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Ammonia Released by *Streptomyces aburaviensis* Induces Droplet Formation in *Streptomyces violaceoruber*

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Abstract *Streptomyces violaceoruber* grown in co-culture with *Streptomyces aburaviensis* produces an about 17-fold higher volume of droplets on its aerial mycelium than in single-culture. Physical separation of the *Streptomyces* strains by either a plastic barrier or by a dialysis membrane, which allowed communication only by the exchange of volatile compounds or diffusible compounds in the medium, respectively, still resulted in enhanced droplet formation. The application of molecular sieves to bioassays resulted in the attenuation of the droplet-inducing effect of *S. aburaviensis* indicating the absorption of the compound. ^{1}H-NMR analysis of molecular-sieve extracts and the selective indophenol-blue reaction revealed that the volatile droplet-inducing compound is ammonia. The external supply of ammonia in biologically relevant concentrations of ≥8 mM enhanced droplet formation in *S. violaceoruber* in a similar way to *S. aburaviensis*. Ammonia appears to trigger droplet production in many *Streptomyces* strains because four out of six *Streptomyces* strains exposed to ammonia exhibited induced droplet production.

Keywords Alkalinisation · Co-cultivation · Microbe-microbe interactions · Morphological differentiation · Volatiles

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Introduction

Streptomyces bacteria have been intensively studied since the middle of the last century because of their structurally diverse, bioactive secondary metabolites. Many of them have become invaluable in medicine, e.g. as antibiotics (Katz and Baltz 2016). With the focus on identifying pharmaceutical leads, streptomycetes have been isolated and grown under optimised conditions in the laboratory. The slow-growing streptomycetes undergo a complex life cycle. They initially produce vegetative hyphae from spores and later on hydrophobic aerial hyphae that typically differentiate into spores within approximately one to two weeks of growth (Flärdh and Buttner 2009). Both morphological differentiation and secondary metabolite production are known to be regulated in streptomycetes by environmental triggers and signalling compounds (e.g. quorum sensing) (Flärdh and Buttner 2009; Polkade et al. 2016).

Microorganisms can sense volatile (Effmert et al. 2012) and diffusible compounds (Straight and Kolter 2009), or directly react to physical contacts (Schroeckh et al. 2009). Co-cultivation of microorganisms is by now commonly used to mimic ecological systems and to induce chemical (Ueda et al. 2000; Yamanaka et al. 2005; Seyedsayamdost et al. 2012; Ueda and Beppu 2016) and morphological differentiation (Hashimoto et al. 2003; Yamanaka et al. 2005; Xu et al. 2008; Nijland and Burgess 2010) in microorganisms. Nevertheless, the underlying mechanisms of microbial interactions often remain unclear.

A variety of streptomycetes (Bonde and McIntyre 1968; Rudd and Hopwood 1979; Okazaki et al. 1983; Schrempf and Merling 2015) and also some fungi (Knoll 1912; Sprecher 1959; Unestam and Sun 1995) are known to exude droplets on their aerial mycelium. *Streptomyces* colonies with droplets on their aerial mycelium are frequently depicted and droplet production was even used as a criterion for the

classification of *Streptomyces* strains (Waksman and Lechevalier 1953). Microbial droplet formation has been compared to the release of droplets by higher plants (guttation) and is favoured at high humidity (Atkinson 1900; Sprecher 1959; McPhee and Colotelo 1977) or during a particular time of the day (Wilson 1948). In addition, droplet formation by fungi and streptomycetes strongly depends on the respective growth stage (Williams and McCoy 1953; Waters et al. 1975; McPhee and Colotelo 1977; Schrempf et al. 2011; Gareis and Gottschalk 2014). The ecological function of the droplets on the aerial mycelium of fungi and streptomycetes is so far virtually unknown.

The high concentrations of sugars and sugar alcohols in these droplets (Sprecher 1959; Arora and Gupta 1993; Sun et al. 1999; Rangel-Castro et al. 2002) suggest a function as a nutrient reservoir. Similarly, the exudates of fungi have been suspected to act as a water reservoir, allowing mycelial growth in the case of a poor water supply of the cells at the top of the aerial mycelium (Jennings 1991). Exudates of both fungi and streptomycetes were reported to contain cellulolytic and catabolic enzymes (Colotelo et al. 1971; Colotelo 1973; Schrempf et al. 2011), which may facilitate the decomposition and subsequent uptake of substrate molecules (Unestam and Sun 1995). Sun et al. even proposed a kidney-like function of fungal droplets, which allows to get rid of harmful waste products and to simultaneously reabsorb valuable nutrients (Sun et al. 1999). The droplets of several fungi were recently found to contain high amounts of cytotoxic secondary metabolites (Grovel et al. 2003; Gareis and Gareis 2007; Hutwimmer et al. 2010; Gareis and Gottschalk 2014), which implies a defensive function of droplets.

Microbial droplet formation occurs in a random way under laboratory conditions, which makes it difficult to study this phenomenon. So far, no biological triggers of droplet formation have been identified. Due to our observation that *Streptomyces violaceoruber* releases much more droplets in co-culture with *Streptomyces aburaviensis* than in single-culture, we investigated how *S. aburaviensis* induces droplet formation in *S. violaceoruber*.

Methods and Materials

Strains and Cultivation Conditions

Unless stated otherwise, bioassays were performed on soy flour mannitol medium (SFM; soy flour 20 g l^{-1}, mannitol 20 g l^{-1}, agar 15 g l^{-1}) (Kieser et al. 2000) in polystyrene petri dishes (Greiner Bio-One GmbH, Frickenhausen, Germany; 94 × 16 mm with vents; 24 ml medium/dish). The analysed *Streptomyces* strains are listed in Table 1. Spore suspensions were prepared as described by Kieser et al. (Kieser et al. 2000). Spore suspensions contained about 5–20 × 10^2 spores per μl. Agar plates were incubated at 28 °C and a relative humidity of about 30–40%.

