Influence of Putisolvin on Membrane Vesicle and Nanotube Formation in *Pseudomonas putida* IsoF

Dissertation zur Erlangung der naturwissenschaftlichen Doktorwürde (Dr. sc. nat.) vorgelegt der Mathematisch-naturwissenschaftlichen Fakultät der Universität Zürich

> von Ratchara Kalawong

> > aus

Thailand

Promotionskommission

Prof. Dr. Leo Eberl (Vorsitz) Asst. Prof. Dr. Masanori Toyofuku Prof. Dr. Jakob Pernthaler

Zurich, 2022

Table of Contents.

Acknowledgments	IV
Summary	VI
Table of figures	х
List of Tables	хv
Chapter 1 Introduction	1
1.1. The genus Pseudomonas	2
1.2. Cyclic lipopeptides (CLPs)	5
1.3. Bacterial membrane vesicle	11
1.4 Objectives of the study	14
Chapter 2 Characterization of genetic and biological functions of putisolvin in	
Pseudomonas putida IsoF	15
2.1. Abstract	16
2.2. Introduction	17
2.3. Results	19
2.4. Discussion	40
2.5. Materials and methods	44
2.6. Supplementary material	50
Chapter 3 Bacterial nanotube formation by a biosurfactant	68
3.1. Abstract	69
3.2. Introduction	70
3.3. Results	72
3.4. Discussion	85
3.5. Materials and methods	88
3.6. Supplementary material	90

Chapter 4 Prophage induction and bacteriophage infection trigger membrane vesicle in	
Pseudomonas putida IsoF	93
4.1. Abstract	94
4.2. Introduction	95
4.3. Results	97
4.4. Discussion	112
4.5. Materials and methods	115
4.6. Supplementary material	117
Chapter 5 Conclusion and future perspectives	131
5.1 Conclusion and future perspectives	132
Chapter 6 General Materials and Methods	136
6.1 Bacterial strains, plasmids, and oligonucleotide	137
6.2 Media and buffer	140
6.3 General Methods	146
References	149
Appendices	162
Appendix 1 : Abbreviations	162
Appendix 2 : Publications	164
Curriculum Vitae	184

Table of figures

Figure 1. 1 Pseudomonas species serve a wide range of roles in nature. Pseudomonads show high versatility in colonizing different environments, leading to the development of numerous biological traits and lifecycles. They demonstrate remarkable metabolic diversity and significantly overlap their roles among the strains. (This chart was modified from Silby et al., 2011 and the graphics were taken from BioRender.) 4 Figure 1. 2 The groups of cyclic lipodepsipeptides produced by NRPSs in Pseudomonas spp. The stereochemistry of amino acid sequences is indicated in different colors; green: D-amino acids, blue: Lamino acids, orange: D-allo amido acids, and white: unknown. This figure was modified from (Geudens Figure 1. 3 The possible self-assembled structures of lipopeptides. The general characteristic of amphiphilic molecules and the possible self-aggregated structures when the concentration is above Figure 1. 4 Types of vesicle generated by various pathways (adapted from Toyofuku et al., 2019). There are two main origins of membrane vesicle formation in Gram-negative bacteria. The first is classic outer membrane blebbing, producing OMVs. The second pathway is explosive cell lysis caused by the activity of phage-derived endolysin. This mechanism produces mostly OIMVs and EOMVs. Both groups of MVs contain cytoplasmic material. In Gram-positive bacteria, the CMVs are generated through 'bubbling cell

