it for 30 min at various temperatures (40 °C, 60 °C, 80 °C and 100 °C). The supernatant was also autoclaved (121 °C at 15 lbs pressure for 20 min). The heat treated supernatant was checked for its ability to inhibit the biofilm formation by *S. pyogenes*. Untreated MC-81 cell free culture supernatant was used as positive control.

**Effect of proteinase K on the antibiofilm activity of active leads**

The CFCS of R60/MC-81 was treated with proteinase K (Sigma Aldrich, Switzerland) to a final concentration of 1 mg mL\(^{-1}\) for 1 h at 55 °C and 20 min at 70 °C. Treated supernatant was evaluated for its ability to inhibit biofilm formation considering the respective untreated CFCS as positive control. Respective medium treated with 1 mg mL\(^{-1}\) of proteinase K as described above was used as negative control.

**Solvent extraction and partial purification of active lead from R60**

The CFCS of R60 was extracted with an equal volume of ethyl acetate. The solvent extract was evaporated to dryness at room temperature under reduced pressure to yield crude extract. The crude extract was partially purified using pre-coated thin layer chromatography (TLC) plates (Merck, USA) using chloroform: ethyl acetate: methanol (9:10:1) solvent system. Distinct bands which were visualized after derivatization of the TLC plates with iodine vapour were scrapped, extracted using methanol and tested for their antibiofilm activity. The active fraction was further
analyzed using Gas Chromatography coupled with Mass Spectrometer (GC-MS) analysis.

**Solvent extraction and partial purification of active lead from metaclone MC-81**

The CFCS of MC-81 was extracted individually using equal volume of various solvents namely ethyl acetate, chloroform, dichloromethane, benzene, hexane and petroleum ether. The solvent extracts were evaporated to dryness at room temperature under reduced pressure to yield crude extracts. Yield of the extracts were calculated as follows: Concentration of the extract = (Dry weight of the tube with extract - Dry weight of the tube). The crude dichloromethane extract, which exhibited significant antibiofilm activity was partially purified by column chromatography using chloroform: ethyl acetate: dichloromethane: methanol (9:7:3:1) mixture as mobile phase in 230-400 mesh silica with 40 mL bed volume. A total of twenty fractions (each 5 mL) were collected. Finally, the column was washed with 50 mL of methanol (10 mL per fraction). All the fractions were concentrated and checked for their antibiofilm activity against *S. pyogenes*. The active fractions were pooled and further analyzed using Gas Chromatography coupled with Mass Spectrometer (GC-MS) analysis.

**GC-MS analysis**

GC-MS analysis was performed using AccuTOF Gcv equipment (SAIF, IITB, Mumbai, India). Compounds were separated in hp1 capillary column, a general purpose column with a length of 30 m and a diameter of 0.25 μm. Temperature was set between the range of 100 to 280 °C with ramp change of 5 °C per minute. The run was carried out in EI+ ionization mode. Helium was used as carrier gas with a flow rate of 1 mL per min. The GC chromatogram was compared with NIST library and the compounds were identified based on the spectral match.
Determination of Biofilm Inhibitory Concentration

Increasing concentration of tested antibiofilm agents/extract was added individually into the wells of sterile microtiter plate containing 1 mL of THYG broth. Overnight culture of *S. pyogenes* (~1 x 10⁵ cells) was used to inoculate each well and the plate was incubated at 37 °C under static condition for 24 h. The biofilm formation was quantified using 0.4 % crystal violet as described above. Microtiter plate wells containing equivalent amounts of methanol were used as vehicle controls. Lowest concentration of compound/extract that showed maximum biofilm inhibition was considered as Biofilm Inhibitory Concentration (BIC) (Subramenium et al., 2015a).

Growth curve analysis

The influence of tested antibiofilm agents/extract on the growth of *S. pyogenes* (SF370 and clinical isolates) was assessed by growth curve assay. Compound/extract (at their respective BIC) and methanol (vehicle control) were individually added to 50 mL of THYG medium to which ~ 1 x 10⁵ cells were introduced. The dynamics of bacterial growth was monitored by measuring the absorbance at 600 nm every hour up to 24 h.

