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Extrusion of extracellular membrane vesicles from hyphal tips of *Streptomyces venezuelae* coupled to cell wall stress

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Abstract

Extracellular vesicle release is a wide-spread and broadly important phenomenon in bacteria. However, not much is known about the mechanism of vesicle release in Gram-positive bacteria. Observations of polarly growing *Streptomyces venezuelae* by live cell time-lapse imaging reveal release of extracellular membrane vesicle from tips of vegetative hyphae. Vesicle extrusion is associated with spontaneous growth arrests, but often the apical cell survives and can re-initiate growth by forming new hyphal branches. Treatment with vancomycin to block peptidoglycan synthesis leads to a high frequency of lysis and vesicle extrusion, where some hyphae can survive growth arrest and vesicle extrusion and reinitiate growth after antibiotic is washed away. The extruded vesicles do not contain nucleoids and do not appear able to proliferate. Vesicle extrusion is not affected by the Ser/Thr protein kinase AfsK that phosphorylates the DivIVA at hyphal tips, nor is it affected by the intermediate filament-like protein FilP that localizes in gradient-like structures at hyphal tips. Notably, hyphae of a *scy* mutant, which has an unstable apical polarisome structure, are prone to spontaneous growth arrests and vesicle extrusion even in the absence of antibiotic treatment, supporting the idea that the nature of the growth zone at the hyphal tips is important for this route of extracellular vesicle formation. We speculate that the propensity for vesicle extrusion is a direct consequence of how polar growth is organized at hyphal tips in *Streptomyces*, with the cell wall sacculus being weak and susceptible to bursting at the apical zones of growth where peptidoglycan synthesis is primarily taking place.

Introduction

Apical growth is a defining feature of organisms that grow as hyphae and form mycelia. Both filamentous fungi and mycelial members of the Actinobacteria, like the genus *Streptomyces*, grow by building their cell walls primarily at the hyphal tips. In both cases, growth depends on systems for control of cell polarity and recruitment of the machinery for cell wall assembly to hyphal tips and branch points, in order to establish and maintain the apical growth zones [1, 2]. However, the underlying molecular mechanisms involved in apical growth have evolved independently in the two types of organisms. Fungal tip extension relies on polarization of the actin and tubulin cytoskeleton, motor protein-driven transport, and regulation of exocytosis at the tip [2]. Driving forces for tip extension in fungi include both turgor pressure and forces generated by the cytoskeleton and associated motor proteins [3]. The control of apical growth in streptomycetes and their relatives is centered around the orchestration of assembly of the peptidoglycan (PG) cell wall, as is typical for control of growth and cell shape in bacteria in general [4]. Thus, growth by tip extension requires recruitment of the machinery for PG assembly to the hyphal tip. Establishment of polarity and control of apical PG assembly in *Streptomyces coelicolor* is dependent on the coiled-coil protein DivIVA [5–7]. Turgor pressure is thought to be important for tip extension also in streptomycetes [8, 9], but the underlying details and the architecture of PG assembly at hyphal tips in streptomycetes remain poorly understood.

The cell polarity determinant DivIVA is essential for growth in *S. coelicolor* and is the main protein of the putative multi-protein complex called the polarisome (also referred to as tip-organizing center, TIPOC) that is found at hyphal tips and is critical for orchestration of hyphal tip extension and branching [5, 6, 10]. New hyphal branches arise by splitting of apical DivIVA-based polarisomes, leaving small daughter clusters behind that appear to seed formation of growth zones and emergence of new hyphal tips [11]. Another part of the

polarisome is the coiled-coil protein Scy, which co-localizes with DivIVA and is involved in scaffolding or stabilizing the polarisome [10, 12]. Deletion of *scy* has pleiotropic effects on hyphal morphology and branching [10], however in *Streptomyces venezuelae* it is not strictly essential for the establishment of seemingly functional (albeit unstable) polarisomes [12]. Immediately behind the polarisome, the protein FilP localizes in gradient-like patterns during active growth [12, 13]. In similarity to Scy, FilP is dispensable for growth, but affects hyphal growth patterns and morphology [13, 14], probably via effects on the shape and positioning of the DivIVA polarisome [12].

One pathway for regulation of polar growth, involving phosphorylation of DivIVA by the Ser/Thr protein kinase AfsK, has been revealed by studies in *S. coelicolor* [15]. DivIVA phosphorylation is detectable at a very low basal level during regular hyphal growth, and this is abolished by deletion of the kinase gene *afsK,* which leads to altered patterns of hyphal branching [15]. Importantly, the AfsK-mediated phosphorylation of DivIVA is strongly up-regulated if PG synthesis is blocked by the antibiotics bacitracin or vancomycin. Ectopic induction of the kinase AfsK, leading to high levels of DivIVA phosphorylation, similar to those observed under inhibition of PG synthesis, leads to growth arrest and re-organization of polar growth [15].

In the course of our studies of hyphal tip extension in the model organism *S. venezuelae*, we have discovered a novel form of release of extracellular membrane vesicles that is intimately associated with polar growth. Release of extracellular membrane vesicles of different types has been observed in both prokaryotes and eukaryotes and is an extensively investigated phenomenon [16, 17]. Vesicle release occurs in both Gram-negative and Gram-positive bacteria [18–20]. In Gram-negatives, vesicles are formed primarily by blebbing from the outer membrane, and such outer membrane vesicles have implications in for example pathogenesis, interactions with host organisms and other microbes, and stress tolerance [19]. Vesicle release

occurs also in a wide range of Gram-positive organisms, such as the clinically relevant actinobacterium *Mycobacterium tuberculosis* [18]. The mechanisms for biogenesis of membrane vesicles in Gram-positives are largely unknown, but are recently suggested to be associated with processes leading to cell death, for example through the action of phage-encoded endolysins or antibiotics targeting the cell wall [20–22].

Here we show that *S. venezuelae* is capable of vesicle release during vegetative growth, where vesicles are extruded at hyphal tips, and that, although this behavior is associated with growth arrests or inhibition of cell wall synthesis, the affected cell often survives and can resume growth again. Further, we argue that the observed vesicle extrusion is a consequence of how apical cell wall assembly and tip extension are organized in streptomycetes.

Methods

Bacterial strains, plasmids, oligonucleotides and growth media. Bacterial strains and plasmids used in this work are listed in Table S1. Oligonucleotide primers used are listed in Table S2. *Escherichia coli* strains were grown at 37°C in lysogeny broth (LB) or on LB agar [23] with or without 10 g L^{-1} of NaCl depending on use of hygromycin. *E. coli* DY380 strain was grown at 30°C. Kanamycin 50 μg ml^{-1}, ampicillin 100 μg ml^{-1}, apramycin 50 μg ml^{-1}, hygromycin 50 μg ml^{-1}, tetracycline 15 μg ml^{-1}, and nalidixic acid 25 μg ml^{-1} were used as appropriate. All *S. venezuelae* strains were grown, unless stated otherwise, at 30°C in maltose-yeast extract-malt extract medium (MYM) [24], made with 50% tap water, 50% Milli-Q water (Millipore) and supplemented with 2 ml L^{-1} trace element solution [25], or on MYM agar. Hygromycin 50 μg ml^{-1} was used as appropriate. All liquid cultures were shaken at 200 rpm during culturing. General DNA manipulation and cloning were done according to standard protocols.

Construction of *S. venezuelae* mutants. *S. venezuelae* deletion strains were constructed using the λRed-based 'Redirect' method, as previously described [26, 27]. In order to construct an *afsK::apr* deletion strain, *afsK* was replaced with an apramycin resistance cassette (amplified using primers KF1392 and KF1393 from pIJ773) on cosmid 3-E07 by λRed-mediated recombination in *E. coli* strain DY380 [28]. The resulting cosmid was conjugated into *S. venezuelae*, as described previously [25]. The *afsK::apr* region was verified with diagnostic PCR using primers KF1401 and KF1406.