Figure 2. 1 Putisolvin biosynthetic cluster of P. putida IsoF. The cluster contains three NRPS genes; psoA, psoB, and psoC (yellow arrows), three exporter genes; macA, macB, and tolC (green arrows), and two Figure 2.2 Putisolvin purification and characterization. (A) Chromatograph of putisolvins I and II produced by IsoF wild-type compared to the putisolvin mutant. The cell-free supernatants of each strain were extracted and purified by HPLC. (B) The two fractions were tested for their ability to reduce the surface tension by drop collapse assay. Control, MQ; Putisolvin I, eluted at 11.43 min; Putisolvin II eluted at Figure 2. 4 Determination of the presence of putisolvin by drop collapse assay. The surface tension of the filtered supernatants of IsoF and the indicated transporter mutants was tested by drop collapse assay. Figure 2. 5 Quantification of putisolvin by Du Nouy ring method. Cell-free supernatants of IsoF and the indicated mutants were collected after growing in ABC medium supplemented with 18 ml l⁻¹ glycerol at 24 and 48 h. The surface tension of each sample was measured using a Du Nouy ring. The graph shows Figure 2. 6 Quantification of putisolvin by HPLC. IsoF and the indicated mutants were cultured in ABC medium with 18ml I⁻¹ of glycerol for 24 h. Cell (A) and supernatant (B) fractions were separated, and putisolvin was extracted from both fractions with ethyl acetate 1:1 v/v. The ethyl acetate fraction was evaporated under vacuum, and the dry compound was dissolved in acetonitrile. To quantify putisolvin, reverse phase C18 HPLC was used. The graphs show data from three biological replicates and error bars represent SD. Significance was assessed using one-way ANOVA (Dunnett) with confidence interval of

Figure 2. 7 Deletion of macB reduces P_{psoABC} and P_{psoR} activity. The activity of the psoABC and psoR promoters was measured using a GFP transcriptional fusion. Activity was measured every 30 min for 36 h (A) psoR (B) psoABC. After 36 h of incubation, the promoter activity was normalized by cell density (C) psoR (D) psoA. The graphs represent data from three replicates, and error bars represent SD. Data were analysed by one-way ANOVA (Dunnett) with confidence interval of 99%, p-value, * < 0.01, Figure 2. 8 Sequence alignment of MacB proteins. The protein sequence of MacB orthologues from different bacteria; A. baumannii (5ws4A), E. Coli (5nikJ), P. putida IsoF putisolvin MacB, and P. putida IsoF pyoverdine MacB were aligned using the Align123 algorithm, which considers the protein secondary structure. The short N-terminal peptide present only in IsoF putisolvin MacB has been Figure 2. 9 Predicted dimeric protein structure of MacB from the putisolvin gene cluster of *P. putida* IsoF. The amino acid sequence of putisolvin MacB was predicted from the nucleotide sequence. The ATP Figure 2. 10 Protein structure alignment of putisolvin MacB against predictions from three other MacBs. Two MacB proteins were from P. putida IsoF (green: putisolvin cluster, orange: pyoverdine cluster), and the other was from A. baumannii (purple). The frame highlights the additional short peptide present Figure 2.11 The electrostatic potential surface of MacB from 4 different sources. The electrostatic surface potential of various MacB proteins was predicted using the Adaptive Poisson-Boltzmann Solver package Figure 2. 12 Swarming assay of IsoF and putisolvin mutants. Bacteria were spotted on ABC plates (0.4 % agar) and incubated at 30 °C. The size of the colonized area was measured after three days. (a) IsoF Figure 2. 13 Biofilm formation of wild-type IsoF and the transporter mutants. All tested strains, (IsoF, ΔpsoABC, ΔmacA, ΔmacB, ΔtolC, and ΔmacAB/tolC) were grown in ABC medium for 48 h. The data illustrate the biofilm index measured at an absorbance of 570 nm. The Biofilm Index (BI) was calculated as follows: BI= OD570 /OD550 * 100. Three biological replicates were tested. The data was evaluated by one-way ANOVA (Dunnett) with confidence intervals of 99 %, Error bars = SD, p-value, *** < 0.0001. Figure 2. 14 Pellicle formation of IsoF and the mutants. Bacteria were cultured in two different media, (A) ABC and (B) LB, at room temperature without shaking. Pellicle formation was monitored after 4 days. Figure 2. 15 Siderophore production by IsoF and transporter mutants. A cell suspension of each tested strain (OD600 of 0.01) was dropped on the CAS agar plate. The plate was incubated at 30 °C, and the Figure 2. 16 Antibacterial activity of putisolvin. Purified putisolvins I and II were spotted and dried on a layer of agar (1.5 % LB agar) at concentrations of 0, 0.5, 1, 5, and 10 mg ml⁻¹. A second layer of agar was then poured, containing the test Gram-negative and Gram-positive bacteria, including P. putida KT2440, E. coli K12, B. cenocepacia H111, S. aureus DSM20235, and B. subtilis 168, to test for susceptibility to Figure 2. 17 Putisolvin influences vesicle production. The wild-type IsoF and psoABC deletion mutant were cultured in ABC broth supplemented with glycerol. MVs were collected at 24 and 48 h and (A) lipid membrane was quantified using the fluorescent dye FM1-43 (B) total protein by BCA protein assay. The graphs show data from three independent experiments. Statistical analysis was carried out Figure 2. 18 SDS-PAGE analysis of proteins (A) Bacterial cell fraction. Lanes 1 and 2, WT and $\Delta psoABC$, respectively. M, PageRulerTM Pre stained 10- 180 kDa (B) MV proteins. Lanes 1-3, three biological replicates of MV samples from IsoF culture. Lanes 4-6, MVs from ΔpsoABC culture. M, PageRulerTM Plus