Light microscopic analysis

In order to visualize biofilms under light microscope, biofilms of *S. pyogenes* were allowed to grow on glass pieces (1 x1 cm) in the absence and presence of tested antibiofilm agents/extract. The glass pieces were placed in 24-well polystyrene plate wells containing 1 mL of THYG and 1 % inoculum from standard cell suspension (~1 x 10⁵ cells). Tested antibiofilm agents/extract (at their respective BIC) and methanol (vehicle control) were added individually to the wells and incubated at 37 °C for 24 h. After incubation, the biofilms formed on glass pieces were washed with sterile distilled water and stained with 0.4 % crystal violet for 10 min. The glass pieces were
washed again with distilled water to remove excess stain; air dried and observed under light microscope (Euromex model: GE3045, The Netherlands) at 400X magnification. Biofilm formation was documented with an attached digital camera (Cmex camera, model: DC 5000, The Netherlands) (Thenmozhi et al., 2011).

**Confocal Laser Scanning Microscopic (CLSM) analysis**

For CLSM analysis, the biofilms were allowed to form over glass, stainless steel and titanium pieces (1×1 cm) in the absence and presence of tested antibiofilm agents/extract (at their respective BIC) as described above and washed with sterile distilled water. The biofilms were stained using 0.1 % acridine orange for 1 min, washed again with distilled water and allowed to air dry. Stained biofilms were visualized under CLSM (model: LSM 710; Carl Zeiss, Germany) using 488 nm argon laser for stain excitation. Image processing and z-stack analysis was done using Zen 2009 image software (Carl Zeiss, Germany). COMSTAT software (kind gift from Dr. Claus Sternberg, Technical University of Denmark) was used for quantifying biomass, maximum thickness and surface to volume ratio of biofilms (Shukla and Rao, 2013).

**Scanning Electron Microscopic (SEM) analysis**

For SEM analysis, the biofilms were allowed to form on glass pieces as described above and fixed by immersing the glass pieces in 2 % glutaraldehyde solution for 8 h at 4 °C. Subsequently, the samples were washed with distilled water and gradually dehydrated using increasing concentrations of ethanol (20, 40, 60, 80 and 100 %) and air dried. The samples were sputtered with gold prior to their visualization under SEM (VEGA 3 TESCAN, Czech Republic) (Subramenium et al., 2015a).
Biofilm formation in petri plates

In order to visualize biofilm formation on glass surfaces, ~ $1 \times 10^5$ cells of *S. pyogenes* were used to inoculate 3 mL of THYG in petri plates (50 mm x 15 mm). Tested antibiofilm agents/extract (at their respective BIC) and methanol (vehicle control) were added individually to the petri plates and incubated at 37 °C for 24 h in static condition. After incubation, planktonic cells were removed and the biofilms formed on the bottom of petri plates were washed with sterile distilled water and stained with 0.4 % crystal violet for 10 min. The petri plates were washed again with distilled water to remove excess stain, air dried and photographed.

EPS quantification

Total carbohydrate quantification was done for the determination of EPS. *S. pyogenes* strains (SF370 and clinical isolates) were grown for 24 h in the absence and presence of tested antibiofilm agents/extract (at their respective BIC). The cells were harvested and washed with sterile PBS and resuspended in 200 µL of the same. To this cell suspension, equal volume of 5 % phenol and 5 volumes of concentrated sulphuric acid containing 0.2 % of hydrazine sulphate were added. The tubes were incubated in dark for 1 h at room temperature, followed by centrifugation at 10,000 rpm for 10 min. The absorbance of supernatants was measured at 490 nm. Cultures grown in the presence of methanol were considered as vehicle control (Shafreen *et al.*, 2011).

Microbial Adhesion to Hydrocarbon (MATH) assay

The effect of tested antibiofilm agents/extract at their respective BIC on the cell surface hydrophobicity of different M serotypes of *S. pyogenes* was measured by MATH assay. The assay measures the ability of cells to adhere to the hydrophobic substrate (toluene). To 1 mL of treated and untreated bacterial culture (OD 600 nm =
0.4), 1 mL of toluene was added and vortexed for 2 min. The tubes were kept undisturbed at room temperature to allow phase separation. After separation, aqueous phase was collected and examined at OD 600 nm. The percentage of hydrophobicity was calculated using the formula: Percentage of hydrophobicity = [1 - (OD 600 nm after vortexing/OD 600 nm before vortexing)] × 100 (Nithyanand et al., 2010).