Microscopy. Microscopy was done with a Zeiss Axio Imager.Z2 upright light microscope or a Zeiss Axio Observer.Z1 inverted light microscope, using a Plan-Apochromat 100x/1.4 Oil Ph3 objective or a Plan-Apochromat 63x/1.4 Oil Ph3 objective. Definite focus and a Zeiss TempModule S with a Heating Unit XL S were used in time-lapse imaging of growing mycelium. Images were captured with a Hamamatsu ORCA-Flash4.0LT sCMOS camera and either Zeiss ZEN 2.3 software or Volocity (Perkin Elmer). mCherry fluorescence was imaged with a Zeiss 63 HE filter set. sfGFP fluorescence was imaged with a Zeiss 38 HE filter set. FM4-64 was imaged with a Zeiss filter set 14. Hyphae were cultivated on 1% agarose in MYM medium in growth chambers, with or without FM4-64 addition (0.5 µg/ml). A growth chamber (3D-printed in PLA, Ultimaker 3 Extended) shaped as a microscope slide with a 13 mm hole in the middle was used, where a volume of 1% agarose in MYM was enclosed between a Lumox Biofoil 25 membrane (Greiner Bio-One) and a cover slip, as previously described [6]. This type of chamber was incubated in a box with moist tissue paper, until imaging, to prevent drying. For microscopy, sterile filtered (0.2 µm pore size) media were used, to avoid insoluble residues that could disturb the imaging. In the microfluidic experiments, cells were grown in MYM with standard conditions [12]. Upon vancomycin induction, 1 µg/ml vancomycin was flushed into the chamber (10 p.s.i. [69 kPa], 30 min), whereupon a 30 min, 10 p.s.i. washing was done with MYM. After this, standard growth

conditions were used. In the osmolarity downshift experiment, cells were cultured in MYM supplemented with 0.5 M NaCl until visibly growing and forming branching hyphae. To switch medium, the system was flushed with MYM for 30 min (10 p.s.i.), whereupon MYM supplemented with 0.5 M NaCl was used to equilibrate the chamber (10 p.s.i., 30 min), followed by culturing in MYM supplemented with 0.5 M NaCl under standard conditions. In the osmolarity upshift experiment, the inverse was done; cells were grown in MYM until visibly growing and forming branching hyphae, treated with MYM supplemented with 0.5 M NaCl for 30 min, upon which MYM was used to wash the chamber followed by culturing in MYM under standard conditions.

Analysis of vesicle formation. Hyphae grown in microfluidic perfusion chambers and subjected to vancomycin treatment or osmolytic upshift were monitored by phase-contrast microscopy and time-lapse imaging. It was determined what percentage of hyphal tips that were subjected to growth arrests, how many of them that showed turgor loss/lysis, how many were able to re-initiate growth after growth arrest, and how many extruded visible vesicles from the arrested hyphal tip.

Hyphal tips of wild type and apparently normal hyphae of the *scy* deletion mutant were investigated during standard growth a microfluidic perfusion system during 4 hours of early exponential growth. The number of total hyphae and the percentage of spontaneous hyphal growth arrests were counted.

Western blot. Total cell lysates were prepared from 12.5 ml culture samples of wild type and *ΔafsK* strains before and after a 30 min treatment with either 50 µg/ml bacitracin or 50 µg/ml vancomycin. The cells were grown until mid-exponential phase before treatment. The samples were harvested by centrifugation (2,800 g, 7 min, 4°C) and washed twice in ice-cold lysis buffer (25 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA) and then resuspended in 0.5

ml lysis buffer with cOmplete Mini protease inhibitor cocktail (Roche). Cells were then mixed with 0.5 ml 0.1 mm Zirconia beads (Biospec products) and bead-beated (Fast Prep 24, MP Biomedical) 5 times 30 sec, 6 m s^{-1}, with 5 minutes on ice between beats. The lysate was then clarified (20,800 g, 30 min, 4°C) and the supernatant was collected. The supernatant was used to determine protein concentrations, using the DC protein assay (Bio-Rad Laboratories) according to the manufacturer's instructions. Volumes of soluble fraction equal to 10 µg of protein was boiled in SDS-PAGE loading buffer for 5 minutes and loaded onto a Mini Protean TGS stain-free gel (BioRad laboratories) and run according to the manufacturer's instructions. The protein was transferred to an Immobilon-P membrane (Merck Millipore) as described by the manufacturer and blocked with "blotto" (5% skimmed milk powder in PBS). Primary antibody incubations were done overnight in blotto with 1:5,000 anti-DivIVA polyclonal rabbit antiserum [29]. A 1:1,000 dilution of secondary horseradish peroxidase-conjugated anti-rabbit swine antibodies (Dako) were used for detection with ECL reagents (Thermo scientific). Phosphorylation of DivIVA was detected as a mobility shift of DivIVA, as described previously [15].

Results

Extrusion of vesicles from hyphal tips upon growth arrest

When monitoring hyphal growth of *S. venezuelae* by time-lapse imaging in a microfluidic cell perfusion system, we have noticed that hyphae occasionally and apparently spontaneously arrest tip extension. These rare growth arrests are typically followed by reprogramming of polar growth, leading to re-initiation of growth in the form of new branches from old tips or lateral walls [12]. By careful observation of the arresting hyphae by phase-contrast microscopy we found an unexpected phenomenon associated with the growth arrests that will be described in this paper: the stalled tips frequently extrude vesicular material when they

stop extending, but apical cells of arrested hyphae survive and are able to initiate outgrowth of new branches (Fig. 1). Although these spontaneous growth arrests (and connected vesicle extrusions) events are rare, we have observed a number of cases over the course of several time-lapse experiments in microfluidic perfusion chambers (see examples in Supplementary Movie 1). The extruded vesicle-like material is heterogeneous in size and shape and most often devoid of strong phase-contrast signal, indicating that the contents do not deviate substantially in refractive index from the surrounding medium and may therefore not contain dense cytoplasmic material. Due to the difficulty in visualizing these expulsions in phase-contrast microscopy and the rarity of growth arrests in most strains, this phenomenon had gone unnoticed when investigating other strains for previous work [12].

Vesicle extrusion is not induced by osmotic stress

Since the release of vesicles appear reminiscent of the recently reported formation of cell wall-deficient S-cells in response to hyper-osmotic stress in the filamentous actinomycete *Kitasatospora viridifaciens* [30], we investigated whether hyper- and hyposomotic stress would induce extrusion of vesicles from hyphal tips in *S. venezuelae*. To determine whether shifts in osmolarity would lead to vesicle extrusion, cells were cultured in microfluidic perfusion chambers and subjected to drastic changes in osmolarity.

When hyphae that were growing in MYM were shifted to MYM supplemented with 0.5 M NaCl, they showed a near immediate cessation of growth (Fig. 2; Supplementary Movie 2). Curiously, very few vesicle extrusions were coupled to these growth arrests (1.4% of surviving hyphal tips expelled vesicles, Table 1), suggesting that the vesicle release that we observe in *S. venezuelae* is not stimulated by hyperosmotic stress. It is unlikely that the increased osmolarity of the medium prohibits imaging of the released vesicles, as a low number of vesicles could still be readily observed. 85.5% of the hyphal tip compartments survived this treatment (Table 1), as indicated by their ability to regrow when the high

osmolarity stress was removed after 30 min and cells were shifted back to regular MYM medium (Fig. 2, Supplementary Movie 2). As previously seen in *S. coelicolor* [31], the shift to high osmolarity medium led to reprogramming of polar growth and cells regrew from new branches (Fig. 2, Supplementary Movie 2).

When the opposite experiment was attempted, with hyphae growing in high osmolarity medium being shifted to standard growth medium, there was a drastic difference in behavior. When grown in high osmolarity medium (MYM supplemented with 0.5 M NaCl), the hyphae appear morphologically distinct, being hyperbranching and irregularly shaped, as well as having lower rate of tip extension, compared to hyphae grown without the NaCl supplementation (Fig. 2, Supplementary Movie 3). When shifting to low osmolarity medium (MYM), the hyphae did not stop growing and no growth arrests or reprogramming of polar growth could be seen. As a result, no vesicle extrusions were observed. When the cells were returned to high osmolarity medium after 30 minutes, the cells behaved similarly to the upshift experiment, with near immediate cessation of extension and reprogramming of polar growth (Fig. 2, Supplementary Movie 3). The 30 min treatment with normal growth medium was enough for the cells to lose the adaptation to the high osmolarity medium and thus an extended growth arrest (approximately 3 hours) was observed before turgor was restored and hyphae regrew. In summary, hyperosmotic stress leads to growth arrests and reprogramming of polar growth but does not stimulate vesicle extrusion form hyphal tips.

Vancomycin treatment leads to vesicle extrusion

Hyphal tips are the main sites for assembly of the cell wall in vegetatively growing *Streptomyces* hyphae [5, 32]. In order to test whether the extrusion of vesicles at hyphal tips could be connected to disturbances of cell wall assembly, we exposed hyphae growing in microfluidic chambers to the peptidoglycan synthesis inhibitor vancomycin. Flushing vancomycin into the system led to immediate growth arrest of most hyphae. 87.2% of the

arrested hyphal tip compartments did not survive the treatment and eventually lost turgor (indicated by sudden shrinkage of hyphal length) and/or lysed. However, 12.8% of the arrested hyphal tip compartments retained viability, as confirmed by their ability to re-initiate growth when vancomycin was washed away. Despite showing growth arrest during the vancomycin treatment, these hyphae retained turgor pressure and could regrow from newly formed branches, similar to what was observed during spontaneous growth arrests. Out of these surviving hyphal tip compartments, 66.7% showed visible extrusion of vesicular material at the tip (Table 1, Fig. 3A, and Supplementary Movie 4). The results indicate that vesicle extrusion is triggered by problems related to cell wall synthesis, and that many hyphae that extrude substantial material in the form of vesicles upon growth arrests are able to avoid lysis, survive, and initiate regrowth when the stress is removed.

