Supplementary Information

The inherent antibiotic activity of myxobacteria-derived autofluorescent outer membrane vesicles is switched on and off by light stimulation

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Supplementary methods

Supplementary figures

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Isolation and purification of OMVs

For OMV isolation, myxobacterial culture supernatants were centrifuged 10 and 15 min at 9,500 × g at 4°C to remove cells and cell debris. Typically, 30 mL of the supernatant was transferred into ultracentrifugation (UC) tubes and centrifuged for 2 h at 100,000 × g at 4°C (Beckman L90K, rotor SW 32Ti) to pellet the OMVs. After carefully removing the supernatant, the pellet was resuspended in 400 µL filtered PBS and transferred to low-binding reaction tubes (Axygen) for analysis, additional purification or stored at -80°C for subsequent experiments.
To separate OMVs from soluble proteins and other low-molecular contaminants, glass columns of 1.5 cm diameter and 30 cm length were packed with Sepharose 2B-CL (GE Healthcare) and equilibrated with PBS. OMV pellets (typically 400 µL) were loaded onto these size-exclusion chromatography columns and eluted with PBS, collecting fractions of 1 mL. When not in use, SEC column were stored in highly purified water with 20% ethanol.

Characterization of OMVs

OMVs were analyzed by nanoparticle tracking analysis (NTA) in terms of size, size distribution and yield. The detection threshold was set to 5, camera level to 13 and 20 – 100 particles per frame were measured. Samples were diluted with freshly filtered (0.2 µm) PBS and typically 100 µL sample volume was used with 3 videos with 30 sec recording. Data were analysed using the NanoSight 3.1 software. Protein concentrations in the SEC fractions or pellets were analysed using a Quanti Pro bicinchoninic acid (BCA) protein assay (Sigma Aldrich) as per the suppliers' instructions. Fluorescence emission of OMVs was recorded using a plate reader (M200, Tecan infinite Pro) in absorbance mode. Subsequently, SBCy050 and Sga15 OMVs were excited at 410 nm and 360 nm, respectively. Emission spectra were recorded between 400-800 nm.
To prepare samples for scanning electron microscopy, a copper grid was placed upwards on a carbon disc. Two microliters of sample were applied, left for drying and sputtered with a thin layer of gold atoms (SEM Quorum Q 150 R ES coater). Scanning electron microscopy images were taken on an EVO HD 15 (Zeiss) with 5.00 kV and 35.00 kX magnification. For cryogen transmission electron microscopy, 3 µL of vesicle suspension was placed onto a holey carbon film (type S147-4, Plano, Wetzlar, Germany) and plotted for 2 s with a Gatan (Pleasanton, CA, US) cryoplunger model CP3. The sample was plunged into liquid ethane at T = 108 K, and vesicles were transferred under liquid nitrogen to a Gatan model 914 cryo-TEM sample holder. Samples were imaged at T = 100 K and 200 kV accelerating voltage via bright field TEM (JEM-2100 LaB6, Jeol, Akishima, Tokio, Japan) and under low-dose conditions.

Cell cytotoxicity assays

THP-1 cells (DSMZ, ACC16) were cultured in RPMI 1640 medium (Gibco, Waltham, Massachusetts, USA). Using 96-well plates, A549 cells were seeded with a density of 20,000 cells/well; THP-1 cells at 100,000 cells/well. THP-1 were additionally stimulated with 7.5 µg/mL phorbol 12-myristate 13-acetate for 48h. After 2 days, cells were incubated with different concentrations of OMVs, ranging from 10 to 10,000 vesicles per cell for 24 h. The cell viability was measured using PrestoBlue Cell Viability Reagent (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and cytotoxicity was assessed with a lactate dehydrogenase assay (cytotoxicity detection kit, Merck) as per the supplier's description. Native OMVs, pure PBS without cells, PBS with cells and cells treated with a 1% (v/v) Triton X (Merck, Darmstadt, Germany) solution were used as controls.

Liquid chromatography coupled mass spectrometry

OMV pellets resuspended in PBS were mixed with ethyl acetate (1:1), vortexed and the organic phase was collected. The organic solvent was subsequently evaporated (Rotavapor R-300, Buchi) and dissolved in 1 mL of acetonitrile:H_2O_{dd} (95:5). Samples were stored at 4 °C until analysis. For

fluorescence analysis, HPLC UltiMate 3000 with an amaZon SL mass spectrometer and a fluorescence detector were used with an EC 100/2 Nucleoshell® RP 18 plus column of 2.7 µm particle size. The detection mass was set between 100 and 2000 m/z and each run was detected for 20 min. Fluorescence detection was undertaken at 365 nm excitation and 515 nm emission wavelength. UHPLC-hrMS analysis was performed using a Dionex UltiMate 3000 rapid separation liquid chromatography (RSLC) system (Thermo Fisher Scientific, Waltham, MA, USA) coupled to a Bruker maXis 4G ultra-high-resolution quadrupole time-of-flight (UHR-qTOF) mass spectrometer equipped with a high-resolution electrospray ionization (HRESI) source (Bruker Daltonics, Billerica, MA, USA). The separation of 2 µL sample was achieved with a linear 5–95% gradient of acetonitrile with 0.1% formic acid in ddH_2O with 0.1% formic acid on an ACQUITY BEH C18 column (100 mm × 2.1 mm, 1.7 µm d_p) (Waters, Eschborn, Germany) equipped with a Waters VanGuard BEH C18 1.7 µm guard column at a flow rate of 0.6 mL/min and 45 °C for 18 min. The LC flow was split into 75 µL/min before entering the mass spectrometer. Mass spectrograms are acquired in centroid mode ranging from 150–2500 *m/z* at an acquisition rate of 2 Hz in positive MS mode. Source parameters are set to 500 V end-plate offset; 4000 V capillary voltage; 1 bar nebulizer gas pressure; 5 L/min dry gas flow; and 200 °C dry gas temperature. Ion transfer and quadrupole parameters are set to 350 V_{PP} funnel RF; 400 V_{PP} multipole RF; 5 eV ion energy; and 120 m/z low-mass cut-off. Collision cell was set to 5.0 eV and pre-pulse storage was set to 5 µs. Calibration was conducted automatically before every HPLC-MS run by the injection of sodium formate and calibration on the respective clusters formed in the ESI source. All MS analyses are acquired in the presence of the lock masses $C_{12}H_{19}F_{12}N_3O_6P_3$, $C_{18}H_{19}F_{24}N_3O_6P_3$ and $C_{24}H_{19}F_{36}N_3O_6P_3$, which generate the $[M + H]^+$ ions of 622.0289, 922.0098 and 1221.9906. The HPLC-MS system was operated by HyStar 5.1 (Bruker Daltonics, Billerica, MA, USA), and LC chromatograms as well as UV spectra and mass spectrograms are analyzed with DataAnalysis 5.3 (Bruker Daltonics, Billerica, MA, USA).