Analysis of Droplet-Promoting Effects

S. violaceoruber DSM 40783 was cultivated either alone or in co-culture with other *Streptomyces* strains. For single-culture and co-culture experiments, drops (1 μl) of a spore suspension of the respective *Streptomyces* strain were spotted either alone

Table 1 Overview of *Streptomyces* strains used in interaction assays with *S. violaceoruber* DSM 40783

	Strain	Droplet production[a]	Droplet-promoting effect[b]
S. aburaviensis	ATCC 31860	N	Y
S. albus	J 1074	N	N
S. ambofaciens	DSM 40053	N	Y
S. antibioticus	DSM 46477	N	Y
S. asterosporus	DSM 41452	N	N
S. avermitils	DSM 46492	Y	Y
S. cattleya	DSM 46488	Y	N
S. coelicolor	M145	Y	N
S. collinus	DSM 40733	N	N
S. griseus	DSM 41080	Y	Y
S. griseus	NBRC 13350	Y	Y
S. odorifer	DSM 40347	Y	N
S. rimosus	DSM 40260	N	N
S. viridochromogenes	DSM 40110	N	N

Yes (Y), No (N)

[a] when grown on SFM medium

[b] on *S. violaceoruber* in interaction assays

or alternately with another *Streptomyces* strain in the shape of a cross onto solid SFM medium (Fig. 1). *S. aburaviensis* was tested for a potential droplet-promoting effect on droplet-producing *Streptomyces* strains, namely *Streptomyces avermitilis* DSM 46492, *Streptomyces cattleya* DSM 46488, *Streptomyces griseus* DSM 41080 and *Streptomyces griseus* NBRC 13350 in an analogous way.

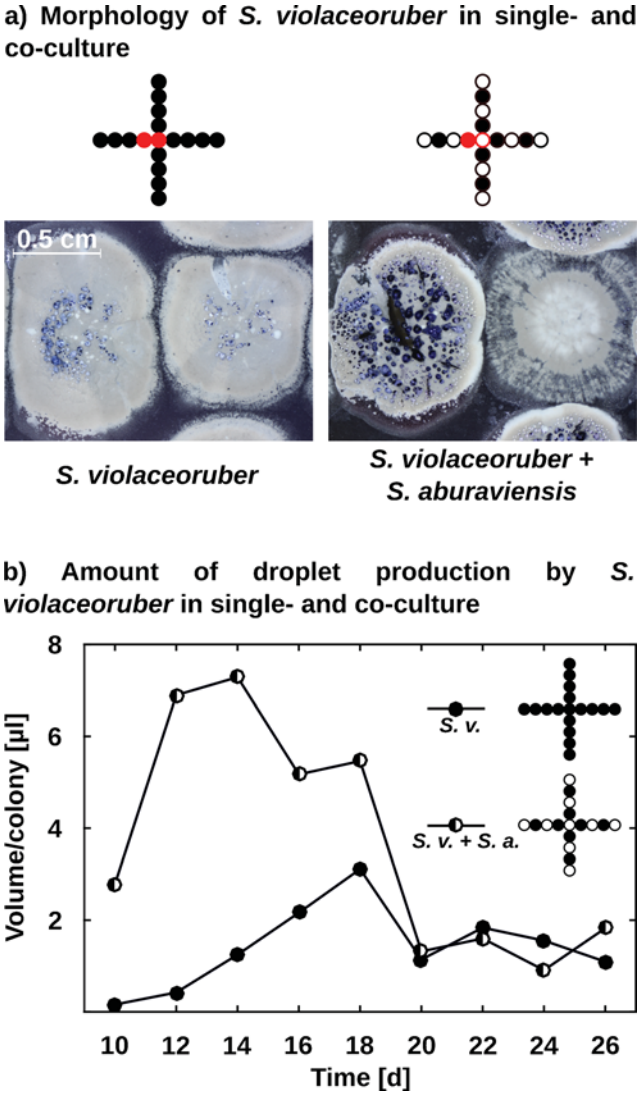


Fig. 1 Droplet formation by *S. violaceoruber* in single- and in co-culture with *S. aburaviensis*. *Points* (●) in the schemes of the bioassays represent *S. violaceoruber* colonies, *circles* (○) represent *S. aburaviensis* colonies. **a** Droplet formation by *S. violaceoruber* cultivated alone and in co-culture with *S. aburaviensis* at a relative humidity of about 30–40% on day 12, when droplet production reached a maximum. In the following days droplets disappeared in parallel to the overall drying of the agar plate. Depicted colonies are highlighted in *red* in the schemes of the bioassays. Each bioassay was performed in six replicates. **b** Amount of droplet production by *S. violaceoruber* colonies cultivated alone (*S. v.*,-●-) and in co-culture with *S. aburaviensis* (*S. v.* + *S. a.*, -◐-) at a relative humidity of 80%. Droplets were collected from bioassays schematically shown in the upper right corner. The volume of droplets produced by a single colony was calculated from droplets collected from at least 16 and a maximum of 255 colonies (for details see Table S1)

