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Bacterial Aggregation Assay in the Presence of Cyclic Lipopeptides

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[Abstract] Lipopeptides is an important class of biosurfactants having antimicrobial and anti-adhesive activity against pathogenic bacteria. These include surfactin, fengycin, iturin, bacillomycin, mycosubtilin, lichenysin, and pumilacidin (Arima *et al*., 1968; Naruse *et al*., 1990; Yakimov *et al.*, 1995; Steller and Vater, 2000; Roongsawang *et al*., 2002; Vater *et al*., 2002). To date, none of these lipopeptides have been reported to possess any anti-motility activity. We isolated, purified and characterized two novel cyclic lipopeptides (CLPs) from *Bacillus* sp. 176 using high performance liquid chromatography, mass spectrometry and nuclear magnetic resonance spectroscopy. CLPs dramatically suppress the motility of pathogenic bacterium *Vibrio alginolyticus* 178, and promote cellular aggregation without inducing cell death. Cell aggregation assay was performed with the modification according to methods described by Dalili for anti-biofilm assay (Dalili *et al*., 2015). In future, this assay can be adapted to test both the cell aggregation and anti-biofilm activity of lipopeptide-like active substances derived from bacteria.

Keywords: Cyclic lipopeptides, Cell aggregation, *Vibrio alginolyticus*, *Bacillus* sp. 176, Anti-motility, Anti-biofilm

[Background] Overuse of broad-spectrum antibiotics and the accompanying proliferation of drug-resistant bacteria have stimulated efforts to develop environment-friendly biocontrol measures to reduce health hazards and environmental pollution (Nam *et al*., 2016; Sajitha *et al*., 2016). In recent years, the anti-microbial properties of biological surfactants have been increasingly recognized and harnessed for antibacterial, antifungal, and antiviral applications (Cameotra and Makkar, 2004; Singh and Cameotra, 2004; Rodrigues *et al*., 2006). Lipopeptides are the most widely reported class of biosurfactants having antimicrobial and 100 anti-adhesive activity against pathogenic bacteria, due to the amphipathic nature of their peptide and fatty acid components (Das *et al*., 2008; Dalili *et al*., 2015). In this study, two cyclic lipopeptides (CLPs) derived from a competing bacterium (*Bacillus* sp. 176) are found to inhibit the motility and promote the aggregation of *V*. *alginolyticus* 178. We purified and characterized the active anti-motility compounds and determined their structural and functional properties. In order to explore the mechanism of action of the CLPs, their impact on cell aggregation, adherence, and the expression of flagellar assembly components in *V. alginolyticus* were also investigated.

Materials and Reagents

1. Pipette tips (Corning, Axygen®, catalog numbers: T-200-Y-STK, T-300-L-R, T-1000-B)
2. 15 ml culture tube (Bomei, catalog number: SGJS15ML)
3. Flat bottom 96-well microtiter plate (Corning, catalog number: 3628)
4. Coverslips (CITOTEST LABWARE MANUFACTURING, catalog number: 80340-1130)
5. Bacterial strains (Identified and stored in our lab)
    a. *V. alginolyticus* 178
    b. *V. anguillarum*
    c. *V. splendidus*
    d. *V. vulnificus*
    e. *Pseudomonas aeruginosa*
    f. *P. stutzeri*
    g. *Staphylococcus aureus*
    h. *Bacillus* sp. 176
    i. *B. subtilis*
6. Methanol (Sinopharm Chemical Reagent, catalog number: 10014108)
7. Ethanol (Sinopharm Chemical Reagent, catalog number: 10009259)
8. Peptone (Solarbio, catalog number: P8450)
9. Tryptone (OXOID, catalog number: LP0042)
10. Yeast extract (OXOID, catalog number: LP0021)
11. Agar powder (Solarbio, catalog number: A8190)
12. Sodium chloride (NaCl) (Sinopharm Chemical Reagent, catalog number: 10019318)
13. Crystal violet (Sinopharm Chemical Reagent, catalog number: 71012314)
14. Glacial acetic acid (Sinopharm Chemical Reagent, catalog number: 10000218)
15. Gelatin (Solarbio, catalog number: G8060)
16. 25% glutaraldehyde solution (Sinopharm Chemical Reagent, catalog number: 30092436)
17. Dimethyl sulfoxide (DMSO) (MP Biomedicals, catalog number: 02196055)
18. Saline LB broth (see Recipes)
19. LB broth (see Recipes)
20. 1% (w/v) solution of crystal violet (see Recipes)
21. 30% (v/v) acetic acid (see Recipes)
22. Modified 2216E broth (see Recipes)
23. 1% (w/v) gelatin solution (see Recipes)
24. 5% glutaraldehyde solution (see Recipes)
25. Sterile saline solution (see Recipes)

