Microbial biofilm formation and degradation of octocrylene, a UV absorber found in sunscreen

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Octocrylene is a widely used synthetic UV absorber of sunscreens and found in several environments. Ecological consequences of the accumulation of UV filters are widely discussed. This is the first report revealing the microbial potential to transform octocrylene. A microbial community comprising four bacterial species was enriched from a landfill site using octocrylene as carbon source. From these microorganisms Mycobacterium agri and Gordonia cho- lesterolivorans were identified as most potent applying a new “reverse discovery” approach. This relies on the possibility that efficient strains that are already isolated and deposited can be identified through enrichment cultures. These strains formed massive biofilms on the octocrylene droplets. GC-MS analysis after cultivation for 10 days with M. agri revealed a decrease in octocrylene concentration of 19.1%. LC-MS/MS analysis was utilized in the detection and quantification of transformation products of octocrylene. M. agri thus represents an ideal candidate for bioremediation studies with octocrylene and related compounds.
Octocrylene (2-ethylhexyl 2-cyano-3,3-diphenylacrylate) is a synthetic organic filter widely used as UV-absorber (in the UVB region) in sunscreens and personal care products. Recently, several studies focused on the extensive use and potential ecological consequences of octocrylene, since the compound was detected in various water and sediment samples in wastewater, oceans, lakes, and rivers. Moreover, octocrylene was found in the liver tissue of Francisca dolphins and accumulation of octocrylene in zebrafish was reported upon exposure. Further, octocrylene was suggested to potentially affect transcription of the respective bands and sequencing. Octocrylene was also proposed for application in environmental bioremediation of aromatic hydrocarbons (PAHs), such as pyrene or oil, and were reported to be associated with degradation of polluting polycyclic aromatic compounds. Recently, several studies focused on the extensive use and accumulation of octocrylene in zebra fish was reported upon exposure. So far, little is known about potential degradation and biotransformation of octocrylene by microorganisms. Hence, this is the first study, to our knowledge, which reports on microbial growth in the presence of octocrylene and its degradation and transformation to other compounds.

Results

Sediment and water samples were taken from an on-site sewage plant and soakage at a landfill site in Singhofen (Germany). Samples were subsequently used for enrichment cultures (inoculation 1% (v/v)) with 0.35% (v/v) octocrylene as carbon source. After 14 days, grown cultures were transferred into fresh medium with two subsequent streakings. Repeatedly, biomass formation on the octocrylene droplet surface could be observed and the medium became turbid compared to the controls. In order to investigate the community composition of the culture, DGGE analysis was performed. The microbial community of the OC-colonizing enrichment culture consisted of four genera, and the distinct bands of the DGGE were obtained and assigned to the bacterial genera Gordonia (100% identity), Mycobacterium (100% identity), and Hydrogenophaga (99% identity) after excision of the respective bands and sequencing (Fig. 1).

Interestingly, Mycobacterium and Gordonia species have been reported to be associated with degradation of polluting polycyclic aromatic hydrocarbons (PAHs), such as pyrene or oil, and were proposed for application in environmental bioremediation. Although different techniques were performed, isolation of pure strains from the enrichment culture was not possible and resulted in mixed cultures. Therefore, the “reverse discovery” approach was integrated. This approach is based on taking use of already isolated and deposited pure strains, showing high identities to the identified 16S rRNA genes amplified from the organisms of the enrichment culture. Highly identical strains can be purchased from culture collections, in order to identify the most efficient strains capable of degrading octocrylene. Likewise, several deposited strains at the German Culture Collection (DSMZ) belonging to the genera Mycobacterium and Gordonia, whose 16S rRNA gene showed high identities to the DGGE-identified ones, were tested for their ability to grow in modified DSM media 645 and 65 at 37 °C and 30 °C in the presence of 0.35% (v/v) octocrylene. Interestingly, M. agri (DSM 44515) and Gordonia cholesterolivorans (DSM 45229) were able to colonize the octocrylene droplet rapidly forming massive biofilms on its surface (Fig. 2).