Mature biofilm dispersion

In order to determine the ability of tested antibiofilm agents/extract to disrupt pre-formed biofilms, *S. pyogenes* strains were allowed to form biofilm in microtiter plate wells for 24 h. The formed biofilms were washed with PBS and overlaid with 1 mL of the same. The biofilms were treated with tested antibiofilm agents/extract at their respective BIC for 24 h and the residual biofilm was estimated using crystal violet assay (Padmavathi et al., 2014).

Hyaluronic acid quantification

*S. pyogenes* strains grown in the absence and presence of tested antibiofilm agents/extract (at their respective BIC) for 24 h at 37 °C were harvested and washed twice with sterile PBS and resuspended in 1 mL of PBS. Cell surface associated hyaluronic acid was extracted by adding equal volume of chloroform. The tubes were vortexed thoroughly and left undisturbed at room temperature for 1 h. The samples were then centrifuged at 12000 rpm for 10 min and the aqueous phase was collected. To 100 µL of the collected aqueous phase, 1 mL of Stains-All reagent (Sigma Aldrich, USA) was added, vortexed and absorbance was read at 640 nm as described earlier (Subramenium et al., 2015b).

Secreted protease activity

To determine the effect of tested antibiofilm agents/extract on secreted protease production in solid agar, standard suspension of *S. pyogenes* was streaked
over tryptose agar medium containing 1 % of skim milk powder in the absence and presence of tested antibiofilm agents/extract (at their respective BIC). Equivalent amount of methanol (vehicle control) was added in control plates. The plates were incubated at 37 °C for 24 h and observed for zone of clearance around bacterial growth (Neely et al., 2003).

For secreted protease quantification in liquid culture, cell free culture supernatant (1 mL) of \textit{S. pyogenes} grown in the absence and presence of tested antibiofilm agents/extract (at their respective BIC) for 24 h at 37 °C were collected. The collected supernatants were combined individually with 200 µL of activation buffer [1mM EDTA and 20mM DTT in 0.1 M sodium acetate buffer (pH 5.0)] and incubated at 40 °C for 30 min. Buffered substrate containing 2 % azocasein (400 µL) was then added and further incubated for 1 h at 37 °C. The reaction was terminated by adding 600 µL of 10 % trichloroacetic acid and incubated at -20 °C for 20 min. The tubes were centrifuged at 3000 rpm for 5 min and 600 µL of supernatants were transferred to fresh tubes. To the collected supernatant, 700 µL of 1M NaOH was added and their absorbance was measured at 440 nm (Hollands et al., 2008).

**Haemolysis assay**

The influence of tested antibiofilm agents/extract (at their respective BIC) on haemolysin production was analyzed by streaking aliquot of standard cell suspension of \textit{S. pyogenes} over tryptose agar supplemented with 5 % sheep blood in the absence and presence of tested antibiofilm agents/extract (at their respective BIC). The plates were incubated at 37 °C for 24 h and observed for zone of clear haemolysis surrounding the colonies (Shelburne et al., 2008). On the other hand, haemolysis was quantified as described previously with slight modifications (Subramenium et al., 2015b). Briefly, freshly collected sheep blood was diluted in sterile THYG medium to
a final concentration of 2 % (v/v) and divided into three aliquots. To the first aliquot, 10 % of standard cell suspension of *S. pyogenes* and methanol (vehicle control) were added. To the second aliquot, 10 % of standard cell suspension and tested antibiofilm agents/extract (at their respective BIC) were added. Tested antibiofilm agents/extract (at their respective BIC) alone was added to the last aliquot and this was considered as blank. All the tubes were incubated at 37 °C for 1 h and subsequently incubated at 4 °C for 1 h. The tubes were centrifuged at 5000 rpm for 5 min and the absorbances of supernatants were measured at 405 nm.

**Blood survival assay**

Blood survival assay was performed to evaluate the effect of tested antibiofilm agents/extract on the ability of *S. pyogenes* to escape opsono-phagocytosis. The assay was performed as described previously by Subramenium et al. (2015b) with slight modifications. Standard cell suspension of *S. pyogenes* was mixed with healthy human blood (1:4 ratio) in the absence and presence of tested antibiofilm agents/extract (at their respective BIC). The tubes were incubated for 3 h at 37 °C with gentle mixing at regular intervals. Following incubation, the assay mixtures were serially diluted and the total number of viable cells was quantified by spread plate method. Percentage of cells that survived in healthy human blood was calculated using the formula: Survival percentage = (CFU of test/ CFU of control) × 100 (Hollands et al., 2008).