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26. 10 mg/ml CLPs (see Recipes)

Equipment

1. 1-10 µl pipettor (Gilson, model: P10N)
2. 20-200 µl pipettor (Gilson, model: P200N)
3. 100-1,000 µl pipettor (Gilson, model: P1000N)
4. Autoclave sterilizer (Zealway Instrument, model: GI80TW)
5. Constant temperature shaker (CRYSTAL, model: IS-RDS3)
6. Centrifuge (Eppendorf, model: 5418 R)
7. SYNERGY-H1 microplate reader (BioTek Instruments, model: Synergy H1)
8. Scanning electron microscope (SEM) (Hitachi, model: S-3400N)
9. Transmission electron microscope (TEM) (Hitachi, model: H-7650)
10. Biological safety cabinet (Heal Force, model: HFsafe 900LC)

Procedure

A. Bacterial strains culture
1. Single colonies of bacterial strains used in this protocol, including *V. alginolyticus* 178, *V. anguillarum*, *V. splendidus*, *V. vulnificus*, *Pseudomonas aeruginosa*, *P. stutzeri*, *Staphylococcus aureus*, *Bacillus* sp. 176 and *B. subtilis*, were selected from their pure culture plates.
2. *V. alginolyticus* 178, *V. anguillarum*, *V. splendidus*, *V. vulnificus*, *Pseudomonas aeruginosa*, *P. stutzeri* and *Bacillus* sp. 176 were cultured in 5 ml saline Luria-Bertani (LB) broth (see Recipes) in tubes at 28 °C overnight with shaking at 170 rpm.
3. *Staphylococcus aureus* and *B. subtilis* were cultured in 5 ml LB broth in tubes at 37 °C overnight with shaking at 170 rpm.

B. Aggregation assay of *V. alginolyticus* 178 in culture tubes
1. After overnight culture, dilute the cell suspension of *V. alginolyticus* 178 at 1:100 with saline LB broth.
2. Prepare six sterilized 15 ml culture tubes.
3. Add 3 ml bacterial suspension into each tube.
4. Treat the cells with 90 µl (10 mg/ml) CLPs with a final concentration of 300 µg/ml or same volume DMSO, respectively, and incubate statically at 28 °C for 24 h after homogenization (Figure 1A).

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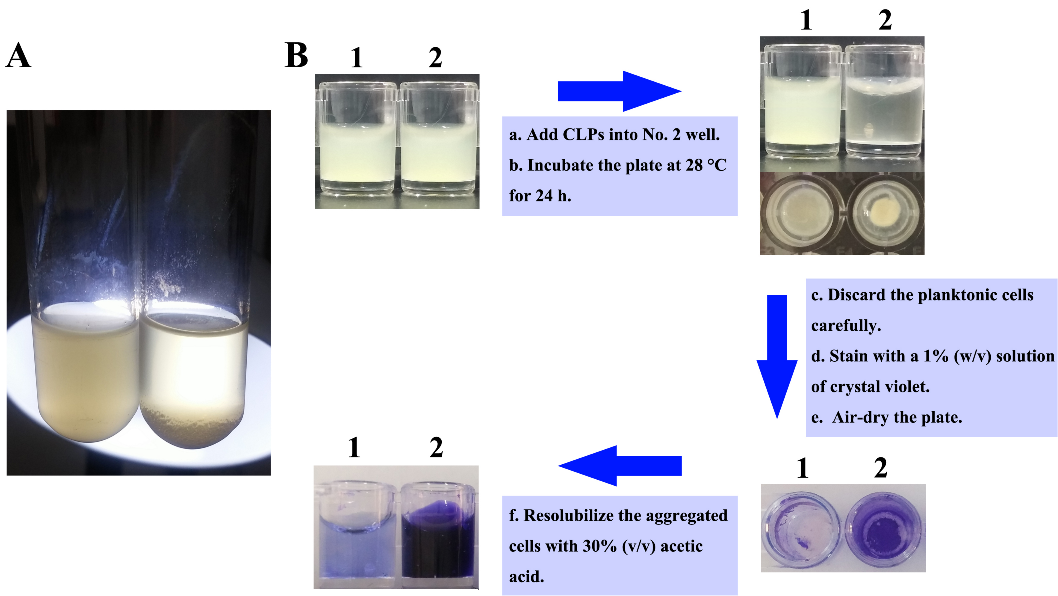

Figure 1. Experimental design of *V. alginolyticus* 178 aggregation in culture tubes and 96-well plates. A. Aggregation of *V. alginolyticus* 178 was observed in culture tubes after CLPs treatment. B. Schematic representation of the steps in aggregation assay using 96-well plates (No. 1 well contains untreated *V. alginolyticus* 178; No. 2 well contains CLPs treated *V. alginolyticus* 178).