Mycobacteria and Gordonia species have been already described to attach to different surfaces that are composed of biomaterials and synthetic compounds initiating the synthesis of an extracellular matrix required for biofilm formation. Since carbon sources can influence biofilm formation, different concentrations of glucose and glycerol in the growth medium were tested. Remarkably, while reduction of carbon sources had no positive influence on biofilm formation of G. cholesterolivorans, M. agri showed fast and massive biofilm formation when carbon sources were significantly reduced (0.05% glucose, 0.05% glycerol). Interestingly, in absence of external carbon sources, no biofilms were formed at all, indicating the necessity of small amounts of accessible energy sources to colonize the octocrylene droplet.

Hence, in order to study transformation of octocrylene by M. agri and G. cholesterolivorans, gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) analyses were performed with the pure cultures grown with 0.35% octocrylene (v/v). Control experiments were conducted with M. agri and G. cholesterolivorans grown in the absence and presence of octocrylene. In addition, the medium containing octocrylene was incubated under the same conditions without inoculation. After 10 days of cultivation, triplicates of cultures and controls were used for GC-MS and LC-MS/MS analyses. Samples were prepared by extraction of the culture using hexane/dichloromethane (1:2).
that in this case biofilm formation does not go automatically along with transformation or degradation of octocrylene.

**Discussion**

Since UV filters such as octocrylene accumulate in nature, the identification of further octocrylene degrading microorganisms is desirable. It has been reported that marine environments are endangered by chemicals and UV-filters of sunscreen3 and solutions for this pollution have to be investigated. Man-made biofilms were proposed previously for bioremediation of sewage effluent contaminated with hydrocarbons16. *M. agri* represents a potential candidate for bioremediation, since it belongs to the rapidly growing *Mycobacteria* and can be found ubiquitously in soil and water systems13,17,18. Therefore, a novel eco-friendly application using *M. agri* or specific microbial consortia could be investigated and applied for degradation of chemical UV filter like octocrylene. This work highlights the potential of heterotrophic microorganisms to transform octocrylene in lab experiments. Due to the omnipresence of *M. agri* in the environment, it is conceivable that *M. agri* will also be able to transform the UV filter octocrylene in nature. Hence, further analysis should be conducted regarding biotransformation of octocrylene by microorganisms in natural sediments and waters. Future studies will reveal the metabolic pathway for octocrylene degradation and the key enzymes involved in this process.

**Methods**

**Sampling and enrichment cultures.** Sediment and water samples were taken from an on-site sewage plant and soakage at a landscaped site in Singen in Switzerland. The samples were subsequently used for enrichment cultures (volume 20 mL) using anoxic medium (0.5 g/L NaCl, 1.0 g/L KCl, 0.1 g/L MgSO₄, 0.1 g/L MgCl₂, 0.5 g/L NH₄NO₃, 0.5 g NH₄NO₃, 0.1 g/L CaCl₂, 40 mM phosphate buffer, trace element and vitamin solution 141, and 0.05% (w/v) yeast extract). Incubation bottles were inoculated with 1% (w/v) of the soil sample and supplemented with 0.35% (v/v) octocrylene as carbon source. After 14 days of incubation, grown cultures were transferred into fresh medium with two subsequent streakings.

**DGGE analysis.** DGGE analysis was performed as described previously.19 Amplification of bacterial 16S rRNA genes was performed using the forward primer 314 F: 5′- CGCCCGGGCCGCTCCGCTCCGGGGCCGCGCGGCGGGCCGCGCCTGGAGCTGTT-3′ and 55 °C and 100 V. The gel was stained in Roti-Gel Stain (Carl Roth GmbH, Karlsruhe, Germany) and afterwards analyzed with a UV transilluminator. In addition, selected bands were cut out and DNA was extracted from the gel by incubation in 10 µL sterile water over night at 4 °C, amplified by PCR and amplified in sequenced (Eurofins Genomics).