**Effect of tested antibiofilm agents/extract on the MIC of antibiotics**

The influence of tested antibiofilm agents/extract on the MIC of antibiotics against *S. pyogenes* strains was evaluated using micro-broth dilution assay in accordance with Clinical and Laboratory Standards Institute guidelines (CLSI, 2006). Briefly, the stock solutions of erythromycin and tetracycline (HiMedia Laboratories,
India) antibiotics were prepared and filter sterilized using 0.22 µm syringe filter. Overnight cultures of *S. pyogenes* strains (1 x 10^3 cells) were used to inoculate THYG broth (200 µL) supplemented with two fold serially diluted antibiotics to give a final concentration ranging from 0.0001 - 16 µg mL⁻¹, in the absence and presence of tested antibiofilm agents/extract (at their respective BIC). The plates were incubated at 37 °C for 24 h under static condition. The lowest concentration of antibiotic which inhibited visible growth after incubation was recorded as MIC.

**Effect of tested antibiofilm agents on *S. pyogenes* growth in solid and liquid media**

To investigate the effect of tested antibiofilm agents/extract on the growth of *S. pyogenes* in solid media, aliquot of standard cell suspension of *S. pyogenes* was streaked over tryptose agar containing tested antibiofilm agents/extract or methanol (vehicle control) and incubated at 37 °C for 24 h. To study its effect on growth of the organism in liquid media, 1 % standard cell suspension was added to THYG broth containing tested antibiofilm agents/extract or methanol and allowed to grow in test tubes and microtiter wells for 24 h at 37 °C. After incubation, the colony morphology of cells in solid media and the growth pattern in liquid media were visually compared and photographed (Green *et al.*, 2012).

**Fourier Transform Infrared Spectroscopic (FTIR) analysis**

To determine the changes in cell wall architecture, control and tested antibiofilm agents/extract (at their respective BIC) treated *S. pyogenes* SF370 were analyzed in FTIR (Nicolet iS5 FT-IR Spectrometer, Thermo Scientific, USA). A total of sixty four scans were taken in the range of 4000 to 400 cm⁻¹ with a spectral resolution of 4 cm⁻¹. KBr was used as reference background. The IR spectra were analyzed using OMNIC software as per manufacturer specifications (Nicolet iS5 FT-
IR Spectrometer, Thermo Scientific, USA). Principal component analysis was done using portable Unscrambler version 9.7.

RNA isolation

Total RNA of *S. pyogenes* grown in the absence and presence of tested antibiofilm agents/extract (at their respective BIC) for 24 h was isolated using standard procedure described by Oh and So (2003) with slight modifications. All the steps were carried out at 25 °C. Briefly, 2 mL of control and treated cells were harvested by centrifugation at 8000 rpm for 10 min and the pellets were resuspended in 1 mL of lysis buffer (0.275 g of sodium acetate, 0.5 g of SDS and 0.034 g of EDTA were dissolved in 100 mL of DEPC water and the pH was adjusted to 5.5 and autoclaved). Glass wool (100 mg) was added to the cell suspensions and the samples were vortexed vigorously for 20 sec to break cell wall. Subsequently, the samples were centrifuged (10,000 rpm for 5 min) to remove cell debris. To the collected supernatants, equal volume of equilibrated phenol was added, mixed well and centrifuged at 10,000 rpm for 10 min. The aqueous layers were collected and mixed with equal volume of chloroform: isoamyl alcohol (24:1) mixture and centrifuged at 10,000 rpm for 10 min. The aqueous layers were collected and treated with 10 units of DNase (NEB) for 1 h at 37 °C. RNA was precipitated using equal volume of isopropanol and centrifuged (10000 rpm; 10 min). The obtained pellets were washed with 70 % ethanol, air dried and RNAs were resuspended in sterile DEPC treated MilliQ water and stored at -20 °C. The quality and quantity of the isolated RNAs were checked by running an aliquot in 2 % agarose gel using 1X TAE buffer (prepared using DEPC water).
Real Time PCR (qPCR) Analysis

The RNA samples were reverse transcribed using High capacity cDNA Reverse Transcription kit (Applied Biosystems) as per the manufacturer’s instructions. Real-time PCR was performed for candidate genes (Table 7) involved in biofilm formation and virulence, using FastStart Universal SYBR Green Master mix (Roche, Switzerland) in 7500 Sequence Detection System (Applied Biosystems Inc., USA).