C. Aggregation assay of *V. alginolyticus* 178 in 96-well microtiter plate
1. Add 200 µl diluted bacterial suspension into each well of the flat bottom 96-well microtiter plate.
2. In experiment group, add 6 µl CLPs (10 mg/ml) with a final concentration of 300 µg/ml in each well, and homogenize with bacterial suspension.
3. Wells containing *V. alginolyticus* 178 cell suspension without treatment, or with DMSO treatment, are employed as controls.
4. Five replicate wells for each treatment, and incubate the plate at 28 °C for 24 h.
5. After incubation, discard the planktonic cells carefully with a pipettor and wash the aggregated cells in each well three times with sterile saline.
6. Fix aggregated cells with 200 µl of methanol (99% purity) per well statically for 15 min, and empty the plates using a pipettor and leave to dry.
7. Then stain the contents of the wells with 200 µl of a 1% (w/v) crystal violet (see Recipes) solution for 10 min at room temperature.
8. Rinse out crystal violet with a pipettor; air-dry the plates after wash with sterile deionized water, and resolubilize the dye bound to the aggregated cells in 200 µl of 30% (v/v) acetic acid (see Recipes) solution.
9. Measure the absorbance of each well in a SYNERGY-H1 microplate reader (BioTek, USA) at 595 nm using 30% acetic acid as the blank (Figure 1B).

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D. Quantitative assay of CLPs' activity

1. Add 200 µl diluted bacterial suspension into each well of the flat bottom 96-well microtiter plate.
2. Add 6 µl CLPs at different concentration (25, 50, 100, 200, 300 µg/ml) into different wells, respectively.
3. Wells containing *V. alginolyticus* 178 cell suspension without treatment, or with DMSO treatment, are employed as controls.
4. Operate the other procedures as described above in Steps C4-C9 (Figure 2).

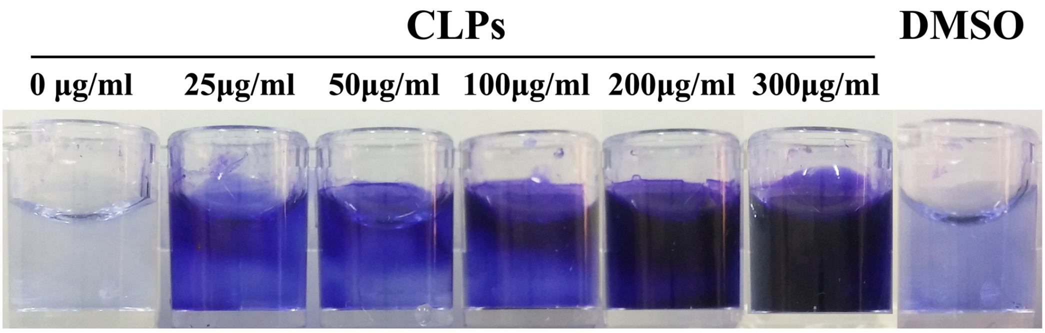

Figure 2. Quantitative cell aggregation assay at different concentrations of CLPs

E. Scanning electron microscope observation

1. Dilute overnight cultured cell of *V. alginolyticus* 178 at 1:100 into 5 ml fresh modified 2216E medium (see Recipes) and culture for another 3 h to OD_{600} 0.2-0.3 with shaking at 170 rpm.
2. Add the cell suspension with 50 µl 50 or 100 µg/ml CLPs or DMSO for additional 3 h.
3. Centrifuge the cells at 1,400 *x g* for 4 min at room temperature, and resuspend the cells with a sterilized saline solution.
4. Drop the resuspended bacterial cells on sterilized coverslips (enveloped by 1% gelation solution, see Recipes), respectively.
5. Dry coverslips at room temperature.
6. Fix samples with a 5% glutaraldehyde solution (see Recipes) for 1 h.
7. Wash with sterile saline solution (see Recipes).
8. Dehydrate samples in a successively graded ethanol series (50%, 60%, 70%, 80%, 90% and 100% ethanol). Incubate the samples for 10 min in each grade ethanol solution. After the final incubation in 100% ethanol, the samples are ready for observation under scanning electron microscope.