**Cultivation of Mycobacterium sp. and Gordonia sp.** Several *Mycobacterium* sp. and *Gordonia* sp. showing within their 16S rRNA genes high similarities compared to the sequenced and identified fragments of the DGGE analysis were chosen and ordered from the DSMZ. *Mycobacterium agri* (DSM 4415), *M. cholesterolivorans* (DSM 44147) and *M. aromaticivorans* (DSM 45407) were incubated in DSMZ medium 645 (per liter: 5 mL glycerol, 0.5 g NH₄NO₃, 1.5 g KH₂PO₄, 1.5 g NaH₂PO₄, 0.4 g Na-citrate, 0.025 g MgSO₄·7H₂O, 0.5 g CaCl₂, 1 mg ZnSO₄·7H₂O, 1 mg CuSO₄·5H₂O, 0.5 g l-glutamic acid, 0.04 g ferric ammonium citrate, 1 mg pyridoxine, 0.5 mg biotin, 0.05 g oleic acid, 0.85 mL NaCl, 5 g BSA, 2 g glucose) supplemented with 0.35% octocrylene at a temperature of 37 °C. *Gordonia caeni* (DSM 45852), *G. cholesterolivorans* (DSM 45229) and *G. defluvi* (DSM 44981) were incubated in DSMZ medium 65 (per liter: 4 g glucose, 4 g yeast extract) supplemented with 0.35% octocrylene at a temperature of 30 °C.

**Medium modification of *M. agri* and *G. cholesterolivorans.** The medium of the positive octocrylene-colonizing strains *M. agri* and *G. cholesterolivorans* was modified in order to enhance the biofilm formation. Therefore, the carbon sources of the medium 645 of *M. agri* were highly decreased to 0.05% glucose and 0.05% glycerol and BSA was also reduced to 0.05%. The carbon sources of medium 65 of *G. cholesterolivorans* were decreased to 1 g/L glucose and 1 g/L yeast extract (no addition of malt extract). Incubation was performed in the presence of 0.35% octocrylene.

**Fig. 3** Octocrylene degradation assay with *M. agri*. 0.35% octocrylene was incubated in liquid medium without *M. agri* (controls, *n* = 3) and in presence with *M. agri* (incubation, *n* = 3) for 10 days. A decrease of 19.1% octocrylene was detected within the incubation time. Mean is shown in gray color, error bars are representing standard error.
Fig. 4 GC-MS scans (a–c) TIC mode, cutout, d–e: Mass spectra) of the incubation experiment with M. agr. a Control sample 0.35% octocrylene (rt 15.58 min) incubated for 10 days without M. agr. b Sample 0.35% octocrylene (rt 15.58 min) incubated for 10 days with M. agr. New metabolite was detected (rt 15.32 min). c Control sample M. agr. incubated for 10 days without 0.35% octocrylene. d Mass spectrum of the peak identified as octocrylene. e Mass spectrum of the unknown metabolite peak (rt 15.32 min).

Chemicals and materials for analytical approaches. Reference standard octocrylene was purchased from Sigma Aldrich Ltd, Saint Louis, MO, USA (≥98%). Reference substances 2-(Carboxymethyl)butyl-2-cyano-3,3-diphenylacrylat (DOCCA), 2-Cyano-3,3-diphenylacrylic acid (CPAA) and 2-Ethyl-5-hydroxyhexyl-2-cyano-3,3-diphenylacrylate (SOH-OC) were provided by V.N. Belov, Max Planck Institute for Biophysical Chemistry, Göttingen, Germany. Preparation and purity (>95%) are well described in ref.

Hexane and dichloromethane were obtained from Merck KGaA, Darmstadt, Germany. Acetonitrile (LC-MS grade) was obtained from Merck, Darmstadt, Germany, formic acid (additive for LC-MS) was purchased from Honeywell Fluka, Muskegon, USA.

All reagents were of analytical grade. Deionized water was purified using a Milli-Q system (Millipore, USA).

Sample preparation. The whole incubation culture of M. agr. and G. cholestr-oilvorum (and controls) (20 mL) was transferred to a centrifuge tube and 10 mL hexane/dichloromethane (1+2) was added. After 30 min stirring the sample was centrifuged (6000 rpm). The lower layer was stirred with NaSO4 and centrifuged (13,000 rpm) again. For GC-MS analysis the liquid was filled in a microbial. For LC-MS/MS analysis 1 mL was filled in a volumetric flask, evaporated and refulled with acetonitrile.

GC-MS analyses. Quantification of octocrylene and qualitative recording of products: The quantitative determination of octocrylene and qualitative recording of the products were carried out on an Agilent Technologies GC-MS system 5977 A MSD with Gerstel KAS4 injection system. The mass selective detector (MSD) was operated in EI mode. All system operations were controlled by MassHunter B.O7.001413. Separation and quantification were performed on an Agilent DB5MS ultra inert capillary column, 30 m × 0.25 mm × 0.25 µm film thickness. Sample volumes of 1 µL were injected in split mode (1:5) at 1 mL/min flow. GC system inlet and MS interface temperatures were set at 70 and 280 °C, respectively. The column temperature was programmed as follows: held at 70 °C for 2 min, then ramped up to 140 °C at 10 °C/min, held for 3 min, then to 300 °C at 100 °C/min. The total runtime was 17.6 min. The carrier gas was helium with constant flow of 1 mL/min, the MSD operating parameters were routinely set by tune file. EM potential was set at –1352 V, MS source temperature at 230 °C and the quadrupoles at 150 °C.