We have previously shown that inhibition of peptidoglycan synthesis by bacitracin or vancomycin triggers phosphorylation of the key protein in the polarisome, DivIVA, mediated by the Ser/Thr protein kinase AfsK in *S. coelicolor* [15]. It was therefore of interest to investigate whether *afsK* affects the vesicle extrusion response and/or ability of hyphae to survive vancomycin treatment. For this reason, we have generated an *afsK* mutant of *S. venezuelae* and demonstrated that *S. venezuelae* shows AfsK-mediated phosphorylation of DivIVA in response to vancomycin and bacitracin treatment (Supplementary Fig. 1), similar to what was reported for *S. coelicolor* [15]. However, in the microfluidic system, the *afsK* mutant is similarly affected as the wild-type parent by vancomycin exposure, showing similar rate of survival (18.2 %) and similar ability to extrude vesicle-like structures upon vancomycin-induced growth arrests (79.2 % of surviving tips showed vesicle expulsions, Table 1, Fig. 3B, Supplementary Movie 5). Further, the *afsK* mutant shows similar infrequent spontaneous growth arrests with vesicle extrusion as the wild-type parent does

(Supplementary Fig. 2). Thus, we do not detect any role of *afsK* in the vesicle extrusion response.

Vesicle extrusion and polarisome remodeling

Next, we investigated how the DivIVA-based polarisome is affected during extrusion of vesicles. A *divIVA*$^{+}$*/divIVA-mCherry* strain behaves like the wild type when treated with vancomycin, extruding vesicle-like structures (Supplementary Figure 3; Supplementary Movie 6). Importantly, the polarisome is typically not expelled together with the vesicles, but rather it is remodeled to form new foci that eventually give rise to new lateral branches, in agreement with what has been described previously for hyperosmotically stressed *S. coelicolor* or spontaneously arrested hyphae of *S. venezuelae* [12, 31].

Effect of polarisome proteins on vesicle extrusions

Next, we investigated the effects of polarisome-associated proteins FilP and Scy on vesicle extrusions. FilP localizes immediately behind the polarisome in gradient-like structures that are highly dynamic and are rapidly remodeled upon growth arrests [12]. FilP also affects the size and position of the DivIVA clusters at hyphal tips and influences hyphal shape [12]. In order to investigate whether *filP* affects the vesicle extrusion response, a *filP* mutant was investigated by live cell imaging. In similarity to the wild-type parent, the *filP* mutant showed very rare spontaneous growth arrests with vesicle extrusions. When treated with vancomycin, the *filP* mutant behaved similarly to the wild type, with 15.7% of the hyphal tip compartments surviving the treatment, and 67.3% of those showing visible extrusion of vesicle-like structures (Table 1; Supplementary Movie 7).

Scy is a coiled-coil protein that co-localizes with DivIVA at hyphal tips and is important for stability of the polarisomes [10, 12]. A *scy* mutant shows frequent splitting of polarisomes, leading to highly branched and dense mycelial structures. However, occasional hyphae are

apparently able to establish a stable polarisome even in the absence of *scy* and grow with normal extension rate and branching pattern [12]. When cultivating the *scy* deletion mutant in microfluidic perfusion chambers, these apparently normal hyphae that protrude from the otherwise dense hyperbranched mycelium are relatively easy to follow and it quickly becomes clear that they are prone to spontaneous growth arrests. The frequent stalling of hyphal extension in the *scy* mutant was typically accompanied by extrusion of vesicle-like structures from the hyphal tips (Fig. 4, Supplementary Movie 8). When following the *scy* mutant for a 4 hour interval during early exponential phase, 7% (n = 71) of the normally extending *scy* mutant hyphae arrested growth, retained turgor, and showed vesicle release and reprogramming of polar growth, compared to 0.3% (n = 337) for the wild type. Only a minor fraction of spontaneously arrested *scy* mutant hyphae showed loss of turgor or lysis during these conditions. In summary, even the apparently normal hyphae of the *scy* mutant show frequent spontaneous growth arrests associated with vesicle extrusion, suggesting that Scy is important for maintaining a stable extending tip and preventing vesicle extrusion.

Vesicle-like expulsions are membranous

In order to confirm that the extruded material is enclosed by membranes, we used membrane dye FM4-64 to stain the material. This was not possible in the CellASIC microfluidic perfusion chambers due to very high background, presumably from FM4-64 interacting with the chambers. However, MYM agarose growth chambers with FM4-64 added to them can be used to visualize membranes during live cell time-lapse imaging (Fig. 5). Due to the scarcity of spontaneous growth arrests in the wild-type strain and the technical limitations in number of imaged cells in each growth chamber compared to the microfluidic chambers, the *scy* deletion strain was used to increase the likelihood of imaging such events with FM4-64 staining. During growth, the extending hyphae are stained with membrane stain outlining the cell and the occasional septa (Fig. 5, Supplementary Movie 9). We did not image any strong

membrane stain in the tips of growing hyphae, such as those described previously [33]. When growth arrest and remodeling of growth occurred, this was typically accompanied by a sudden increase in extracellular FM4-64 signal at the hyphal tips (Fig. 5, Supplementary Movie 9). This confirms that that the material extruded from the hyphal tips is enclosed by membranes.

Nucleoids are not extruded in vesicles upon vancomycin treatment

It has recently been reported that *K. viridifaciens* and *S. venezuelae* can form cell wall-less S-cells [30]. For *K. viridifaciens*, data were presented indicating strikingly similar apical expulsion of vesicle-like structures that we describe here for *S. venezuelae.* In order to investigate if the expulsions we see here could represent S-cells, we asked whether the vesicles may contain DNA and for this reason visualized nucleoids in live hyphae using a fluorescent derivate of the nucleoid-associated HU protein HupA [34]. The distribution of HupA-sfGFP in growing hyphae is fully consistent with nucleoid-association (Fig. 6). When treating the HupA-sfGFP strain with vancomycin, vesicle-like structures were extruded but do not appear to contain any HupA-sfGFP (Fig. 6; Supplementary Movie 10; no signal observed in any of the vancomycin-induced expulsions, n = 18), suggesting that they are devoid of nucleoids, and therefore that the majority of the extruded vesicles do not represent viable cells. Even in cases where extruded material appeared dark in the phase-contrast microscope, it did not contain any fluorescence signal indicating nucleoid material.

Discussion

The ability of bacteria to release extracellular membrane vesicles attracts great interest [16, 18, 19], but the mechanisms of vesicle biogenesis are often unclear. This is particularly true for Gram-positives, in which vesicles would have to traverse the thick cell wall in order to be secreted. Alternatively, and more well-documented, formation and release of vesicles may be associated with processes leading to loss of cell integrity and cell death [20]. Vesicle

formation have for example been reported as result of phage or prophage-encoded endolysin activity, autolysins, or cell wall-targeting antibiotics in Gram-positive bacteria [20, 21, 22]. In this paper, we demonstrate another route for vesicle extrusion that occurs at the sites of cell wall assembly at cell poles in apically growing Gram-positives, and that is correlated with cell survival, even though it is stimulated by agents that block PG synthesis. We also identify a genetic determinant, the *scy* gene, which affects the frequency of spontaneous vesicle extrusion by this route.

The propensity to extrude membrane vesicles is likely an inherent feature or consequence of the way *Streptomyces* hyphae grow by tip extension. In analogy to hyphae of filamentous fungi, cell wall synthesis occurs in *Streptomyces* primarily in a restricted zone at the hyphal tip [35, 36]. The architecture of this mode of PG assembly remains poorly understood, and it is for example not known how glycan strands are oriented and how the transglycosylases, transpeptidases, and other proteins involved in PG assembly are positioned at the tip. However, the PG sacculus at the very tip is likely to be susceptible to damage. Models of tip extension also assumes a high degree of flexibility of the cell wall at the apex of hyphae, while the lateral wall is thought to be strengthened and rigidified by further cross-linking just below the tip [8, 9, 35]. In this view, it is not surprising if the wall at the tip is susceptible to breakage and extrusion of membrane blebs, particularly when there is disturbance or imbalance in PG synthesis. We find that osmotic shock alone does not lead to excessive vesicle release. However, cell wall damage or polarisome damage, combined with the turgor pressure of the cell, is likely causing the observed vesicle release phenomenon.