Table 7. List of genes and primer sequences used for real-time PCR

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<tr>
<th>Gene</th>
<th>Primer sequence (5’-3’)</th>
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<td>Forward</td>
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Data was collected using ABI sequence detection 1.3 software (Applied Biosystems Inc., USA). The expression patterns of candidate genes were normalized against Gyrase gene expression (House-keeping gene) and quantified by calculating $2^{-\Delta\Delta CT}$.

**Caenorhabditis elegans survival assay**

To analyze the effect of tested antibiofilm agents/extract on the virulence of *S. pyogenes*, in vivo studies were performed using simple eukaryotic model organism, *C. elegans*. The toxicity of the tested antibiofilm agents/extract were preliminarily assessed by measuring the survival rate of countable numbers (~10) of worms grown in 1 mL of M9 medium in the absence and presence of tested antibiofilm agents/extract. Worms grown in the presence of equivalent amount of methanol served as vehicle control. Nearly 1000 CFU mL$^{-1}$ of *Escherichia coli* OP50, laboratory food source of *C. elegans*, was also supplemented in the medium.

On the other hand, the effect of tested antibiofilm agents/extract on the virulence of GAS was assessed by comparing the survival rate of *S. pyogenes* SF370 (~1 x 10$^3$ CFU mL$^{-3}$) infected *C. elegans* (~10) grown in the presence of methanol (vehicle control) with those grown in the presence of tested antibiofilm agents (at their respective BIC). The survival rate was monitored every 4 h till complete killing was observed in control. The animals were considered to be dead when it showed no response to external stimuli.

**In vivo biofilm formation in C. elegans**

Worms infected with *S. pyogenes* SF370 in the absence and presence of tested antibiofilm agents/extract as described in survival assay were taken at LT$^{50}$ (Lethal Time 50 - time point at which half of the infected worms in control were dead) and washed with M9 buffer containing 0.01 % sodium azide. The worms were subsequently washed with 0.5 µg mL$^{-1}$ tetracycline to remove bacteria adhered to the
external surface. The worms were stained with 0.1 % acridine orange, washed thrice with M9 buffer and viewed under CLSM to measure the intensity of bacterial colonization.

**Microscopic examination of C. elegans**

The impact of tested antibiofilm agents/extract on the virulence of *S. pyogenes* was further evaluated by directly visualizing its effect on *C. elegans* physiology. Nematodes infected with *S. pyogenes* in the absence and presence of tested antibiofilm agents/extract for LT$_{50}$ were washed thrice with M9 buffer. The washed nematodes were placed on glass slides with 0.01 % of sodium azide (for immobilization) and viewed under light microscope (NIKON SMZ, Japan) (Garsin et al., 2001).

**CFU assay**

The effect of tested antibiofilm agents/extract on the internal colonization of *S. pyogenes* inside *C. elegans* was determined using CFU assay wherein, nematodes were infected with *S. pyogenes* in the absence and presence of tested antibiofilm agents/extract (at their respective BIC) for LT$_{50}$ as described in survival assay. The nematodes were washed with M9 buffer containing sodium azide (0.01 %) to paralyse them. Subsequently, the nematodes were washed with tetracycline (0.5 µg mL$^{-1}$) to kill the bacterial cells adhered to the external surface of nematodes. Sodium azide treatment (which paralyses the nematodes) was given prior to tetracycline wash to prevent antibiotic intake and killing of internalized *S. pyogenes*. Silicon carbide (0.4 g) was added to the nematode suspension and vortexed well to crush the nematodes and release the internalized bacteria into the solution. The colony forming units of the released bacteria were quantified using spread plate method (Durai et al., 2013).
Statistics

All the experiments were performed at least three times in triplicates. The data were presented as mean values ± standard deviation. The differences between control and test were analyzed using Student’s t test.