The T-ReX-3D molecular feature finder of MetaboScape 2021b (Bruker Daltonics, Billerica, MA, USA) was used to obtain molecular features. Detection parameters are set to intensity threshold 5×10^3 and minimum peak length of five spectra. Annotation of bacterial features was performed with the in-house myxobacterial metabolome database (*MXbase*) at the Helmholtz-Institute for Pharmaceutical Research Saarland containing analytical data obtained from isolated standards using the same instruments and methods as described before. Bacterial features are annotated if their measured data lies within all of the following tolerances: *m/z* deviation < 5 ppm, retention time deviation < 0.3 min and mSigma (isotopic pattern fit) score < 20. Extracted ion chromatograms are generated for all annotated features (*m/z* ± 0.05) and the respective peaks are automatically integrated using DataAnalysis 5.3 to calculate the maximum peak intensity as well as peak area for each compound.

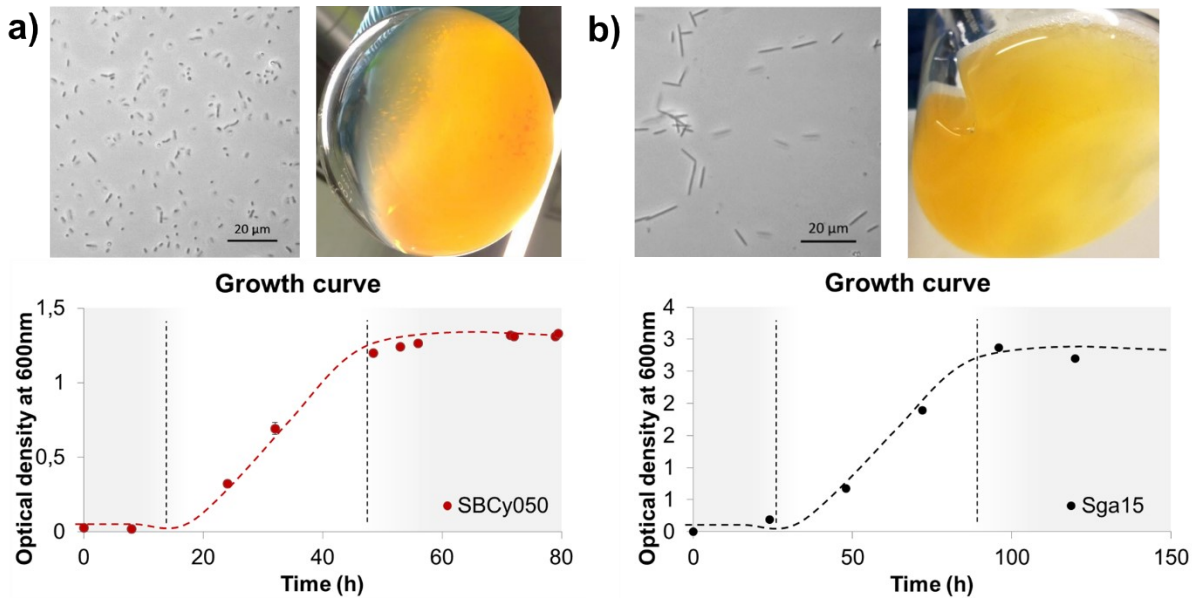


Figure S1. **Growth behaviour of myxobacteria (a) SBCy050 and (b) Sga15**. For both bacterial strains, microscopic and macroscopic images are shown. Mean growth curves were recorded by measuring optical density (OD) at 600 nm for up to 120 h.