Even though the apical cell often survives substantial vesicle extrusion and blebbing from the tip, as shown by the ability to re-initiate polar growth after growth arrest and vesicle release, the re-initiation of growth is via establishment of new branches. Even when growth restarts at the old tip, it appears to be a branch emerging from the old pole rather than just continuation

of growth from the same growth zone as in the arrested tip. Thus, the tips that extrude vesicles have not only arrested tip extension, but it also seems like their polarisomes and systems for PG assembly are disabled. The polarisomes must be reassembled before regrowth, either close to the original position, leading to outgrowth form the old tip, or at other sites, leading to one or several new branches. Similar re-initiation of growth by formation of new branches after growth arrest at the original tip have been observed previously [12, 15, 31]. The regulatory systems directing disassembly of old polarisomes and re-establishment of cell polarity and growth elsewhere are not understood, but the AfsK-mediated phosphorylation of DivIVA and other proteins is likely part of such regulation [1, 15]. It is also conceivable that the expulsion of material from the old and damaged tip may help to purify the system by getting rid of toxic intermediates or non-functional protein assemblies.

It should be noted that mycobacteria and corynebacteria use a similar mode of polar growth as streptomycetes, directed by clusters of DivIVA [37–39]. Therefore, the route for vesicle release that we describe here may be valid and important also for biogenesis of the extracellular vesicles that have been observed in for example *Mycobacterium tuberculosis* [18, 40]. Mycobacteria have a complex and multilayered cell envelope. Although vesicles have been observed adhering to cells at many positions along the cell surface, the cell poles where the envelope is assembled appear to be the most feasible places where they could be extruded, perhaps similarly to what we describe here.

Extracellular vesicles have previously been reported in *Streptomyces* spp. in conjunction with specialized metabolites. Droplets of exudates on the surface of sporulating lawns of *S. coelicolor* and *Streptomyces lividans* contain the antibiotics actinorhodin and undecylprodigiosine, respectively, and were shown to be enriched in membrane vesicles [41, 42]. Further, production of lipid-like polyene polyketides linearmycins by *Streptomyces* sp. Mg1 is associated with generation of extracellular membrane vesicles that facilitate the

383 diffusibility of the linearmycins [43]. It is difficult to decipher how vesicles that are observed
384 at late growth stages are formed. As discussed, they could be generated by cell death and lytic
385 processes in aging *Streptomyces* colonies [43, 44], but it is also possible that extrusion of
386 vesicles at growth zones at hyphal tips and new branch sites may contribute [42]. Without
387 direct observation of vesicle release, it is difficult to determine how they are formed.

388 What could the function be, if there is any, of the extrusion of membrane vesicles that we
389 observe at stalled hyphal tips in this study? Formation of cell-wall deficient S-cells has been
390 reported in *K. viridifaciens* and also in several streptomycetes [30]. However, three
391 observations suggest that the apical vesicle extrusion in *S. venezuelae* is not part of this
392 phenomenon: (*i*) The vesicle extrusion is not stimulated by hyperosmotic stress, like S-cell
393 formation in *K. viridifaciens*, (*ii*) we did not observe any nucleoids being extruded in vesicles,
394 making it impossible for the vesicles to exist as S-cells, and (*iii*) the extruded structures do not
395 appear to grow on their own. It cannot be excluded that occasional vesicles may contain
396 DNA, but we do not think this is the main function of the vesicle extrusion reported here.

397 As discussed above, vesicle extrusion may contribute to the dismantling of the growth
398 apparatus at the hyphal tip upon growth arrest, or even detoxification by getting rid of toxic
399 intermediates or antibiotic-poisoned enzymes. It is also possible that vesicle extrusion may
400 simply act to get rid of excess membranes, resulting from a decoupling of membrane and cell
401 wall synthesis at growth arrest. Extensive internal membranous structures at hyphal tips and
402 elsewhere, referred to as cross-membranes, have been observed in *Streptomyces* hyphae [33,
403 45]. However, another report failed to observe these structures in growing hyphae and showed
404 that they appear in non-growing cells or hyphae that have suddenly shrunk, presumably due to
405 loss of turgor or a lytic event [46]. Similarly, we fail to see any evidence of cross-membrane-
406 like structures in growing FM4-64-stained cells. Possibly, the formation of such internal
407 membrane invaginations reflect that excessive membrane material form upon growth arrest.

Also in outer membrane vesiculation in Gram-negatives, an unbalanced biosynthesis of cell envelope components may lead to membrane blebbing [20]. Overall, an unbalanced biosynthesis of cell wall and membranes, and a weak cell wall at the hyphal apex giving way to the turgor of the cytoplasm may explain the observations made here, but mechanistic details are still needed to elucidate the mechanism of vesicle release.

Author statements

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Author contributions. MJF and KF conceived and designed the study, MJF acquired and analyzed the experimental data, and MJF and KF wrote the manuscript.

Conflicts of interest. The authors declare that there are no conflicts of interest.

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561 **Tables and figure legends**

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564 **Table 1.** Vesicle release and survival in hyphae of wild type, *ΔfilP* (NA1225) and *ΔafsK*

565 (LUV029) strains of *S. venezuelae* during vancomycin treatment or osmotic upshift.

Strain and treatment	Hyphae that stop growing (n)[1]	Lysis or loss of turgor (%)[2]	Survivors (%)[3]	Fraction of survivors that showed vesicle extrusion (% of survivors)
Wild type				
Vancomycin	375	87.2	12.8	66.7
Osmotic upshift	172	14.5	85.5	1.4
ΔafsK				
Vancomycin	132	81.8	18.2	79.2
ΔfilP				
Vancomycin	313	84.3	15.7	67.3

566 [1] Number of analyzed hyphae that showed growth arrest in response to the treatment.

567 [2] Fraction of counted hyphae that either lysed or showed visible loss of turgor (sudden
568 shrinkage of hyphal length). None of these hyphae showed any re-initiation of growth from
569 the apical compartment, which appeared to have lost viability.

570 [3] Fraction of counted hyphae that were able to re-initiate growth from the apical compartment.
571 None of these hyphae showed signs of sudden loss of turgor.

Figure 1: Spontaneous growth arrests in wild-type *Streptomyces venezuelae* are coupled to extrusion of vesicle-like structures.

Montage of images from time-lapse movies of wild-type hyphae undergoing growth arrest and vesicle release. Cells re-establish growth from a new polar growth center. Vesicles indicated by arrowheads. Images assembled from Supplementary Movie 1. Relative times in minutes shown. Scale bar, 5 μm.

Figure 2: Effect of osmotic shock on growth of hyphae of *Streptomyces venezuelae*.

Montages of images from time-lapse movies of hyphae subjected to osmotic upshift (A) or downshift (B), assembled from Supplementary Movies 2 and 3, respectively. In panel A, the osmotic upshift from MYM medium to MYM with 0.5% NaCl occurs at approximately minute 12, whereupon growth ceases and the hyphae shrink due to loss of turgor (indicated by arrowheads). Turgor is rapidly recovered after re-introducing normal growth medium (approximately at minute 42) and new branches appear roughly 30 min after removal of the high osmolyte medium (approximately at minute 72). In panel B, the downshift from MYM with 0.5% NaCl medium to regular MYM occurs at approximately minute 11, whereupon growth continues without apparent hindrance until the high osmolyte medium is returned at approximately minute 41, shortly upon which growth is arrested and turgor is lost. Turgor is slowly regained and growth resumes after a prolonged growth arrest from new lateral branches. The cells growing in high osmotic medium display an aberrant and irregular morphology. Scale bars, 5 μm.

Figure 3: Vancomycin causes growth arrests and vesicle extrusions in wild-type and Δ*afsK* strains of *Streptomyces venezuelae*.

S. venezuelae wild type (top panel) and Δ*afsK* (bottom panel) were cultured in a microfluidic perfusion system, whereupon vancomycin (1 μg mL^{-1}) was added to the chamber for 30 min,

and then washed away. Upon vancomycin addition, hyphal extension is arrested and many cells lose turgor or lyse (arrow). However, some cells can maintain turgor and resume growth from new growth centers upon removal of vancomycin. This survival is often coupled to the expulsion of vesicle-like structures from the hyphal tip compartments (arrowheads). Scale bar, 5 μm.

Figure 4: A *scy* deletion mutant of *Streptomyces venezuelae* is prone to growth arrest and extrusion of vesicle-like structures from the hyphal tip.