Quantification of Droplet Production

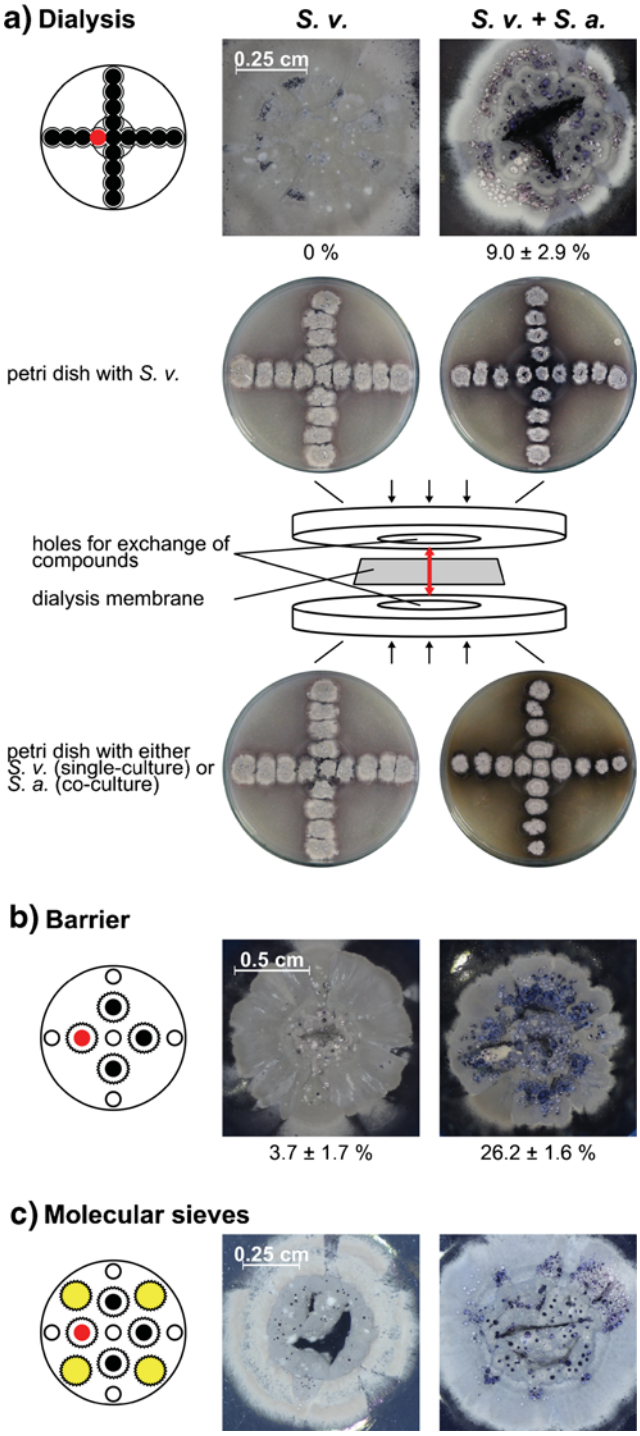
For the quantification of the volume of droplets produced by *S. violaceoruber* in single-culture and in co-culture with *S. aburaviensis* assays were incubated over 26 d at a relative humidity of approximately 80% and without ventilation to achieve a high droplet yield. Droplets of single- and co-cultures were collected with pulled soda glass capillaries ($Ø_{in}$ 0.96 mm, Hilgenberg, Germany) on days 10, 12, 14, 16, 18, 20, 22, 24 and 26. Droplets were stored in 1.5-ml glass vials at −20 °C. The volume of the collected droplets was calculated from the quotient of the weight of the collected droplets and their density. The density was determined in triplicate for each single- and co-culture sample collected at the specified time points by weighing 5 µl of the collected droplets on an analytical balance.

Determination of the Droplet-Wetted Area from Colony Photographs

Droplet production was further estimated from the droplet-wetted area of a *S. violaceoruber* colony referenced to the entire area of the colony. Values represent means and standard deviations of three analysed photographs. The areas were measured from photographs with the manual selection tools of the software ImageJ (National Institutes of Health, Bethesda, USA; Fig. S1).

Co-cultivation of *S. violaceoruber* and *S. aburaviensis* Separated by a Dialysis Membrane

The amount of mannitol or soy flour was reduced to 10, 5, 2.5 and 1.25 g l^{-1}, respectively, in order to optimise the nutritional conditions for droplet formation by *S. violaceoruber*. The other components of the medium were kept constant. A reduced soy flour concentration of 10 g l^{-1} (1SF2M) was chosen to analyse how *S. violaceoruber* and *S. aburaviensis* interact because the droplet-promoting effect was strongest for this medium. In order to assess whether the interaction between both strains was mediated by a compound which was secreted into the medium, *S. violaceoruber* and *S. aburaviensis* were cultivated in separate glass petri dishes (20 × 100 mm; Anumbra®, FGH plus, Šumperk, Czech Republic) filled with 19 ml of 1SF2M agar (Fig. 2a). The dishes were facing away from each other and were only connected by a centred hole in the bottom of both dishes (Ø 30 mm), which was covered by a single-layered pre-wetted and sterilised regenerated cellulose membrane with a molecular weight cut-off (MWCO) of 12–14 kDa (Spectra/Por® 5, Spectrum Laboratories Inc., Rancho Dominguez, USA). Drops (1 µl) of a *S. aburaviensis* or of a *S. violaceoruber* spore suspension, respectively, were spotted on each petri dish, forming the shape of a cross.



◀ **Fig. 2** Investigation of the mode of interaction between *S. violaceoruber* and *S. aburaviensis*. *Points* (●) in the schemes of the bioassays represent *S. violaceoruber* colonies, *circles* (○) represent *S. aburaviensis* colonies. Images depict colonies which are highlighted in *red* in the schemes of the bioassays. **a** Droplet formation by *S. violaceoruber* (*S. v.*) separated from *S. aburaviensis* (*S. a.*) by a dialysis membrane on day 14. For illustration of the dialysis assays a schematic top view is depicted on the left side. The hole in the bottom of the petri dish is marked by the *inner circle*, colonies of *S. aburaviensis* are on the opposite dish. Petri dishes were assembled as indicated by the *black arrows* in the scheme. The *red arrow* indicates the exchange of diffusible compounds between both dishes. **b** Droplet formation by *S. violaceoruber* separated from *S. aburaviensis* by a mechanical barrier (centrifugal tube lids) on day 17. Lids of centrifugal tubes, which act as mechanical barriers, are displayed by jagged-edged circles. **c** Droplet formation in the presence of molecular sieves (*yellow*), which trapped released volatile compounds, on day 17. Each bioassay was performed at least in three replicates. The percentage of the droplet-wetted area of a colony is shown below the photographs as the mean of three analysed photographs

Co-cultivation of *S. violaceoruber* and *S. aburaviensis* Separated by a Physical Barrier

In order to investigate if volatile compounds were responsible for a droplet-promoting effect, four lids of 15-ml centrifugal tubes (Cellstar®, Greiner Bio-One, Frickenhausen, Germany) were placed into glass petri dishes (20 × 100 mm; Anumbra®, FGH plus, Šumperk, Czech Republic) in the shape of a square (Fig. 2b). Lids were filled with 2 ml 1SF2M medium and the rest of the glass petri dish with 24 ml of 1SF2M agar. Drops (1 μl) of a *S. violaceoruber* spore suspension were spotted into the medium-filled lids and drops (1 μl) of a *S. aburaviensis* spore suspension on each side of each lid, forming the shape of a cross. Thus, *S. violaceoruber* and *S. aburaviensis* colonies were physically separated to allow the induction of droplet formation by volatile compounds only.