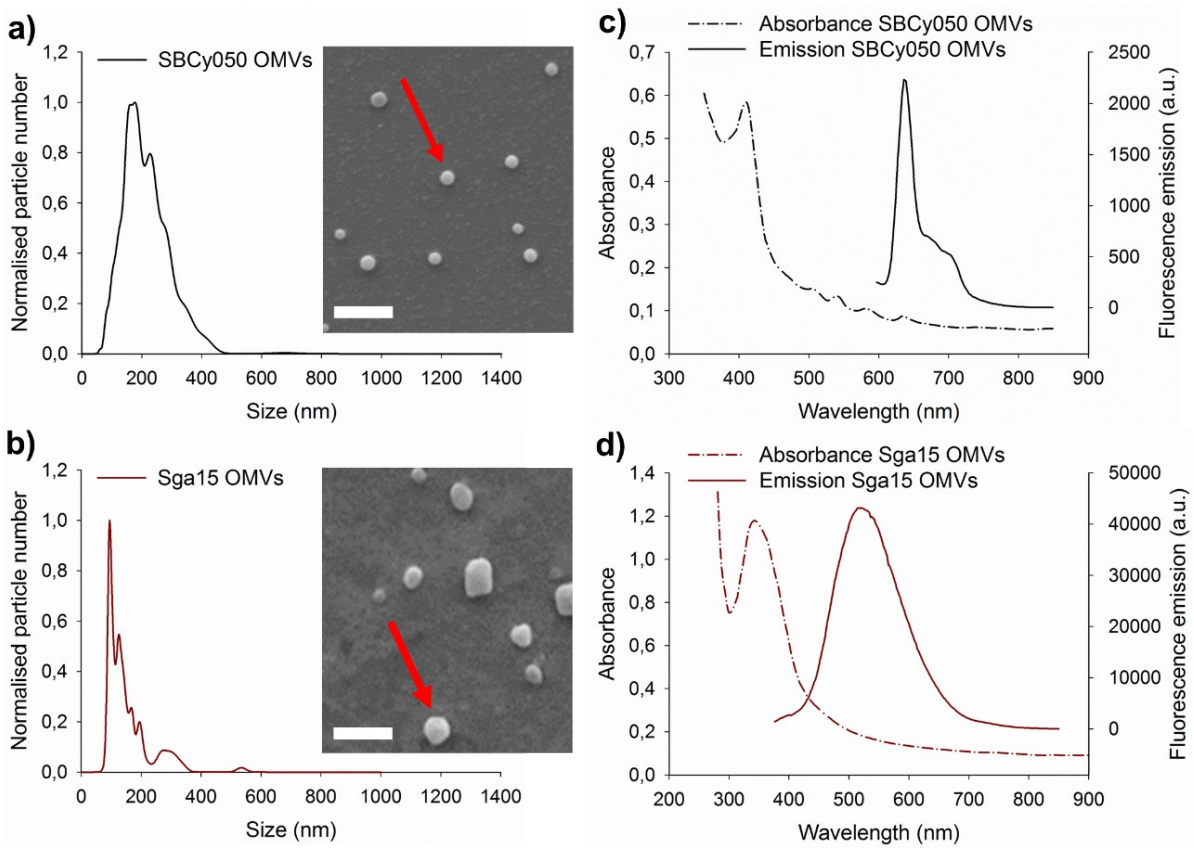


Figure S2. **Characterisation of OMVs obtained from myxobacteria.** Size distribution of OMVs from **(a)** SBCy050 and **(b)** Sga15 was measured using nanoparticle tracking analysis and their morphology was studied by scanning electron microscopy. Representative data from n=3-6, scale bars represent 1 µm. Absorbance and fluorescence emission spectra of purified OMVs obtained from **(c)** SBCy050 and **(d)** Sga15 myxobacteria. OMVs were isolated and their absorbance spectrum measured using a plate reader. OMVs were subsequently excited at the absorbance maximum to obtain their emission peak. SBCy050 showed fluorescence at excitation at 410 nm and emission at 636 nm, while Sga15 were fluorescent at excitation 340 nm and emission at 515 nm. Representative spectra.

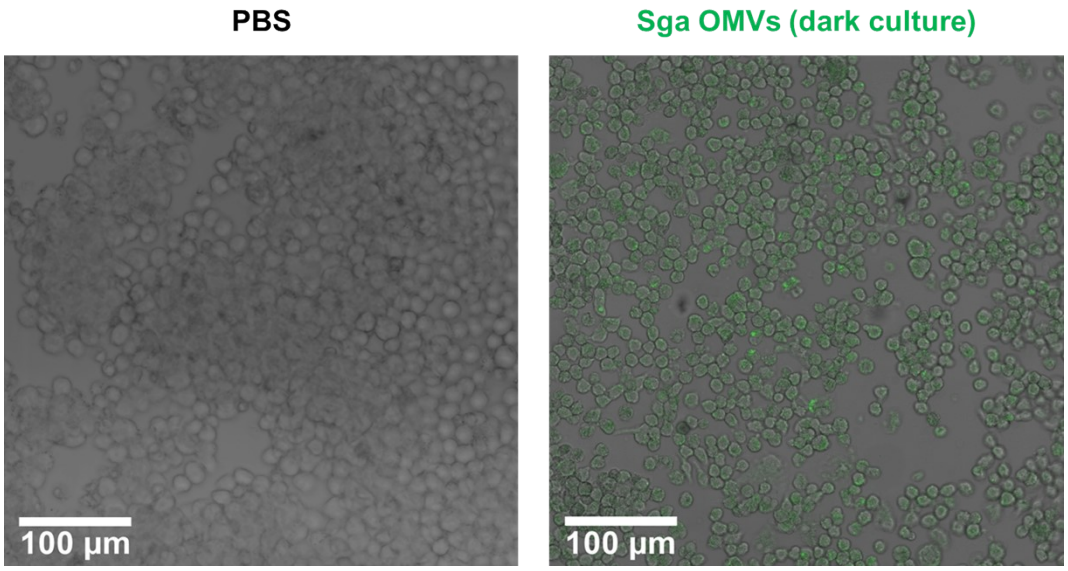


Figure S3. **Uptake of OMVs from Sga15 myxobacteria in RAW cells.** Vesicles were incubated with murine monocyte/macrophage-like cells RAW 264.7 for 24 h, and imaged by confocal fluorescence microscopy. The RAW cells were seeded 2 d prior to the experiment in a 96-well plate and at a density of 20,000 cells/wells. Cells were kept at 37 °C and 5% CO_2 during the entire experiment. PBS was used as control; representative images. Magnification 25x, objective Fluotar VISIR 25x/0.95 WATER, numerical aperture 0.95, Refraction Index 1.33, Zoom 1, Pinhole 55.8 µm, Laser Diode 405 nm, Detector HyD (500nm-578nm) Gain 70.