Figure S2. 3 Quantification of putisolvin extracted from the supernatant by HPLC. Bacteria were grown in ABC medium supplemented with 18ml I⁻¹ glycerol at 30 °C for 24 h. Cell-free supernatant was extracted with ethyl acetate (1:1 volume). The solvent fraction was evaporated, and dry compounds were Figure S2. 4 Quantification of putisolvin extracted from bacterial cells by HPLC. Bacteria were grown in ABC medium supplemented with 18ml ⁻¹ glycerol at 30 °C for 24 h. Cell pellets were sonicated for 20 min, and putisolvin was extracted with ethyl acetate (1:1 volume). The solvent fraction was evaporated, Figure S2.5 Sequence alignment of MacAB/TolC transporter proteins. The protein sequence of (A) MacA, (B) MacB and (C) TolC orthologues from P. putida IsoF putisolvin and pyoverdine cluster were aligned using the ClustalW. The subunits share high amino acid similarity (60.1 % for MacA, 63.6 % for MacB, Figure S2.5 Sequence alignment of MacAB/TolC transporter proteins. The protein sequence of (A) MacA, (B) MacB and (C) TolC orthologues from P. putida IsoF putisolvin and pyoverdine cluster were aligned using the ClustalW. The subunits share high amino acid similarity (60.1 % for MacA, 63.6 % for MacB,

Figure 3. 1 Nanotubes were present in the IsoF MV fraction but not in that from the putisolvin deficient strain. MVs isolated from (A) IsoF and (B) $\Delta psoABC$ cell culture supernatants were visualized by negative stain TEM. Scale bars represent 200 nm......72 Figure 3.2 Cryo tomograms of the IsoF MV fraction showed interactions between nanotubes and vesicles. A, B: Vesicles and nanotubes observed in the tomograms., C: Small vesicles were inside the nanotube, D-E: nanotube capped with rougher membranes., F: Vesicle was at the nanotube tip., G: Vesicle fused to the nanotube. H-I: Vesicle attached to the structure tip., J-K: Vesicle in proximity to a nanotube. Figure 3. 3 Cryo-ET images showing the nanotube fused vesicle structure in IsoF (Lollipop). Bacteria were grown on an EM grid which were placed on cellulose membrane on LB agar plate for 5 h. The samples were frozen by plunging into liquid nitrogen and visualized by ECT. (A) Tomograms of IsoF showed tubular structures (NT) connected to the outer membrane (black arrows) tipped with a vesicle (MV) (white arrows). (B) Only MV production by classical blebbing was observed in the putisolvin mutant. The tomograms were captured by Dr. Bastien Casu......75 Figure 3. 4 Bacterial nanotube network present in IsoF and flagellum mutant biofilms. Bacteria were grown on a coverslip placed in ABC broth without shaking for two days. The samples were fixed by 2.5% GA in 0.1 M Cacodylate buffer and visualized by SEM (A) IsoF (B) ΔpsoABC and (C) GC25 (flagellum mutant). The IsoF and GC25 strains built nanotubes that connected neighboring cells. Such tube structures were absent from the putisolvin mutant. The cell morphology of putisolvin deficient strains Figure 3. 5 Observation of nanotube formation in bacterial culture. All strains were grown in ABC broth for 24 h at 30 °C (A) IsoF, (B) ΔpsoABC, and (C) GC25 (flagellum mutant). Cell cultures were negatively