Extraction of the Droplet-Inducing Compound(s)

In order to extract the volatile compound(s) responsible for the droplet-promoting effect, the lids of eight 15-ml centrifugal tubes (Cellstar®, Greiner Bio-One, Frickenhausen, Germany) were placed into glass petri dishes (20 × 100 mm; Anumbra®, FGH plus, Šumperk, Czech Republic). Thereby, four of them each were arranged in the shape of a smaller and a bigger square being shifted by 45 degrees (Fig. 2c). The petri dish was filled with 18 ml and the lids of the inner square were each filled with 2 ml of 1SF2M agar. The lids of the outer square were filled with either activated charcoal powder, Diaion® HP-20 (Sigma Aldrich Chemie GmbH, Steinheim, Germany), Amberlite® IRA-96 (Sigma-Aldrich Chemie GmbH, Steinheim, Germany), Amberlite® IRA-410 (Sigma-Aldrich Chemie GmbH, Steinheim, Germany), Amberlite® IRC86 (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) or molecular sieves (0.5 nm pore size, Merck, Darmstadt, Germany) to test them for the absorption of any volatile droplet-promoting compound(s).

NMR Analysis of Molecular-Sieve Extracts

Molecular sieves (0.5 g) from single- and co-culture bioassays were extracted with 1.5 ml 250 mM HCl, sonicated for 30 min and centrifuged for 10 min at 11800 g. ^{1}H-NMR spectra were

recorded with water suppression using a Bruker Avance III 600 spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany). Samples were locked and shimmed to DMSO-d6, which was added to the samples in a sealed capillary, and referenced to 3-trimethylsilyl propionic acid (TMSP).

Supplementation of *Streptomyces* Strains with Ammonia via the Gas Phase

In order to evaluate the role of ammonia as the droplet-promoting compound, 2 ml of aqueous ammonia dilutions (1, 2, 4, 8, 16, 32, 64 mM) were added to *S. violaceoruber* single-cultures. The experiments were prepared as described for the extraction of volatile compounds. Two of the outer centrifugal lids were left empty and the other two were filled with 1 ml of the respective ammonia solution after 4 d of growth (Fig. 3b). Lids of single- and co-culture controls were filled with water instead. Other droplet-producing *Streptomyces* strains, namely *S. avermitilis* DSM 46492, *S. cattleya* DSM 46488, *S. griseus* DSM 41080 and *S. griseus* NBRC 13350, were tested for a potential response to ammonia and the ammonia production by *S. aburaviensis* analogously.

Quantification of Ammonia in Molecular-Sieve Extracts

The amount of ammonia collected with molecular sieves from single- and co-cultures of *S. violaceoruber* and *S. aburaviensis* was determined and compared to an ammonia reference curve. Ammonia-supplemented assays and controls were prepared as described above. Molecular sieves (1 g) were added to the two remaining empty lids of the outer square right at the day of inoculation (Fig. 3b). After 17 d of growth molecular sieves were extracted with 10 ml 4 M KCl and sonicated for 30 min. Ammonia was quantified using the indophenol-blue method adapted from the DIN 38406–5 norm (DIN-Normenausschuss Wasserwesen 1983). 100 µl of solution A (0.65 g sodium salicylate, 0.65 g trisodium citrate dihydrate and 10 mg sodium nitroprusside dihydrate in 5 ml ddH_2O) and 100 µl of solution B (10 mg sodium dichloroisocyanurate in 5 ml NaOH (32 g l^{-1})) were added to 1 ml of an ammonium standard (0, 25, 50, 50, 75, 100 µM) or the molecular-sieve extracts. Solutions A and B were freshly prepared on the day of measurement. Reaction mixtures were incubated for 3 h in the dark. The absorption was measured at 655 nm.

Quantification of Ammonia in Interaction Assays and pH Measurements

In order to reduce possible background from the medium, defined zones around the colonies growing in the middle of the cross of single- and co-culture assays were cut out with a stencil in the shape of a Swiss cross (Ø 50 mm, 15 mm beam thickness) after 3, 5, 7, 10, 12, 14 and 17 d of growth. Samples were weighed, uniformly sliced into squares of about 1 cm size, and kept at −80 °C until further analysis.

For pH measurements, samples were thawed and centrifuged at 11800 g to collect the liquid. The pH was measured with a pHenomenal® LS 221 glass electrode (VWR International GmbH, Darmstadt, Germany). For ammonia quantification, 2 M HCl was added to the thawing samples to a weight of 10 g. Samples were sonicated for 20 min at room temperature and centrifuged for 10 min at 11800 g to collect the supernatant. Samples were neutralised with an equal amount of 2 M KOH. Ammonia was quantified using the indophenol-blue method as described above.

Supplementation of Bioassays with Ammonia/Ammonium Salts and Alteration of pH

S. violaceoruber was grown in the shape of a cross as described above. NH_4Cl, $(NH_4)_2SO_4$ or NH_3 concentrations of growing bioassays were increased by 3 mM on days 3, 5, 7 and 10. Solutions (240 µl, 300 mM) were applied to the centre of the petri dish underneath the agar in a way that the solutions were distributed beneath the medium and growing colonies did not get into direct contact with them. The influence of the added compounds on droplet formation was analysed at the time point of maximum droplet production, day 12. The critical time point of ammonia addition was determined by the addition of ammonia to the medium from day 3 or only from days 5, 7 or 10 on. The pH of growing bioassays was changed by the single addition of either ammonia, KOH or NaOH to final concentrations of 3 mM in an analogous way. All alkaline solutions were applied once on day 3, 5, 7 or 10.

Results

Induction of Droplet Formation in Co-culture

S. violaceoruber DSM 40783 occasionally forms dark blue-coloured droplets on soy flour mannitol (SFM) medium. In co-culture some *Streptomyces* strains strongly affected droplet production by *S. violaceoruber*. *S. aburaviensis* ATCC 31860 strongly induced droplet production by *S. violaceoruber* when both strains were grown together (Fig. 1a). In addition, *Streptomyces avermitilis* DSM 46492, *Streptomyces ambofaciens* DSM 40053, *Streptomyces griseus* DSM 41080 and *Streptomyces griseus* NBRC 13350 induced droplet formation in *S. violaceoruber*, but less pronounced than *S. aburaviensis* (Fig. S2).