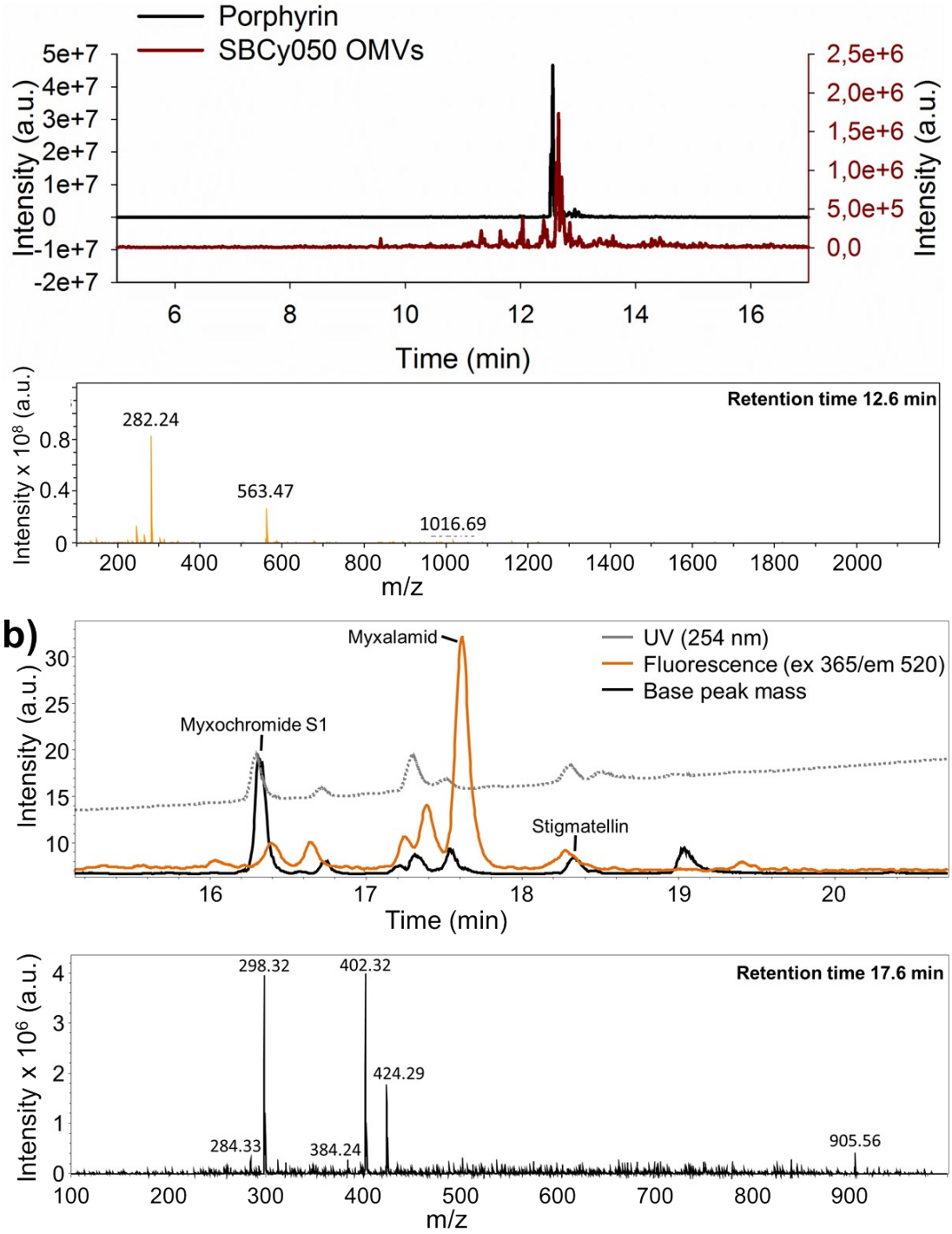


Figure S4. **Liquid chromatography analyses of OMVs from (a) SBCy050 and (b) Sga15 myxobacteria**. In **(a)**, SBCy050 OMVs were injected into a HPLC coupled to a mass spectrometer in comparison to protoporphyrin IX. At 12.6 min the major eluting peak showed the m/z of 563.47, corresponding to the molecular weight of protoporphyrin (562 Da). In **(b)**, Sga15 OMVs were analysed by HPLC coupled to a fluorescence detector and a mass spectrometer. Identified compounds in the sample were myxochromide S1, myxalamid B and stigmatellin as indicated in the chromatogram. At 17.6 min, myxallamid B was identified with an m/z of 402. Representative chromatograms.