Quantitative determination of octocrylene was done by SIM mode, with quantifying ion with m/z 249 and qualifying fragment ions m/z 360 and 204. Recording of products was done by TIC mode (from m/z 4–450, solvent delay 5 min, dwell time 0.2 ms).

Table 1 Characteristics of metabolites of octocrylene tested in this study.

| Analyte   | Polarity | Q1  | Q3  |
|-----------|----------|-----|-----|
| DOCCA     | Positive | 364 | 250 |
| CPAA      | Negative | 248 | 204 |
| SOH-OC    | Positive | 378 | 232 |

Preparation of analytical standards (GC-MS). Stock solution was prepared with a concentration of 5300 mg/L using dichloromethane as solvent. By diluting the stock solution with hexane-dichloromethane (1+2) calibration standards were prepared with levels from 5.0 mg/L to 200 mg/L. While the stock solution was stored at –18 °C, the calibration standards had to be newly made for each analytical run.

LC-MSMS analyses. Instrumentation and parameters: The quantitative determination of metabolites was carried out on an Agilent 1260 HPLC system (Agilent Technologies Deutschland GmbH and Co. KG, Waldbronn, Germany), equipped with binary pump, degasser, thermostat autosampler, column oven, which is coupled to the Sciex Qtrap 5500 tandem mass spectrometer (AB Sciex Germany GmbH, Darmstadt, Germany).

Separation was achieved by using a Phenomenex Synergi Fusion RP 80 A column (4 µm, 50 × 2 mm) with a Phenomenex standard C18 guard column. The mobile phase consisted of Milli-Q water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B), the flow rate was set to 0.7 mL/min. The gradient elution was programmed as follows: 0–3.5 min, 40% to 95% B (acetonitrile), 3.5–7.0 min 95% B, 7.1–10 min, 40% B.

The injection volume was 5 µL, the column temperature was set to 35 °C. To detect all three available metabolites with at least two characteristic mass transitions per analyte in one run, the electrospray source of the tandem mass spectrometer operated simultaneously in positive and negative ion mode (Table 1). Mass transitions, declustering potentials and collision energies for the compounds were obtained by direct infusion experiments with single compound standard solutions.
The following mass spectrometer settings were used: Curtain gas 40 psi, source temperature 450°C, ion spray 4500 V, −4500 V respectively, Gas 1/Gas2: 40/60 psi.

Preparation of analytical standards. Stock solutions were prepared with a concentration of 100 mg/L using acetonitrile. By diluting a mix standard solution (concentration 10 mg/L) calibration standards were prepared with acetonitrile/ water (60/40) at levels from 2.0 mg/L to 100 mg/L. While the stock solutions were stored at −18°C, the calibration standards had to be newly made for each analytical run.

Quantification of metabolites with LC-MS/MS. Due to a substantial peak separation, the identity of peaks could be confirmed using retention times and mass transitions.

Data were acquired and processed on Analyst 1.6.3. Quantification was carried out with an external calibration. The calibration curves were calculated by linear regression with a RSD of <20% and a coefficient of determination (R2) of >0.99.

Statistics and reproducibility. All biodegradation assays were performed in biological triplicates. The dot-plot format of Fig. 3 was built using the software Interactive Dot Plot Tool.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

All data are shown in the figures and tables. Raw data generated during this study are available from the corresponding author on reasonable request. All Mycobacteria and Gordonia strains tested in this study were purchased at the German collection of Microorganisms and Cell cultures GmbH (DSMZ).

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Author contributions

M.S. performed all microbiological experiments; A.Si. and A.St. carried out the mass spectrometry analyses; M.S., C.S. and H.F. drafted the paper. M.S., C.S., H.F., M.K., and G.A. designed the experimental setup.

Competing interests

The authors declare no competing interests.

Additional information

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