   Note: You can stop when the samples are placed in 80% ethanol.
9. Observe the samples under a scanning electron microscope (Figure 3).

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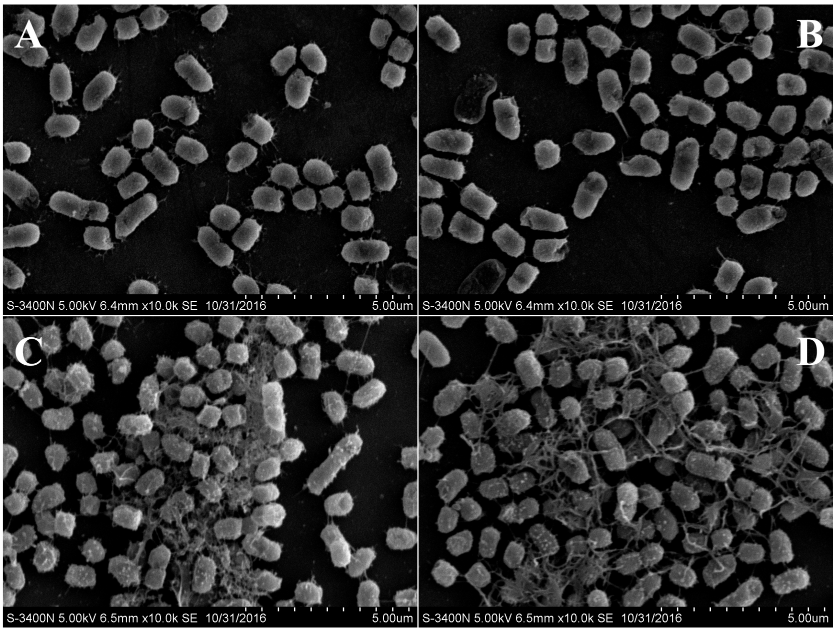

Figure 3. SEM images of *V. alginolyticus* 178 cells following treatment with CLPs. *V. alginolyticus* 178 cell morphology without any treatment (A), with DMSO treatment (B), with 50 µg/ml CLPs (C), and with 100 µg/ml CLP treatment (D).

F. Transmission electron microscope observation
1. Perform the procedures as described above in Steps E1-E3.
2. Fix samples with a 5% glutaraldehyde solution for 1 h.
3. Drop samples on copper grids, and dried at room temperature.
4. Observe the samples under a transmission electron microscope (Figure 4).

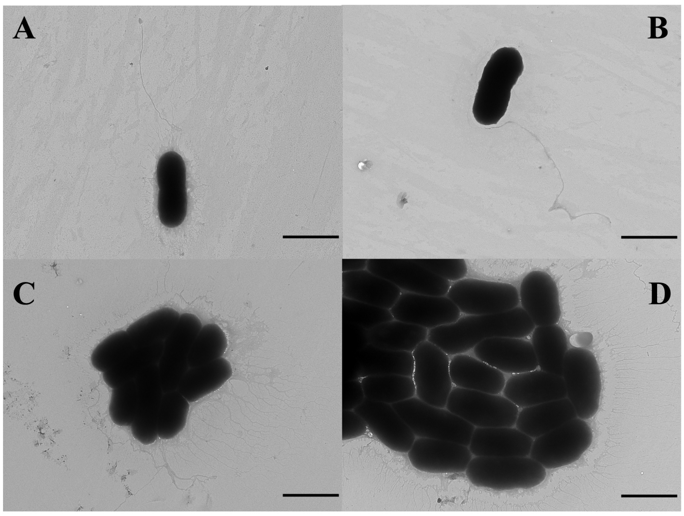

Figure 4. TEM images of *V. alginolyticus* 178 cells following treatment with CLPs. *V. alginolyticus* 178 cell morphology without any treatment (A), with DMSO treatment (B), with 50 µg/ml CLPs (C), and with 100 µg/ml CLP treatment (D). The scale bars are 2 µm.