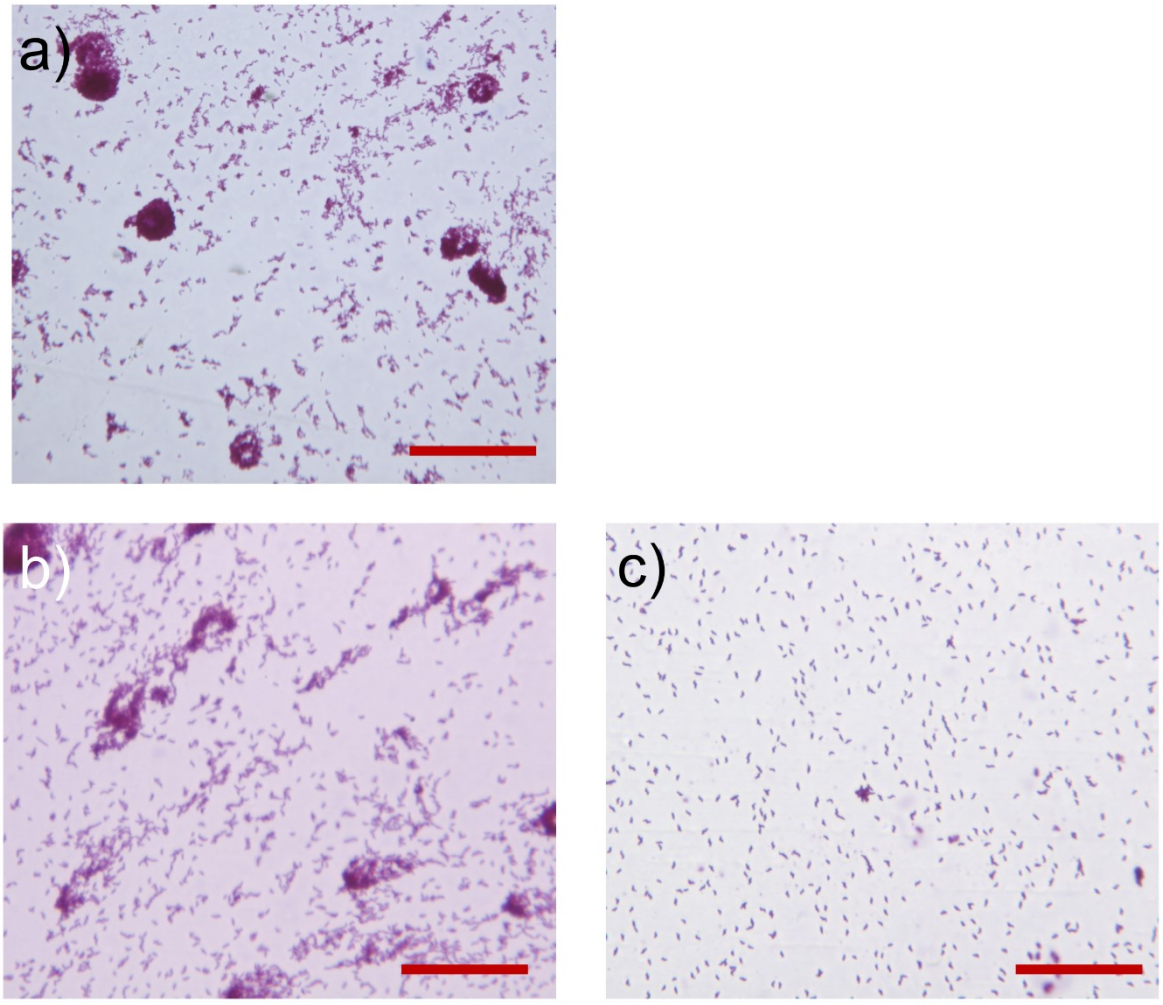


Figure S5. Gram staining of the Sga15 cultures. Sga bacteria were diluted to an OD_{600} of 0.1 and incubated at 30°C and 180 rpm for 3 days with **(a)** no light (control culture), or **(b)** blue and **(c)** red light. The bacterial suspensions were added to glass slides and fixed with heat. Then, the slides were soaked with crystal violet, iodine solution, decolouriser and fuchsine, with washing steps in between. The slides were imaged with an Axio Zoom.V16 Zeiss microscope equipped with an Axiocam 506 color camera. Scale bar = 100 µm.

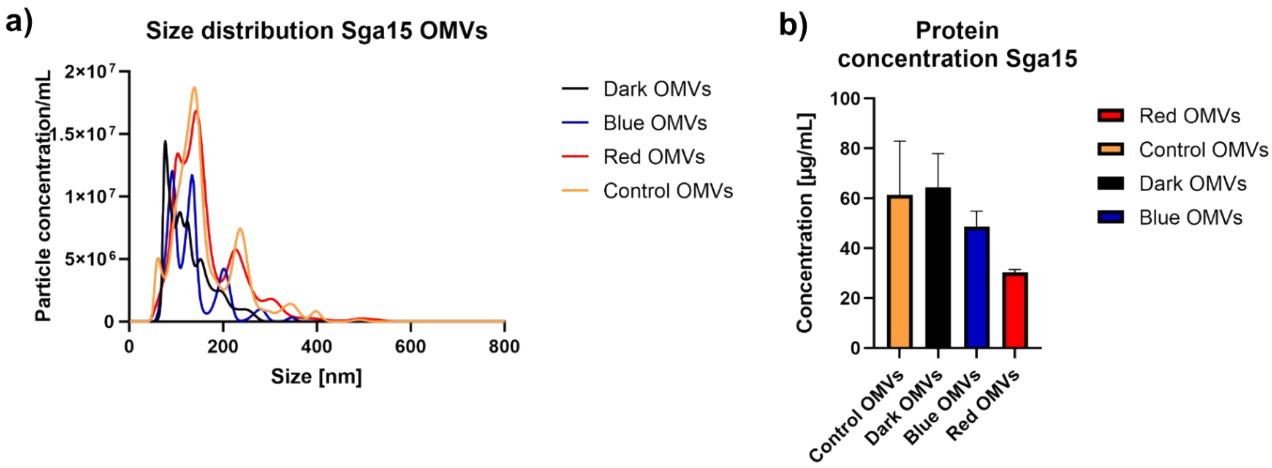


Figure S6. Size and protein concentration of OMVs from Sga15 bacteria. OMVs from Sga15 bacteria cultured under blue or red light, kept in the dark, or from control cultures were assessed by nanoparticle (a) tracking analysis and (b) regarding their protein content.