Montage of time-lapse images showing a representative example of growth arrest and vesicle release in *S. venezuelae Δscy* strain NA1255 grown microfluidic perfusion chamber. Black arrowheads indicate vesicle-like structure released from the hyphal tip upon growth arrest, which is followed by reprogramming of polar growth and establishing a new branch (white arrowhead). Scale bar, 5 μm.

Figure 5: Extrusions released from a *scy* deletion mutant of *Streptomyces venezuelae* are membranous.

Montage of time-lapse images showing a representative growth arrest and vesicle release in *S. venezuelae Δscy* strain NA1255 culture in an agarose growth chamber in the presence of FM4-64 membrane staining dye. Images extracted from Supplementary Movie 9. Fluorescence channel shown in inverted grayscale. Arrowhead indicates release of FM4-64 staining material from hyphal tip, accompanied by growth arrest and later re-initiation of new branch from the old tip. Inset at the 40 min panel is a section of the corresponding phase contrast image, with an arrow indicating the newly formed branch. Scale bar, 5 μm.

Figure 6: Extruded vesicle-like structures do not contain HupA-sfGFP.

A *S. venezuelae hupA*$^{+}$*/hupA-sfGFP* strain was cultured in a microfluidic perfusion system, whereupon vancomycin was added to the chamber. Upon vancomycin addition (minute 0),

growth arrests and some hyphal tip compartments manage to survive and are able to regrow from new growth centers once vancomycin has been removed (minute 30, arrowhead). Coupled to this survival is the expulsion of vesicle-like structures from the hyphal tips. The expulsions are heterogeneous in phase contrast signal. No expulsions appear to contain the DNA-associated HupA-sfGFP protein. Phase-contrast channel is shown on the left and fluorescence channel shown on the right. Times are relative time after addition of vancomycin. Scale bar, 5 μm.

Figures and tables

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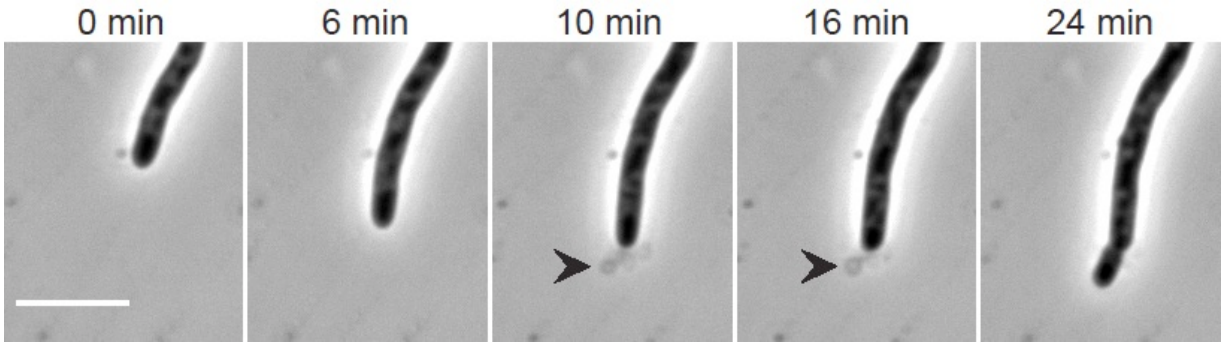

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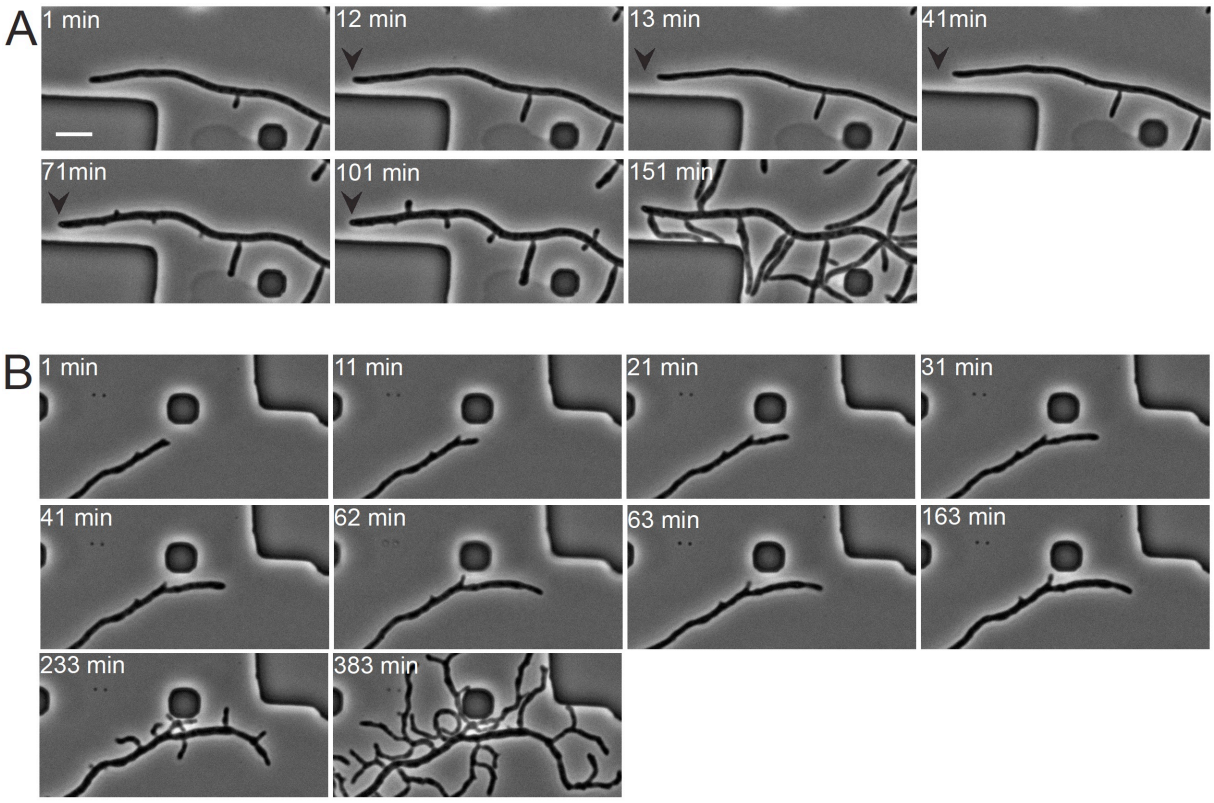

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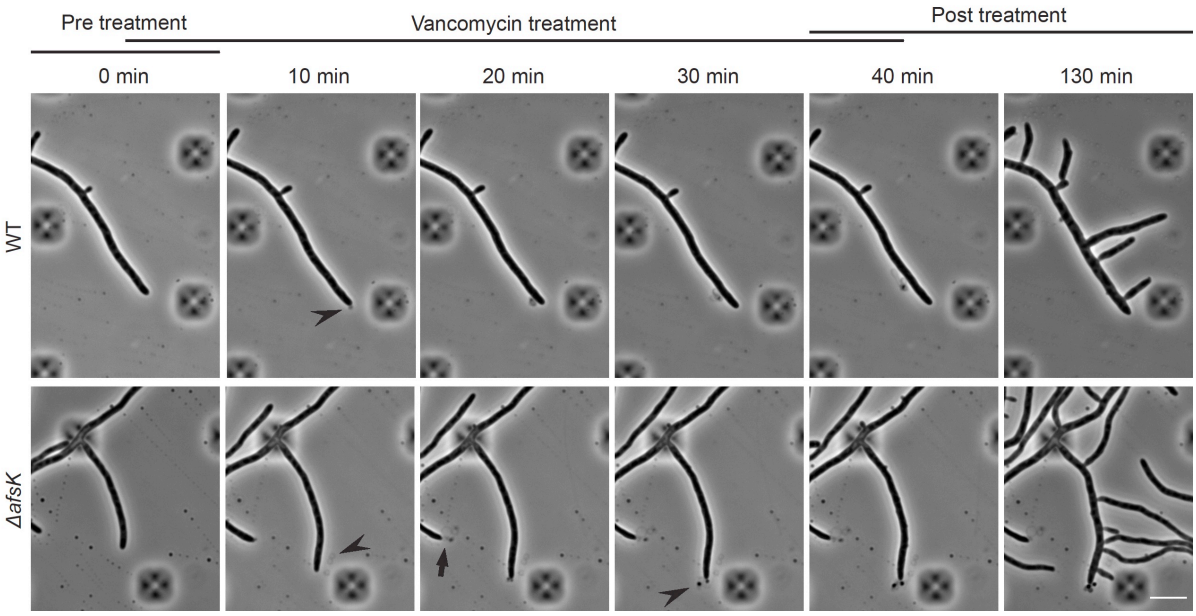