In co-culture with *S. violaceoruber*, both *S. aburaviensis* and *S. avermitilis* most strongly induced droplet formation after 12–14 d. For *S. ambofaciens* and the two *S. griseus*

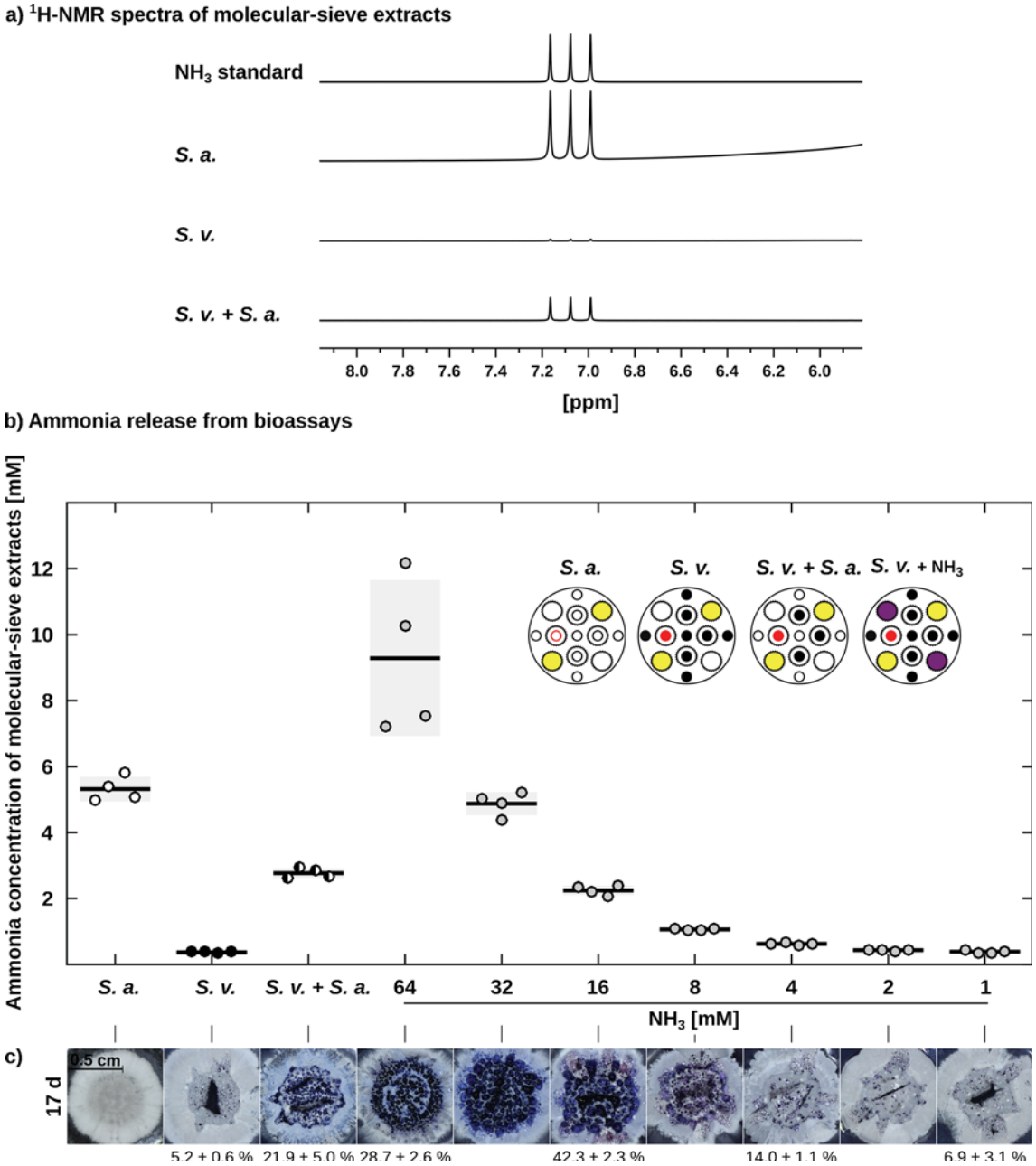


Fig. 3 Identification of ammonia as the droplet-inducing compound. **a** ^{1}H-NMR spectra (600 MHz, 250 mM HCl) of molecular-sieve extracts of an ammonia standard, single-cultures (*S. a.*, *S. v.*) and a co-culture (*S. v.* + *S. a.*). **b** Release of ammonia from single- (*S. a.* ○; *S. v.*, ●) and co-culture (*S. v.* + *S. a.*, ◐) experiments and from ammonia-supplemented single-cultures (●) to the headspace of the agar plate. *Black horizontal bars* represent mean values, *grey bars* indicate standard deviations ($N = 4$). The schemes of the bioassays are depicted in the upper right corner. Ammonia dilutions in centrifugal lids (*jagged-edged circles*) are displayed in *purple*. Lids either filled with molecular sieves or left empty in control experiments are shown in yellow. *Black points* (●) represent *S. violaceoruber*, *circles* (○) *S. aburaviensis* colonies. **c** Colonies of *S. violaceoruber* from control experiments without molecular sieves. Depicted colonies are highlighted in red in the schemes of the bioassays. The percentage of the droplet-wetted area of a colony is shown below the photographs as the mean of three analysed photographs

strains the droplet-promoting effect was observed at earlier time points around 5 to 7 d. In the presence of all other *Streptomyces* strains which were analysed (Table 1) *S. violaceoruber* formed droplets to a similar or even a reduced extent compared to when cultivated alone. Out of the droplet-promoting strains, the two strains of *S. griseus* and *S. ambofaciens* inhibited the growth of *S. violaceoruber*.

Because droplet formation of *S. violaceoruber* was induced most in co-culture with *S. aburaviensis*, the effect was further analysed for this strain combination. Droplet production by

S. violaceoruber was highest after about 12 d of growth, both when cultivated alone and in co-culture with *S. aburaviensis* (Fig. 1a). For the quantification of droplet production, the bioassays were incubated at a relative humidity of 80% and without ventilation in order to achieve a higher droplet yield compared to when incubated at ambient humidity. At a relative humidity of 80%, the maxima of droplet production were observed at later time points (single-culture: 18 d, co-culture: 14 d) than at ambient humidity (Fig. 1b, Table S1). At the point of maximum droplet production, *S. violaceoruber* had an about 17-fold higher volume of droplets in co-culture compared to when grown alone.

How Does *S. aburaviensis* Influence Droplet Formation by *S. violaceoruber*?