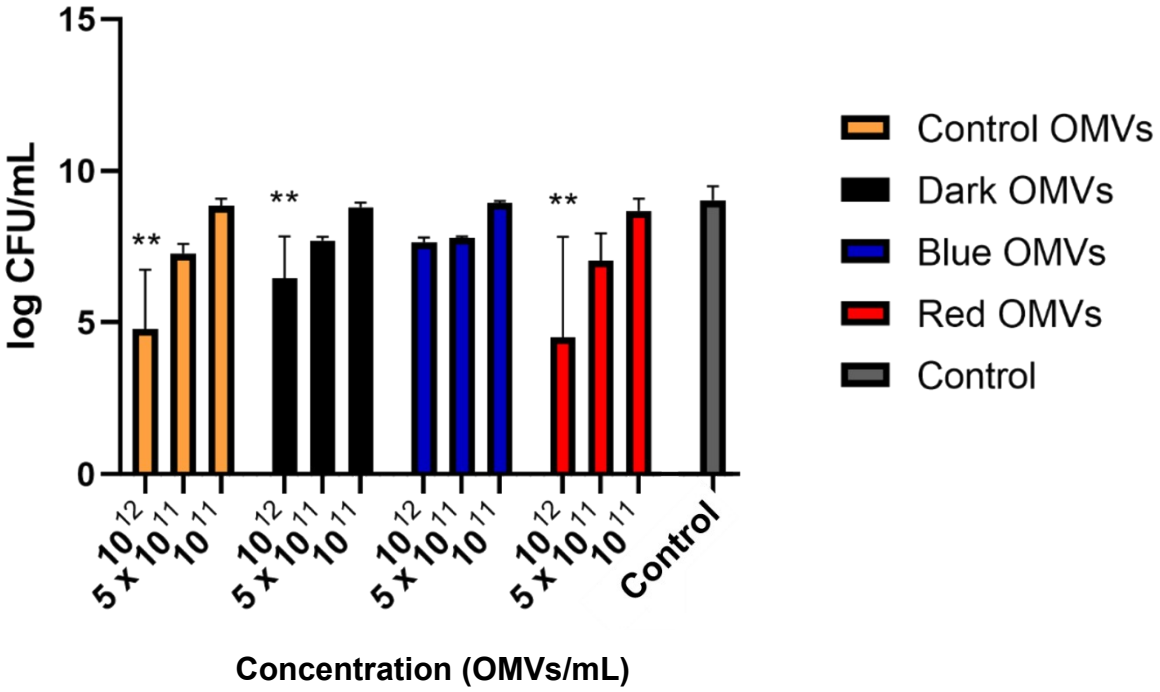


Figure S7. **Colony forming unit-reducing effects of OMVs from Sga15**. Colony forming units of *Staphylococcus aureus* upon incubation with different concentrations of OMVs obtained from dark, blue and red light radiated cultures. PBS was used as negative control, mean ± SD, n = 3-4.

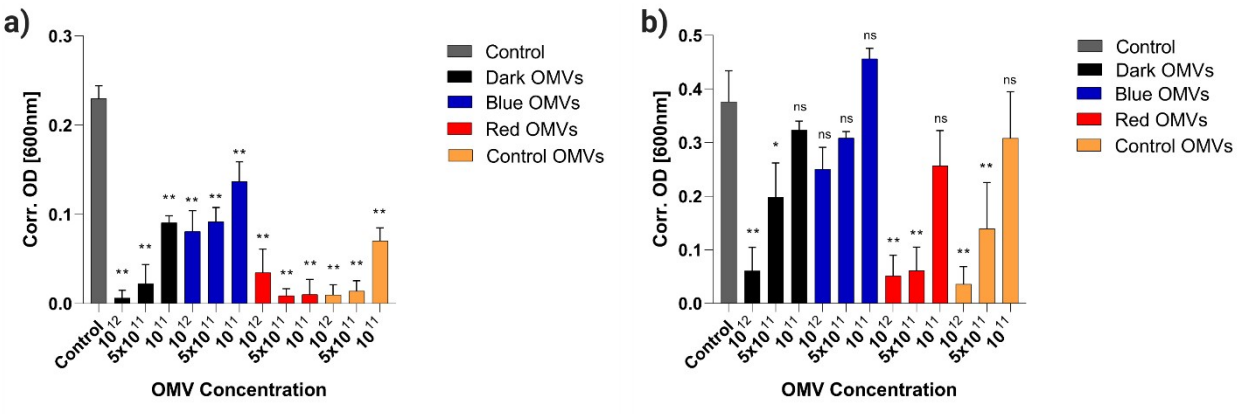


Figure S8. **Antimicrobial effects of OMVs from Sga15**. Optical density measurements *Staphylococcus aureus* upon incubation with different concentrations of OMVs obtained from dark, blue and red light radiated cultures. Bacterial growth was studied after (a) 4h and (b) 20 h of incubation, PBS was used as negative control, mean ± SD, n = 3-4, OMV concentration is in particles/mL.

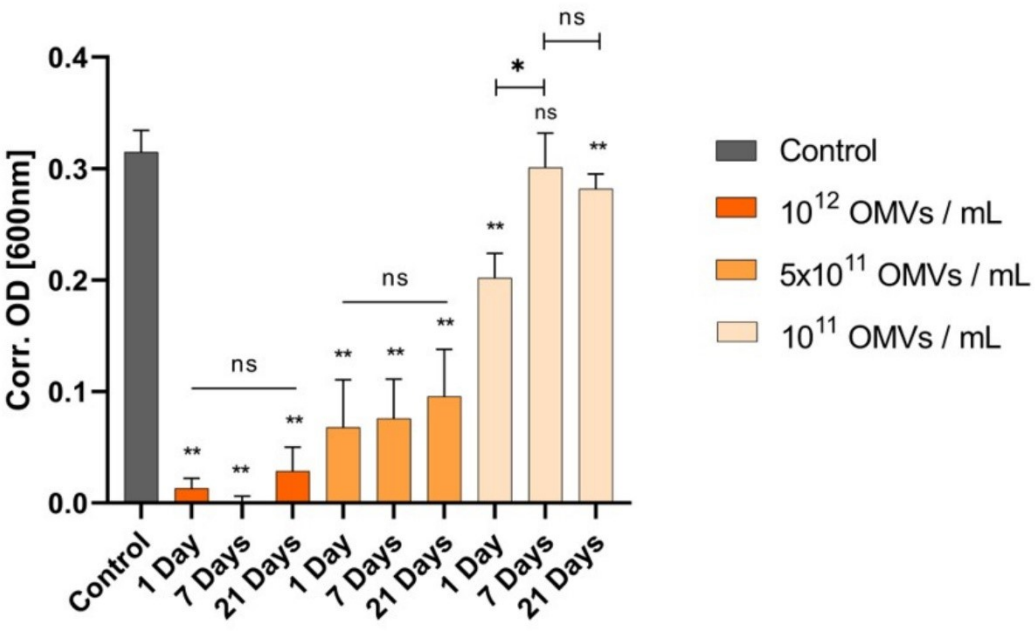


Figure S9. Stability of Sga15 OMVs from dark controls upon storage. Vesicles were kept at 4°C for 7 and 24 days and their antibiotic activity against *Staphylococcus aureus* were assessed by optical density measurements. PBS was used as negative control, mean ± SD, n = 3-4.