Figure 3. 6 Biosurfactant putisolvins I and II self-assemble into nanotube structures of various diameters and lengths. Putisolvins I and II produced from IsoF were extracted and purified by HPLC. The purified compounds were dissolved in MQ. The resuspended samples were dropped on a copper EM grid and inspected by cryo-EM (A-D) Putisolvin I (E-G) Putisolvin II. The tomograms were captured by Dr. Bastien Figure 3. 7The three-dimensional (3D) reconstruction of purified putisolvin tube. The tomograms of purified putisolvin tubes were collected by cryo-EM. The EM serial sections were reconstructed in 3D by an IMOD......78 Figure 3.8 The average diameter of nanotubes found in bacterial culture compared to those from purified putisolvins I and II. Cell culture of IsoF was collected after growing in ABC medium for 24 h. Putisolvins I and II were dissolved in PBS to a concentration of 0.1 mg ml⁻¹. All samples were negatively stained and observed by TEM. The graph shows no significant difference between each group. Error bars display SD, and one-way ANOVA was performed (Dunnett)......79 Figure 3. 9 The critical micelle concentration (CMC) of different surfactants. Each surfactant's CMC was determined using a 1,6-diphenyl-1,3,5-hexatriene (DPH) fluorescent probe. The fluorescence intensity of a serial dilution was plotted. The CMC was the intersection point of the straight line at low concentration with the straight line at the region of rapid increase. (A) TritonX-100, (B) SDS, (C) Figure 3. 10 Visualization of putisolvin nanotubes at different concentrations. Putisolvin I suspension was collected at three different concentrations (upper graph). The samples were imaged by negative staining TEM (A) below CMC (0.0005 % w/v, (B) at CMC (0.004 % w/v), and (C) above CMC (0.125 % Figure 3. 11 Stability of nanotubes in different ranges of pH. Putisolvin nanotubes at 0.004 % w/v putisolvin were incubated in 20 mM universal buffer (Britton-Robinson) at various pHs ranging from 4 to 12. The samples were visualized by TEM after 24 h incubation. The scale bar represents 500 nm. . 82 Figure 3. 12 Observation of nanotubes in pellicle of IsoF. The wild-type IsoF and putisolvin mutant ApsoABC were cultured at RT in ABC medium without shaking for 2 days. Pellicles of both strains were stained by the membrane probe FM1-43 for 30 min. Visualization was done by CLSM using the 100x Figure 3. 13 Non-conjugative plasmid transfer assay. The donor strains were IsoF and $\Delta psoABC$ carrying the pIN25-gfp plasmid with the Km resistance gene, and the recipients were IsoF and putisolvin mutant genomically tagged with mcherry and a Gm resistance marker. After co-culture of the donor and the recipient on an LB agar plate for 8 h, the mixture was serially diluted and spotted on LB plates containing appropriate antibiotics to select for the recipient strain bearing the plasmid. The plates were grown at