The induction of droplet formation in *S. violaceoruber* by *S. aburaviensis* could either be mediated by volatile compounds, non-volatile compounds or by direct cell-cell contacts. In order to establish whether a potential droplet-inducing compound was released into the growth medium, *S. violaceoruber* and *S. aburaviensis* were initially prevented from forming cell-cell contacts and an interaction via compounds in the gas phase. Both strains were cultivated on separate petri dishes facing away from each other (Fig. 2a). The dishes were connected via a hole in the bottom of each plate and only separated by a dialysis membrane with a molecular weight cut-off (MWCO) of 12 to 14 kDa. Since the effect could conceivably be attenuated when relying on one possible interaction mode only, strains were cultivated on soy flour mannitol (SFM) medium with a reduced soy flour concentration of 10 g l^{-1} (1SF2M), the medium composition which led to most pronounced droplet formation. In the dialysis membrane experiments, *S. aburaviensis* clearly enhanced droplet-formation (Fig. 2a; *S. v.*: 0%, *S. v.* + *S. a.*: 9.0 ± 2.9% droplet-wetted area/colony), but the effect was weaker compared to the effect in co-cultures.

In a second experiment, *S. aburaviensis* and *S. violaceoruber* were prevented from cell-cell contacts and an interaction via diffusible compounds in the growth medium by a mechanical barrier. Droplet formation was, as well, enhanced (Fig. 2b; *S. v.*: 3.7 ± 1.7%, *S. v.* + *S. a.*: 26.2 ± 1.6% droplet-wetted area/colony).

Extraction and Identification of the Compound Promoting Droplet Formation

The droplet-promoting compound could not be extracted using solvents such as hexane, diethyl ether or ethyl acetate. The droplet-inducing compound could not either be extracted from the headspace of *S. aburaviensis* single-cultures or of co-cultures by closed-loop stripping (CLS). Analysis of the gas phase of single- and co-cultures by solid-phase microextraction (SPME) and gas chromatography-mass spectrometry (GC-MS) did not reveal any compounds present in single-cultures of *S. aburaviensis* or co-cultures to which enhanced droplet formation by *S. violaceoruber* could be attributed.

With the aim to trap any volatile droplet-promoting compound from the gas phase, barrier assays of co-cultures were supplemented with different absorbents and monitored for reduced droplet production. Out of the absorbents tested (activated charcoal, Diaion® HP20, Amberlite® IRA-96, Amberlite® IRA-410, Amberlite® IRC-86, molecular sieves 0.5 nm), molecular sieves most strongly decreased the droplet-promoting effect of *S. aburaviensis* (Fig. 2c). In order to identify the trapped compound(s), molecular sieves were removed from co-cultures and extracted with water. The pH of the extracts turned out to be alkaline. So, we screened for volatile amines. Molecular sieves from single- and co-cultures were extracted with 250 mM HCl and analysed by ^{1}H-NMR using water suppression. A triplet at 7.08 ppm with a coupling constant of 52.3 Hz was observed (Fig. 3a). This signal is characteristic of ammonia (Hoffmann 2016) and it matched perfectly to the signal of an ammonia standard. Moreover, when the extracts were tested for ammonia using the selective indophenol-blue method all extracts developed a dark blue colour (Patton and Crouch 1977; DIN-Normenausschuss Wasserwesen 1983).

Determination of Volatile Ammonia Enhancing Droplet Formation

The role of volatile ammonia in the induction of droplet formation by *S. violaceoruber* was evaluated by the addition of 2 ml of ammonia solutions (1, 2, 4, 8, 16, 32, and 64 mM) to falcon tube lids in single-culture barrier assays of *S. violaceoruber*. Ammonia concentrations higher than 8 mM promoted droplet formation in a similar way to *S. aburaviensis* (Fig. 3c). Thereby, *S. violaceoruber* single-cultures supplemented with 32 and 64 mM ammonia were most similar to *S. violaceoruber* grown in co-culture. To verify whether quantities of ammonia in co-cultures resembled those in ammonia bioassays with similar morphology, ammonia was both collected from ammonia-supplemented single-cultures of *S. violaceoruber* and from unsupplemented single- and co-cultures with equal amounts of molecular sieves (1 g). Ammonia was extracted from the molecular sieves and quantified by the indophenol-blue method (Patton and Crouch 1977; DIN-Normenausschuss Wasserwesen 1983) after 17 d of growth. The ammonia concentrations of molecular-sieve extracts of co-cultures lay between those of bioassays supplemented with 16 and 32 mM ammonia (Fig. 3b; *S. v.* + *S. a.* co-culture: 2.77 ± 0.15 mM, administration of 16 mM NH_3: 2.24 ± 0.15 mM, administration of 32 mM NH_3: 4.88 ± 0.35 mM). Single-cultures of *S. violaceoruber*, which

had not been supplemented with ammonia, showed ammonia concentrations lower than ammonia-supplemented bioassays and only produced few droplets.

Monitoring Ammonia Formation

The release of ammonia into the growth medium of single-cultures and co-cultures, which were grown without any physical barrier (Fig. 4), was determined from day 3 to day 17. Therefore, the liquids of both single- and co-cultures were collected from solid media at different time points after a freeze-thaw cycle by syneresis. Ammonia in the media of single- and co-culture bioassays was quantified using the indophenol-blue method (Patton and Crouch 1977; DIN-Normenausschuss Wasserwesen 1983). Single-cultures of *S. aburaviensis* and co-cultures contained about 12-times and 9-times higher ammonia concentrations, respectively, compared to single-cultures of *S. violaceoruber* on day 12 (Fig. 4; *S. v.*: 1.31 ± 0.06 mM, *S. a.*: 15.96 ± 0.94 mM, *S. v.* + *S. a.*: 12.38 ± 1.78 mM). Ammonia concentrations of single-cultures of *S. aburaviensis* and co-cultures showed two main increases between days 3 and 5 and days 12 and 14. Ammonia concentrations in single-cultures of *S. violaceoruber* exhibited a wave-like character with decreasing concentrations between 7 d and 12 d (Fig. 4 -●-).

Is Droplet Formation Specifically Induced by Ammonia or by the Resulting Alkalinisation?