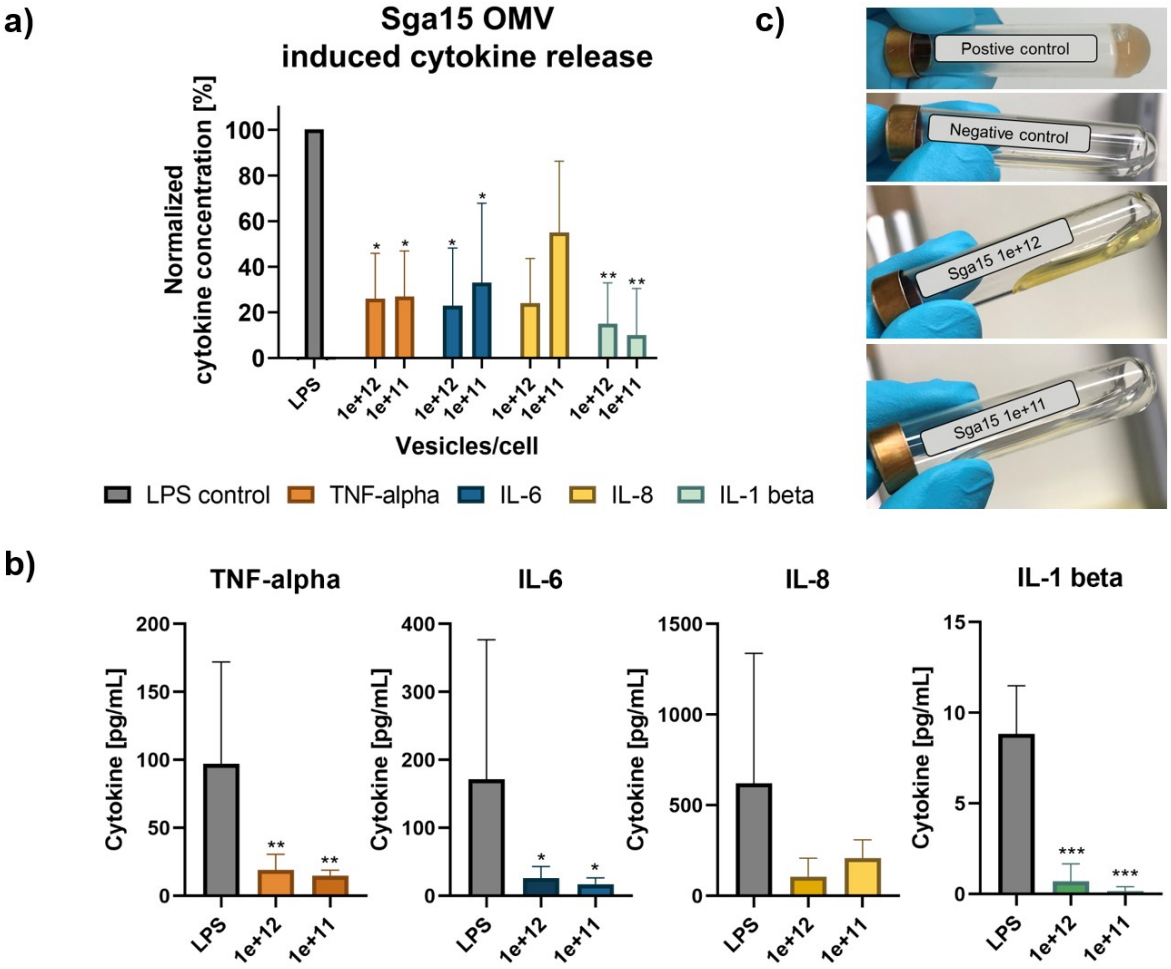
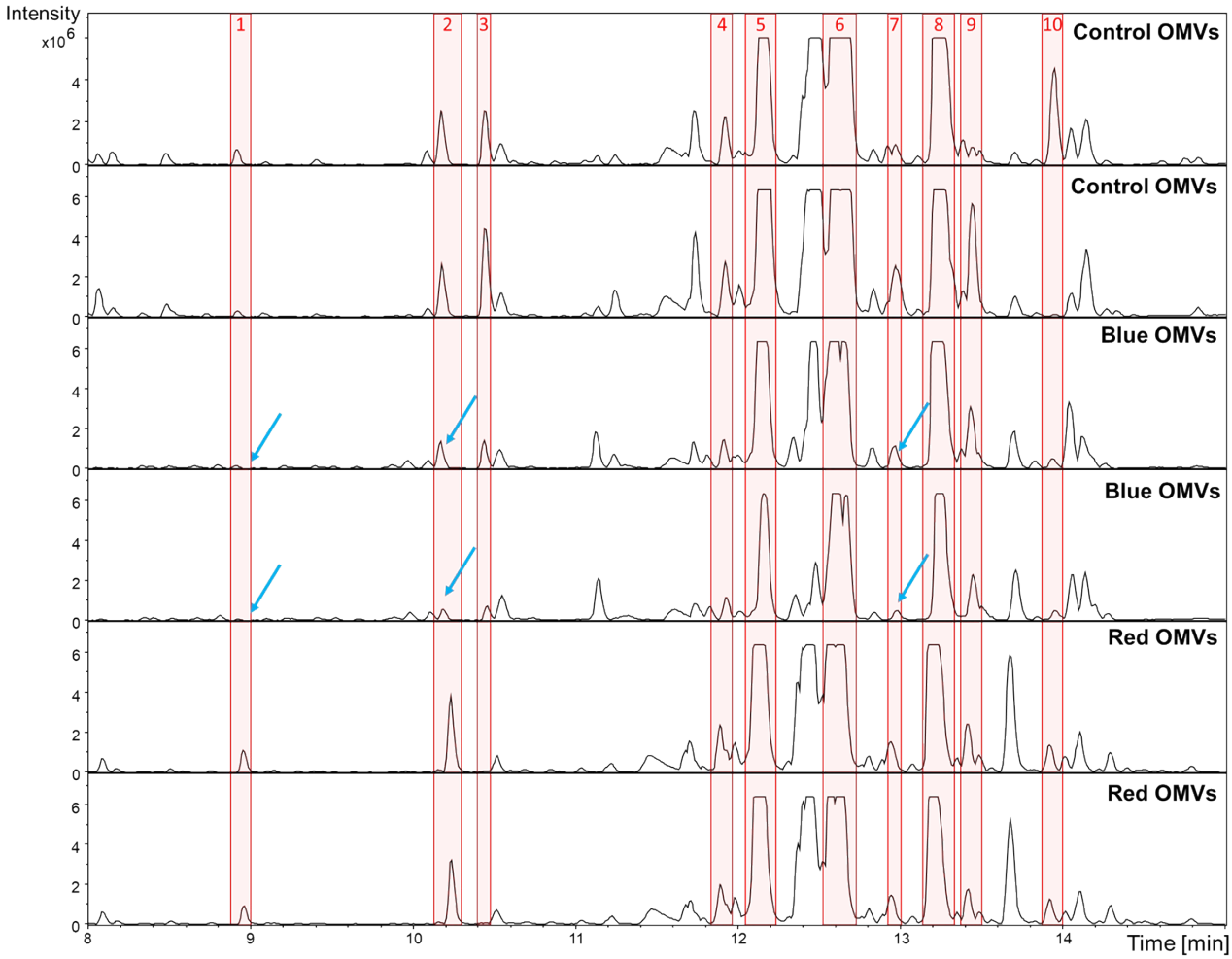