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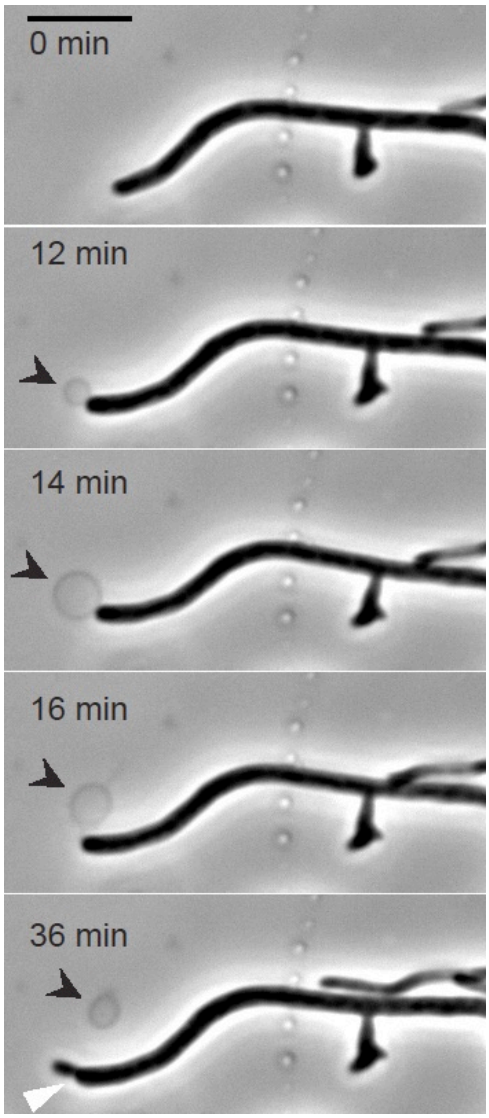

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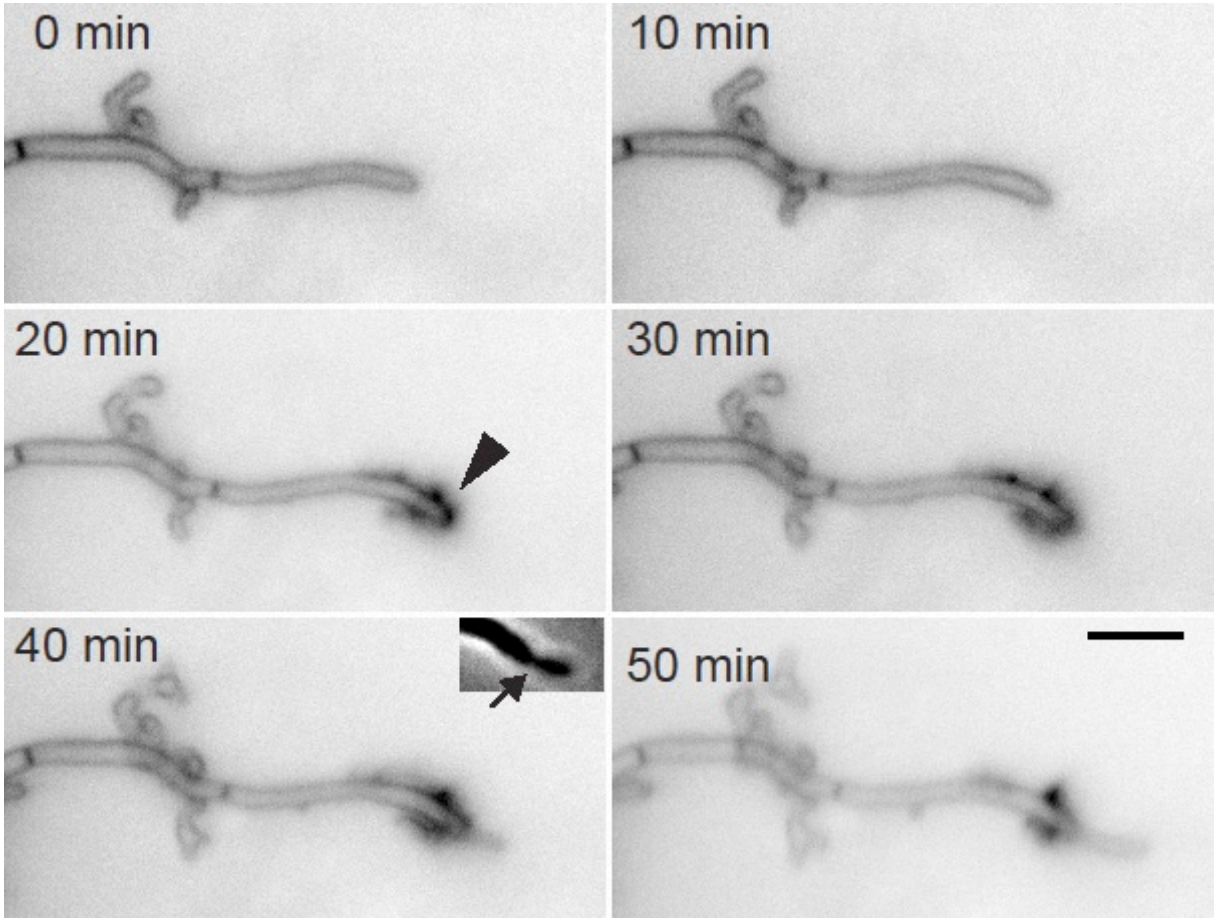

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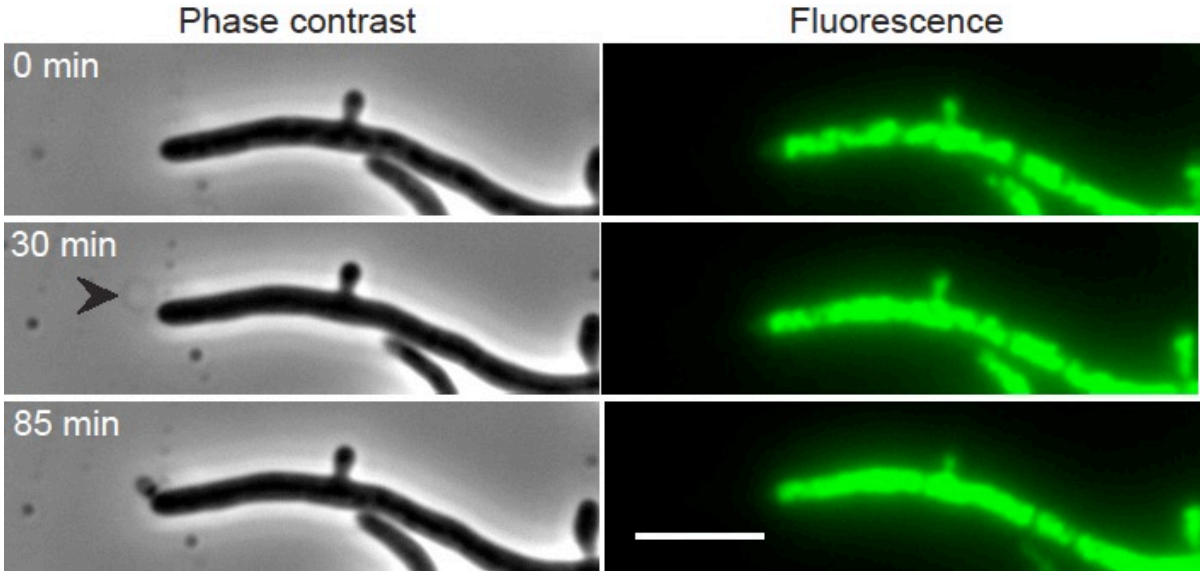

Figure 6: Extruded vesicle-like structures do not contain HupA-sfGFP.

S. venezuelae hupA^{+}*/hupA-sfGFP* was cultured in a microfluidic perfusion system, whereupon vancomycin was added to the chamber. Upon vancomycin addition (minute 0), growth arrests and some hyphal tip compartments manage to survive and are able to regrow from new growth centers once vancomycin has been removed (minute 30, arrowhead). Coupled to this survival is the expulsion of vesicle-like structures from the hyphal tips. The expulsions are heterogeneous in phase contrast signal. No expulsions appear to contain the DNA-associated HupA-sfGFP protein. Phase-contrast channel is shown on the left and fluorescent channel shown on the right. Times are relative time after addition of vancomycin. Scale bar, 5 µm.