When the ammonium concentration in bioassays of *S. violaceoruber* was incrementally increased by 3 mM at each time on days 3, 5, 7 and 10 by the addition of ammonium either as its chloride or sulphate salt, the droplet-promoting effect could not be reconstituted (Fig. 5b). Instead, droplet formation in the presence of ammonium salts was diminished compared to controls without ammonium addition (*S. v.* control: 10.4 ± 1.9%, NH_4Cl: 1.1 ± 0.2%, $(NH_4)_2SO_4$: 1.7 ± 0.4% droplet-wetted area/colony). Only when ammonia was added to bioassays in an analogous way, droplet formation was enhanced (*S. v.* control: 10.4 ± 1.9%, NH_3: 41.2 ± 0.4% droplet-wetted area/colony). Thereby the droplet-promoting effect of ammonia strongly depended on its early addition to bioassays and was only pronounced when ammonia was added from day 3 on (Fig. 5c). When ammonia was supplemented later than day 3, droplet formation was considerably less enhanced (*S. v.* control: 10.4 ± 1.9%, NH_3 from day 3: 41.2 ± 0.4%, NH_3 from day 5: 21.8 ± 1.0%, NH_3 from day 7: 19.4 ± 1.7%, NH_3 from day 10: 16.4 ± 1.9% droplet-wetted area/colony).

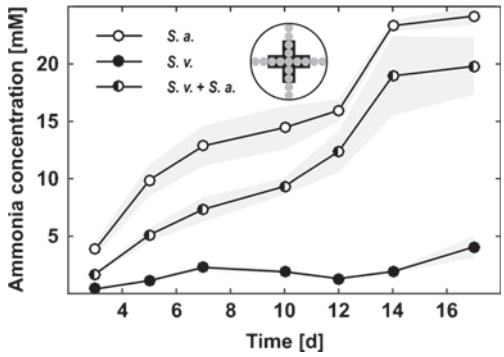


Fig. 4 Ammonia production by single-cultures of *S. aburaviensis* (*S. a.*, -○-) and *S. violaceoruber* (*S. v.*, -●-) as well as by co-cultures (*S. v* + *S. a.*, -◐-). Points represent mean values ($N = 5$), standard deviations are shaded in *grey*. Ammonia was determined in the inner zone of the agar plate, which is marked in *black* in the scheme of the bioassays

These observations raised the question whether ammonia specifically enhances droplet formation or if alkalinisation of the medium was sufficient to trigger induced droplet formation. Indeed, co-cultures exhibited a significantly higher pH than single-cultures of *S. violaceoruber* throughout the whole growth, even at early growth stages (Fig. S3). However, unlike the addition of ammonia, single changes in pH on day 3 by the addition of either NaOH or KOH to final concentrations of 3 mM did not enhance droplet formation (Fig. S4b, Table S2; *S. v.* control: 10.4 ± 1.9%, NH_3: 22.6 ± 2.9%, NaOH: 6.7 ± 2.9%, KOH: 4.6 ± 2.2% droplet-wetted area/colony). Only when the growth medium was alkalised by NaOH and KOH on days 5 and 7, droplet formation was enhanced to a similar extent as by the addition of ammonia (Fig. S4c; *S. v.* control: 10.4 ± 1.9%, NH_3: 18.4 ± 2.5%, NaOH: 18.4 ± 0.8%, KOH: 18.4 ± 4.1% droplet-wetted area/colony).

S. aburaviensis and Ammonia Induce Droplet Production in Other *Streptomyces* Strains

To investigate whether *S. aburaviensis* induces droplet formation in other *Streptomyces* strains, it was cultivated side-by-side with *S. avermitilis*, *S. cattleya*, *S. odorifer, S. coelicolor* M145 and two *S. griseus* strains (Table 1). *S. aburaviensis* strongly enhanced droplet formation in *S. cattleya* and *S. coelicolor* (Fig. S5). Nevertheless, droplet production of both strains was only affected at a late time point, namely after 17 d. Similarly, slight changes in the droplet production of *S. avermitilis* were observed after 17 d in co-culture with *S. aburaviensis*, when small droplets irregularly appeared. Droplet production by the *S. griseus* strains and by *S. odorifer* was not clearly enhanced when they were grown together with *S. aburaviensis*. The application of ammonia via the gas phase enhanced droplet formation in four of the six tested *Streptomyces* strains (Fig. S6).

Discussion

Streptomycetes produce a large variety of volatile organic compounds, among others terpenoids, such as geosmin and 2-methylisoborneol, the biological functions of which are