Figure S10. Potential immunogenic effects of OMVs. (a) Normalized cytokine release, and (b) individual cytokine release from primary blood mononuclear cells incubated with different concentrations of Sga15 OMVs. Cytokines studied were TNF-alpha, IL-6, IL-8 and IL-1 beta. LPS was used as positive control. In (a), individual cytokine release was normalized to the lipopolysaccharide control. In (b), absolute concentrations are shown. Untreated control cells incubated with PBS showed cytokine releases of TNF=0 pg/mL, IL6=0 pg/mL, IL1 beta=0 pg/mL and IL8=12.5±7.6 pg/mL., mean ± SD, n = 3, *$p > 0.05$, **$p > 0.01$, ***$p > 0.001$. (**c**) Limulus amebocyte lysate clot test to assess the presence of endotoxins in OMV samples. LPS and sterile water were used as positive and negative controls.



1: Imidacin A1
2: Imidacin A2
3: Unknown compound
4: Myxalamid C
5: Myxochromide S1
6: Aurachin A (left)+ Myxalamid B (right)
7: Myxochromide S3
8: Stigmatellin A
9: Unknown compound (probably Aurachin derivative „Aurachin-388“)
10: Aurachin B

Figure S11. Representative mass-spectrometry spectrum of differently treated Sga15 OMVs. OMV samples from controls, blue light stimulated or red light stimulated Sga15 bacteria were analysed using mass spectrometry. Annotated peaks were identified by retention time and m/z ratio. Peaks saturating the detector were not taken into consideration during the quantification.

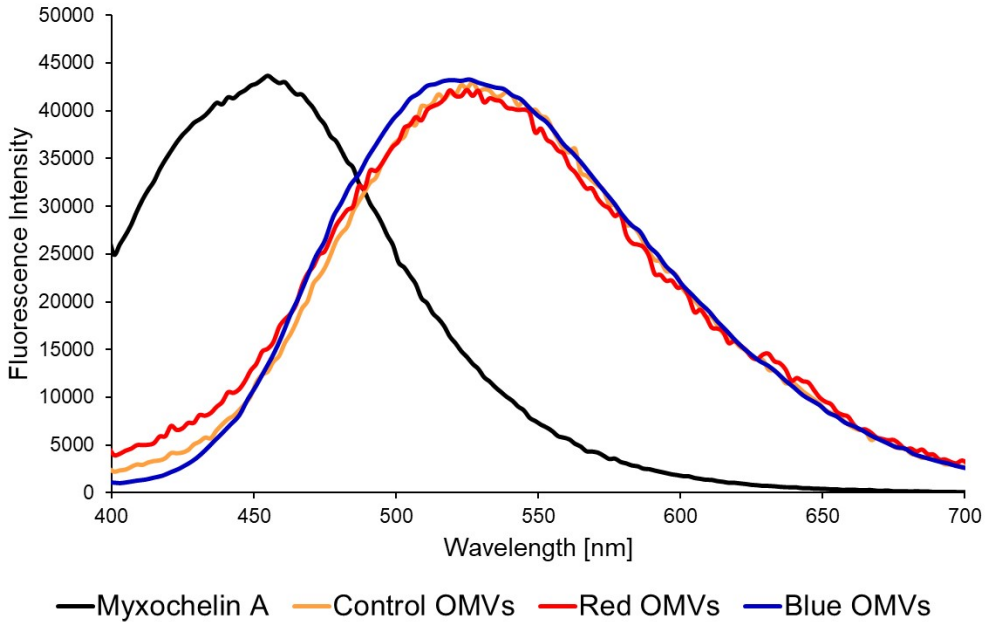


Figure S12. Fluorescence scan of different Sga15 OMVs and Myxochelin A. Representative samples of control OMVs and vesicles obtained under red and blue culture conditions were excited at 338 nm. Fluorescence emission spectra were measured in comparison to myxochelin A.

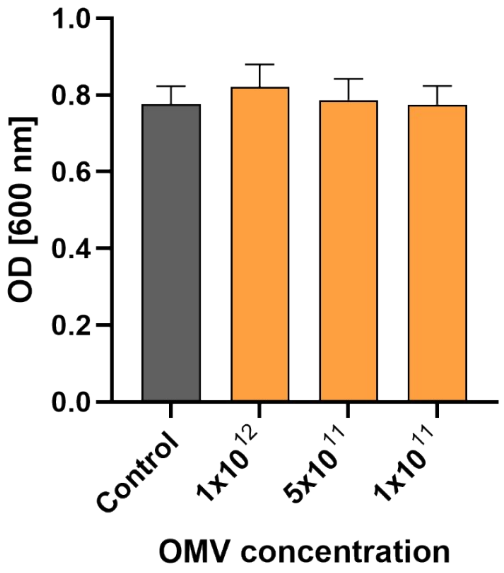


Figure S13. **Antimicrobial assay of OMVs from Sga15 with gram-negative bacteria**. Optical density measurements *Pseudomonas aeruginosa* (DSM 50071) upon incubation with different concentrations of OMVs obtained from dark control cultures. Bacterial growth was studied after 24h of incubation, PBS was used as negative control, mean ± SD, n = 3, OMV concentration is in particles/mL.