Supplementary materials

Extrusion of extracellular membrane vesicles from hyphal tips of *Streptomyces venezuelae* coupled to cell wall stress

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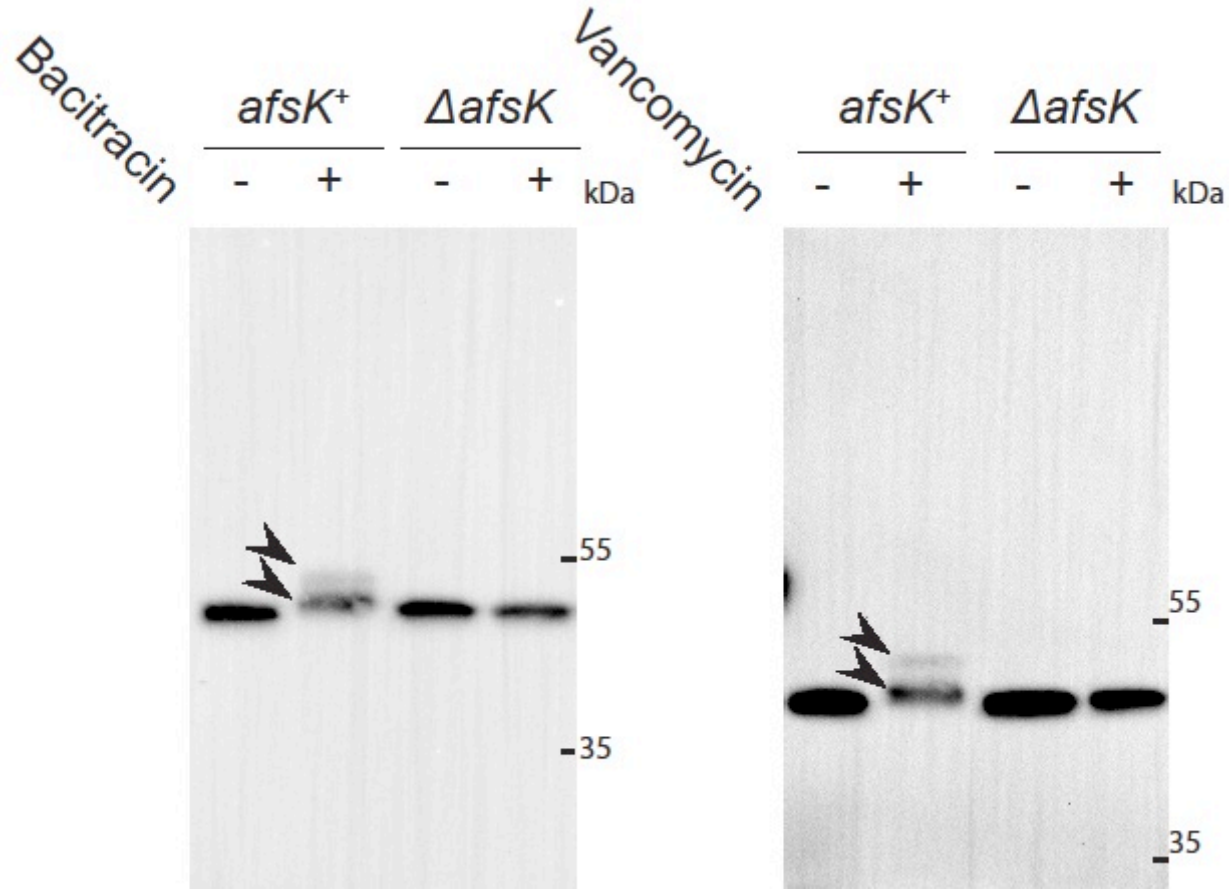
Keywords: hyphae, *Streptomyces*, cell wall, peptidoglycan, cell membrane, cell polarity

Supplementary table 1. Strains and plasmids used in this work.

Strain or plasmid	Genotype and/or description	Source or reference
Strains		
S. venezuelae		
NRRL B-65442	Wild-type *S. venezuelae* strain	[1]
NA1225	Δ*filP::frt*	[2]
NA1255	Δ*scy::frt*	[2]
NA1279	$attB_{\varphi BT1}$::pSS204 (contains *divIVA-mCherry*)	[2]
LUV029	Δ*afsK::apr*	This work
E. coli		
DH5α	Cloning strain	[3]
DY380	DH10B λcl857 *Δcro-bio < > tet*	[4]
ET12567/pUZ8002	*dam-13*::Tn*9 dcm-6 hsdM*, carries RK2 derivative with defective *oriT,* for plasmid mobilization	[5]
Plasmids		
3-E07	Cosmid containing *afsK*	
pSS48	pIJ10770-*hupA-sfGFP*	Susan Schlimpert, JIC, Norwich, UK

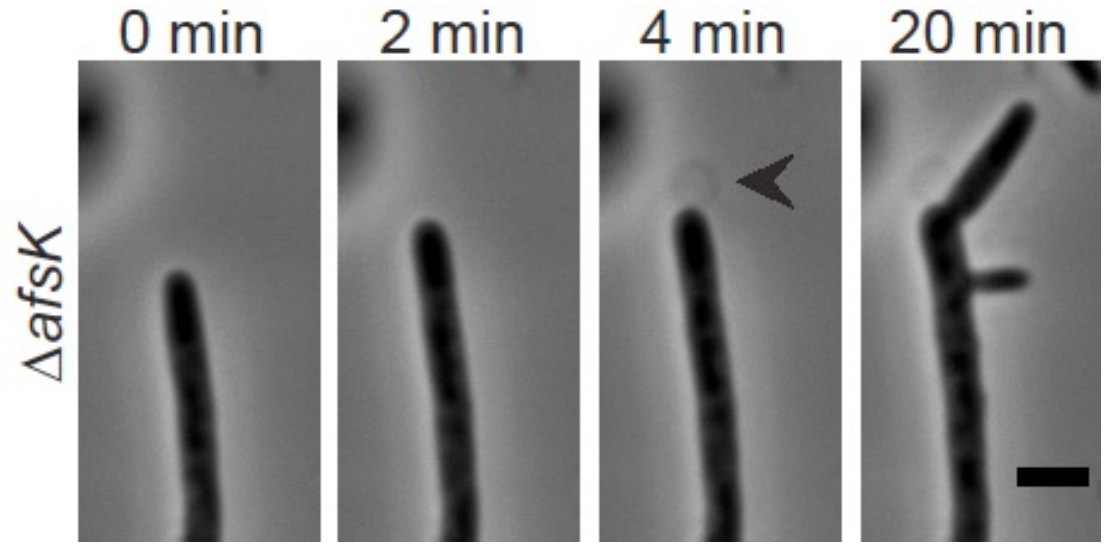
Supplementary table 2. Oligonucleotide primers used in this work.

Oligonucleotide	Sequence
KF1392	CGCGCGGCGAGGGGTGAGGCATCCGTTCCGGATGGCTCCGGTATGA
	GGTATTCCGGGGATCCGTCGACC
KF1393	GAACCCGCCGCCCAGGAGCTAGGGCAGCGGGTCCTCGGCAGGAGAA
	GCATGTAGGCTGGAGCTGCTTC
KF1401	CGAGACCCAGACGTACGAC
KF1406	ACCATTCTTCAGGATGGCAAG



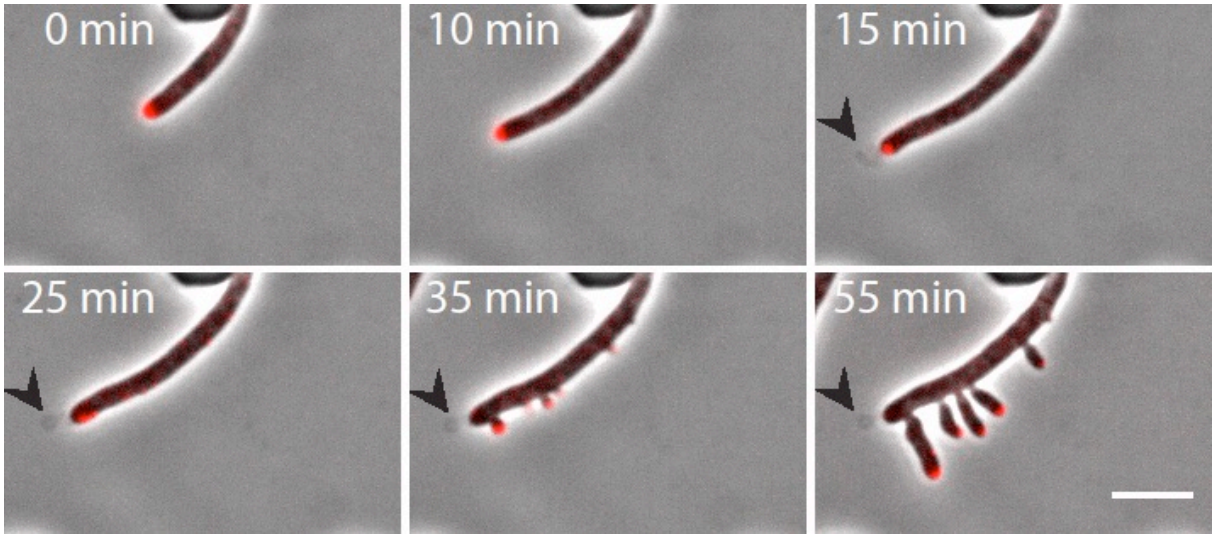
Supplementary figure 1: Addition of bacitracin or vancomycin causes *afsK*-dependent phosphorylation of DivIVA in *Streptomyces venezuelae*