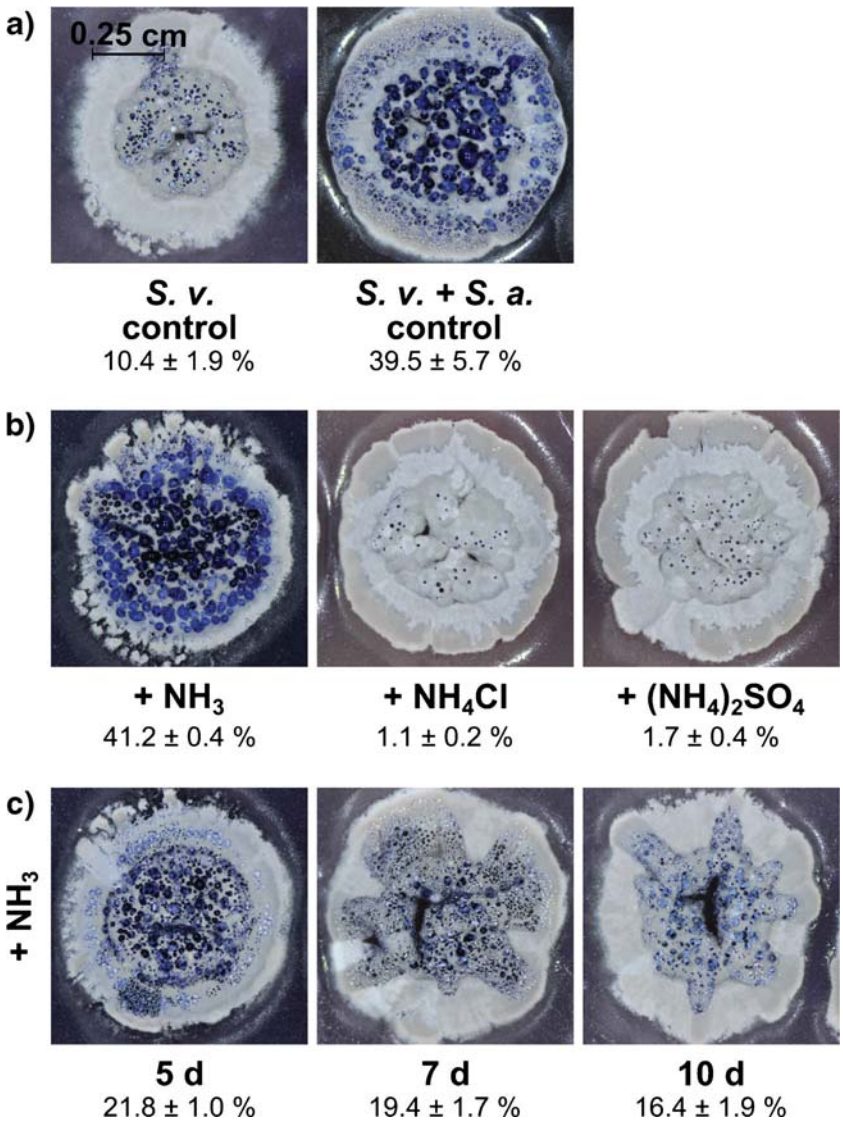


Fig. 5 Influence of ammonia and ammonium on droplet formation by *S. violaceoruber*. **a** *S. violaceoruber* controls grown for 12 d in single-culture and in co-culture with *S. aburaviensis*. **b** Single-culture assays of *S. violaceoruber* on day 12 when the ammonia or ammonium concentration in bioassays was incrementally increased by 3 mM a time on days 3, 5, 7 and 10 by the addition of either NH_4Cl, $(NH_4)_2SO_4$ or NH_3, respectively. **c** Droplet formation by *S. violaceoruber* on day 12 when starting ammonia supplementation (3 mM) from days 5, 7 or 10 on, respectively. Each bioassay was performed in four replicates. The percentage of the droplet-wetted area of a colony is shown below the photographs as the mean of three analysed photographs

often still unknown (Schulz and Dickschat 2007). Volatiles trapped from *S. aburaviensis* single-cultures or *S. aburaviensis* and *S. violaceoruber* co-cultures by closed-loop stripping (Grob 1973; Grob and Zürcher 1976) did not induce droplet production by *S. violaceoruber*. Instead, volatile ammonia strongly induced the droplet production of *S. violaceoruber* in co-culture with *S. aburaviensis* (Fig. 1). Ammonia production was reported for many microorganisms (Schulz and Dickschat 2007), such as yeasts (Palková et al. 1997), rhizobacteria (Weise et al. 2013) or *Bacillus licheniformis* (Nijland and Burgess 2010). In the yeast *S. cerevisiae*, ammonia serves as an intraspecies signal to coordinate growth with neighbouring colonies (Palková et al. 1997). The bacterium *Bacillus licheniformis* shows increased biofilm formation in the presence of ammonia (Nijland and Burgess 2010). Ammonia released by rhizobacteria was observed to hamper the growth of plants due to an alkalinisation of the growth medium (Weise et al. 2013). Bernier et al. found ammonia to induce antibiotic and stress resistance in a variety of microorganisms (Bernier et al. 2011). Jones et al. recently established that volatile trimethylamine serves *Streptomyces venezuelae* as a signal to induce a so far unknown exploratory filamentous growth over long distances. Interestingly, this exploratory growth was also observed in the presence of ammonia (Jones et al. 2017).

The excretion of ammonia might in the first place serve *Streptomyces* strains to get rid of excess ammonia. In addition, streptomycetes might profit from the toxicity of ammonia in outcompeting other bacteria. Droplets may serve to store water and nutrients and thereby foster the formation of differently differentiated microenvironments in *Streptomyces* colonies. This way a colony could both produce spores and preserve growing filaments in order to flexibly react to changing growth conditions.

The wave-like character of ammonia concentrations suggests that ammonia is emitted both by *S. violaceoruber* and by *S. aburaviensis* in pulses (Fig. 4). Similarly, this was observed for the yeast *Saccharomyces cerevisiae* (Palková et al. 1997). Referred to the colony number of both strains in co-culture (*S. aburaviensis*: 9 colonies, *S. violaceoruber*: 8 colonies), ammonia concentrations were in fairly good agreement with ammonia concentrations predicted from single-culture bioassays of *S. violaceoruber* and *S. aburaviensis* (17 colonies) until day 10 (< 25% deviation). However, from day 12 on ammonia concentrations in co-cultures were more strongly elevated (12 d: 37%, 14 d: 43%, 17 d: 35%). Induced ammonia production by *S. aburaviensis*, and possibly also by *S. violaceoruber*, might be a specific response by any of both strains to the presence of another strain. Alternatively, it might be a rather unspecific reaction by *S. aburaviensis* to compensate for lower ammonia concentrations in co-culture.

In the presence of ammonium ions, no increased droplet formation was observed (Fig. 5b). This suggests that ammonia and not ammonium induces droplet formation in *S. violaceoruber*. Droplet formation could either be induced specifically by ammonia or by the mere alkalinisation of the medium due to its basic properties. Experiments with buffered medium (data not shown) led to a changed growth morphology and unclear droplet formation. Hence, we tried to address the influence of the pH by an alkalinisation of the growth medium of *S. violaceoruber*, comparing the effect of ammonia with the effects of NaOH and KOH when added on different days. At early growth stages (3d), an alkalinisation of the growth medium by NaOH or KOH did not enhance droplet formation in a similar way to ammonia (Fig. S4b). Droplet formation was only induced by the addition of hydroxide bases on day 5 or 7 (Fig. S4c).

The alkaline character of ammonia is therefore probably not sufficient to induce droplet formation. Still, pH effects may depend on more subtle factors, such as the right timing of pH changes or a variable potential of *S. violaceoruber* to compensate for pH changes induced by the addition of different bases. Thus, although we clearly demonstrated that ammonia induces droplet formation on the aerial mycelium of *S. violaceoruber*, the exact mechanism by which ammonia exhibits this effect remains to be elucidated.

Since volatile ammonia induced droplet formation in four out of six *Streptomyces* strains tested (*S. vialoceoruber*, *S. coelicolor*, *S. griseus*, *S. avermitilis*; Fig. S6) it seems to act as a widespread trigger of droplet formation. However, other droplet-inducing compounds can be expected for *Streptomyces* strains that form droplets, but do not respond to ammonia with increased droplet formation, as for example *S. odorifer*. Continued work is needed to reveal the biological function of microbial ammonia release and to further understand how and why some *Streptomyces* strains form droplets on their aerial mycelium. With ammonia identified as a reliable trigger of droplet formation, such experiments now have become well amenable.

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Compliance with Ethical Standards

Conflict of Interest The authors declare no conflict of interest.

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