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G. The spectrum of action of the CLPs
   1. After overnight culture, dilute the cell suspension of *V. anguillarum*, *V. splendidus*, *V. vulnificus*, *Pseudomonas aeruginosa*, *P. stutzeri*, *Staphylococcus aureus*, *Bacillus* sp. 176 and *B. subtilis* at 1:100 with saline LB broth or LB broth, respectively.
   2. Operate the other procedures as described above in Steps C2-C9 (Figure 5).

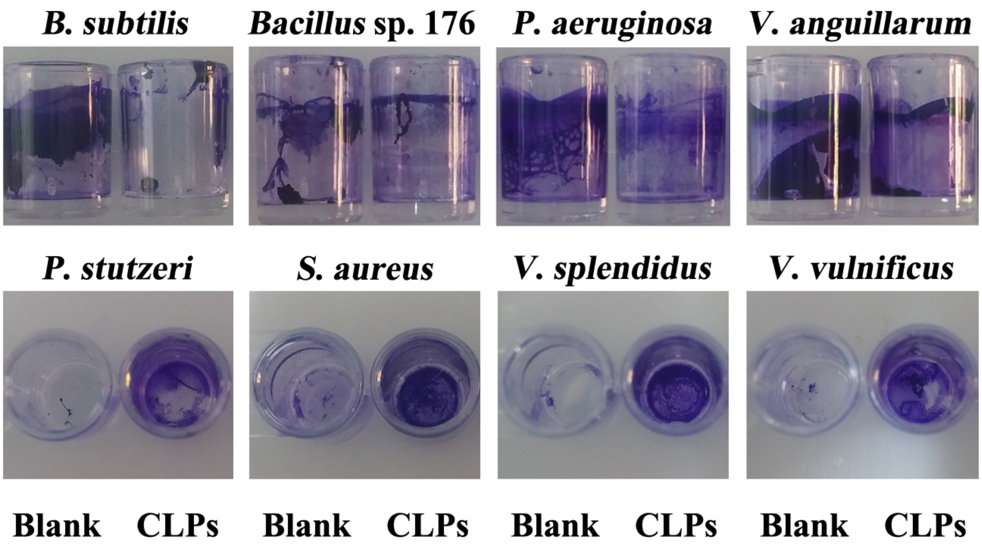

Figure 5. Action spectrum assays of CLPs. Blank: untreated strains; CLPs: CLPs treated strains.

Data analysis

All data were analyzed by the Statistical Package for Social Sciences (SPSS) 18.0 software. The statistically significant differences among groups were calculated using one-way analysis of variance (one-way ANOVA) followed by a post hoc multiple-comparisons (Tukey's) test.

Notes

1. To avoid other bacteria contamination, microbial operations must be carried out under aseptic conditions.
2. All bacterial mediums and reagent solutions are sure to be prepared freshly and sterilized by autoclaving.
3. It is necessary to homogenize the cell suspension with CLPs before incubation.
4. Notice that the operation in discarding the planktonic cells and washing the aggregated cells with pipettor must be careful and gentle.

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Recipes

1. Saline LB broth (1 L)
   10 g peptone
   5 g yeast extract
   1 L filtered seawater
   pH 7.4-7.6
   Sterilize by autoclaving at 121 °C for 20 min, and store at 4 °C
2. LB broth (1 L)
   10 g peptone
   10 g NaCl
   5 g yeast extract
   1 L deionized water
   pH 7.4-7.6
   Sterilize by autoclaving at 121 °C for 20 min, and store at 4 °C
3. 1% (w/v) solution of crystal violet (100 ml)
   1 g crystal violet
   100 ml sterile water
4. 30% (v/v) acetic acid (100 ml)
   30 ml glacial acetic acid
   70 ml sterile water
5. Modified 2216E broth (1 L)
   5 g tryptone
   1 g yeast extract
   1 L filtered seawater
   pH 7.4-7.6
   Sterilize by autoclaving at 121 °C for 20 min, and store at 4 °C
6. 1% (w/v) gelatin solution (10 ml)
   0.1 g gelatin
   10 ml deionized water
   Sterilize by autoclaving at 121 °C for 20 min, and store at 4 °C
7. 5% glutaraldehyde solution (10 ml)
   2 ml 25% glutaraldehyde solution
   8 ml sterile water
8. Sterile saline solution (100 ml)
   0.85 g NaCl
   100 ml deionized water
   Sterilize by autoclaving at 121 °C for 20 min, and store at 4 °C

9. 10 mg/ml CLPs (1 ml)
   10 mg CLPs
   1 ml DMSO

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