Western blot visualizing the effect of bacitracin and vancomycin on the phosphorylation state of DivIVA. Phosphorylation affects the mobility of DivIVA, as demonstrated previously [6]. Shifted bands are indicated by arrowheads. Consistent with observations in *S. coelicolor*, more than one mobility-shifted species is observed, presumably due to different degree of phosphorylation of several possible Ser and Thr residues [7]. 10 µg total protein from soluble fractions of lysates of cells treated with (+) or without (-) bacitracin or vancomycin. Wild type (*afsK*$^{+}$) and *ΔafsK* (LUV029) strains were used.



Supplementary figure 2: Extrusion of vesicles is not affcted by *afsK*.

Montage of time-lapse images showing an example of a spontaneous growth arrest in an *S. venezuelae* Δ*afsK* mutant strain (LUV029). Extrusion of vesicles (arrowhead) occurs shortly after growth arrest. Relative times in minutes shown. Scale bar, 2 μm.



Supplementary figure 3: DivIVA-mCherry is remodeled as a result on growth arrests coupled to vesicle extrusions.

Spontaneous growth arrest in a *S. venezuelae divIVA*$^{+}$*/divIVA-mCherry* strain NA1279 lead to reprogramming of polar growth coupled to extrusion of vesicle from the hyphal tip (arrowheads). Relative time in minutes. Cells cultured in microfluidic perfusion chambers. Scale bar, 5 μm.

Supplementary movie 1: Vesicle release from hyphal tips associated with spontaneous growth arrests in wild-type *Streptomyces venezuelae.*

Examples of vesicle release events occurring when tip extension is spontaneously arrested in individual hyphae of *S. venezuelae*. Time in hours::minutes. Cells cultured in microfluidic perfusion chambers.

Supplementary movie 2: Subjecting *Streptomyces venezuelae* to an osmotic upshift causes growth arrests but not vesicle extrusion.

Wild type *S. venezuelae* cultured in MYM in a microfluidic perfusion system were subjected to a 30 min treatment with MYM supplemented with 0.5 M NaCl, followed by a return to the normal medium. During the osmotic shift (approximately at time point labeled 10 min) the cells undergo drastic changes in turgor and growth is arrested. However, these growth arrests do not lead to excessive vesicle extrusion, nor do the hyphae permanently lose turgor or lyse. Upon return to normal growth medium, the cells quickly regain turgor and resume growth from reprogrammed polar growth centers. Relative time in minutes shown.

Supplementary movie 3: Subjecting *Streptomyces venezuelae* to an osmotic downshift does not cause growth arrests.

Wild type *S. venezuelae* cultured in MYM supplemented with 0.5 M NaCl in a microfluidic perfusion system were subjected to a 30 min treatment with standard MYM, followed by a return to the high osmolyte medium. During the osmotic downshift (approximately at time point labeled 12 min) the cells do not appear to arrest growth or reprogram polarity. However, the cells do arrest growth and have drastic changes in turgor after returning to the high osmolyte medium, apparently having lost adaptation to the high osmotic medium during the downshift. Relative time in minutes shown.

Supplementary movie 4: Subjecting *Streptomyces venezuelae* to vancomycin leads to growth arrests and vesicle extrusions.

Wild type *S. venezuelae* cultured in MYM in a microfluidic perfusion system were subjected to a 30 min treatment with MYM supplemented with 1 μg ml^{-1} vancomycin, followed by a return to MYM. Immediately after vancomycin has been added to the microfluidic chamber (approximately at time point labeled 50 min) cell growth is arrested and a majority of hyphal tip compartments lose turgor and/or lyse. However, a subpopulation survives the treatment, retaining turgor, and can regrow from reprogrammed polarity centers upon removal of the vancomycin. Most of the surviving hyphal tip compartments extrude vesicles, examples of which are shown here. Relative time in minutes shown.

Supplementary movie 5: *afsK* does not affect the ability of hyphae to extrude vesicles upon growth arrests caused by addition of vancomycin.

S. venezuelae Δ*afsK* strain LUV029 cultured in MYM in a microfluidic perfusion system was subjected to a 30 min treatment with MYM supplemented with 1 μg ml^{-1} vancomycin, followed by a return to normal medium 30 min later. Immediately after vancomycin has been added to the microfluidic chamber (approximately at time point labeled 42 min) cell growth is arrested and a majority of hyphal tip compartments lose turgor and/or lyse. However, a subpopulation survives the treatment, retaining turgor, and can regrow from reprogrammed polarity centers upon removal of the vancomycin. Most of the surviving hyphal tip compartments extrude vesicles, examples of which are shown here. Relative time in minutes shown.

Supplementary movie 6: DivIVA-mCherry is remodeled and not extruded upon vancomycin-induced vesicle release.

S. venezuelae strain NA1279, expressing a *divIVA-mCherry* fusion, was grown in MYM in a microfluidic perfusion chamber. At approximately time point labeled 12 min MYM supplemented with 1 μg ml^{-1} vancomycin was flushed into the chamber for 30 minutes. Following this treatment, the medium was switched back to regular MYM and growth was monitored in standard growth conditions. Upon vancomycin treatment the hyphae generally arrest growth, often coupled to release of vesicle-like structures, whereupon the cells occasionally regrow from new polar growth centers. Relative time in minutes shown.

Supplementary movie 7: *filP* does not affect the ability of hyphae to extrude vesicles upon growth arrests caused by addition of vancomycin.

S. venezuelae Δ*filP* strain NA1225 cultured in MYM in a microfluidic perfusion system were subjected to a 30 min treatment with MYM supplemented with 1 μg ml^{-1} vancomycin, followed by a return to normal medium. Immediately after vancomycin has been added to the microfluidic chamber (approximately at time point labeled 10 min) cell growth is arrested and a majority of hyphal tip compartments lose turgor and/or lyse. However, a subpopulation survives the treatment, retaining turgor, and can regrow from reprogrammed polarity centers upon removal of the vancomycin. Most of the surviving hyphal tip compartments extrude vesicles, examples of which are shown here. Relative time in minutes shown.

Supplementary movie 8: Spontaneous growth arrests in a *Streptomyces venezuelae scy* mutant are coupled to extrusion of vesicle-like structures.

Collection of examples of vesicle release events occurring when growth is spontaneously arrested in a *S. venezuelae Δscy* strain NA1255. Time in hours::minutes. Cells cultured in microfluidic perfusion chambers.

Supplementary movie 9: Extruded vesicle-like structures are membranous.

Representative spontaneous growth arrest and vesicle release in a *S. venezuelae Δscy* strain NA1255 cultured in an agarose growth chamber in the presence of FM4-64 membrane staining dye. Fluorescence channel (left) and phase contrast channel (right) show that growth arrest and remodeling of polar growth is accompanied by release of strongly FM4-64 staining material from the hyphal tip. Scale bar, 5 μm.

Supplementary movie 10: HupA-sfGFP is not present inside extruded vesicles upon vancomycin treatment.

S. venezuelae expressing a *hupA-sfGFP* fusion (wild type containing plasmid pSS48) cultured in MYM in a microfluidic perfusion system were subjected to a 30 min treatment with MYM supplemented with 1 μg ml^{-1} vancomycin, followed by a return to normal medium. Immediately after vancomycin has been added to the microfluidic chamber (approximately at time-point labeled 30 min) cell growth is arrested and a majority of hyphal tip compartments lose turgor and/or lyse. However, a subpopulation survives the treatment, retaining turgor, and can regrow from reprogrammed polarity centers upon removal of the vancomycin (approximately at time point 60 min). Most of the surviving hyphal tip compartments extrude vesicles, examples of which are shown here. No HupA-sfGFP signal was detected in the extruded material. Relative time in minutes shown.

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