Figure 4. 1 MV production by P. putida IsoF under different conditions. (a) IsoF was cultured in 5 different media; LB, TSB, King's B, M9 glucose, and ABC. After 24 h, MVs from each condition were isolated and quantified by staining with a lipid fluorescent dye FM1-43. (b) Vesicle yield after culture in LB and ABC medium at 12, 24, 48, and 72 h. (c) IsoF was grown in LB and ABC with and without Figure 4. 2 Comparison of growth and MV formation between P. putida IsoF and KT2440 after treatment with MMC. The indicated MMC concentrations (ng ml⁻¹) were added after 4 h incubation and the OD₆₀₀ measured to generate a growth curve. MV production was quantified by lipid fluorescent dye FM1-43. (a) IsoF growth is not affected by MMC dose. (b) KT2440 growth curve displaying does-dependent cell lysis after 6 h. (c) MV liberation from IsoF was not significantly altered by MMC concentration after 24 Figure 4. 3 Identification of prophages in the IsoF and KT2440 genomes by PHASTER. Prophage regions found in (a) IsoF and (b) KT2440. Prophage region analysis and illustration were obtained by the phage search tool, PHASTER...... 101 Figure 4.4 Plaque formation from different soil bacteriophages. Isolated phages that were able lyse IsoF were screened and purified. Isolate that showed different plaque formations (distinguished by size and pattern) were streaked on bacterial lawn plates (a) phage Nr.1 (b) phage Nr.2 (c) phage Nr.4 (d) phage Figure 4. 5 TEM micrographs of bacteriophages. The 6 soil phage isolates which can infect IsoF, which were collected by ultracentrifugation and visualized using negative staining TEM. Scale bars present 50 nm.... 104 Figure 4. 6 Lysogens show tolerance to lysis by the integrated phage in a spot assay. Cell-free supernatant from an IsoF lysogen was spotted at the centre of a first agar layer, with a second layer containing IsoF (a) or the same lysogen (b-d) poured on top. The halo, representing cell lysis, was observed after 16 h incubation...... 105 Figure 4. 7 MMC treatment in P. putida IsoF and the lysogens. MMC (500 ng ml⁻¹) was added to the culture after 4 h of incubation, and MVs were harvested at 24 h. Membrane-specific dye FM1-43 quantified MVs liberated from IsoF and lysogens.....106 Figure 4. 8 Bacteriophages induce cell lysis. IsoF was cultured in LB medium for 4 h to reach the beginning of the log phase. Bacteriophage isolate 1 was added into the culture (10⁶, 10⁷, 10⁸ PFU ml⁻¹). (a) The OD_{600} was measured every hour to observe cell lysis. (b) Cells were checked at 4 h after bacteriophage treatment. Propidium iodide (PI) was used for staining nucleic acids, indicating cell death. The scale bar Figure 4. 9 MV formation in the presence of bacteriophage. IsoF was cultured in LB medium for 4 h, and phage isolate 1 was added to the culture (10⁶, 10⁷, 10⁸ PFU ml⁻¹). MVs were collected at 24 h and quantified by several methods. (a) MV concentration (NanoSight NS300 particle counter), (b) Vesicle size distribution by NanoSight NS300, (c) Lipid membrane by specific FM1-43 staining, (d) Total protein Figure 4. 10 Lysogenic strains showed increased biofilm formation. (a) IsoF and lysogens were grown in ABC medium in 96-well plates for 48 h. Biofilm was stained with 1 % crystal violet solution and dissolved in DMSO and OD₅₇₀ measured. WT; IsoF, L1; lysogen 1, L16; lysogen 16; L23; lysogen 23 (b) The Biofilm Index (BI) was calculated as follows: BI= OD570 /OD550 * 100. 109 Figure 4. 11 Host specificity of phage isolates among Pseudomonas spp. Phage isolates 1, 2, 4, 16, 20, and 23 were tested in bilayer assay against ten different *Pseudomonas* strains. The results were recorded

List of tables

Table 2. 1 Potentia	al secondary metabolic gene	clusters in IsoF,	analyzed by antiSMASH 5.2.	20
---------------------	-----------------------------	-------------------	----------------------------	----

Table S2. 1 Transporters showing homology at the amino acid level in GenBank, found by NCB BLAST
Table S2. 2Proteins present in MVs with > 2-fold change in abundance between wt and putisolvir mutant cultures. (1) 53
Table S3. 1 Size and shape analysis of purified putisolvin nanotubes observed by ETC 92
Table 4. 1 Comparison of prophage regions identified within IsoF and KT2440 genome
Table S4. 1 Prophage identification of P. putida IsoF.117Table S4. 2 Prophage identification in P. putida KT2440119