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Published in:
Microbiology (United Kingdom)

DOI:
[10.1099/mic.0.000836](https://doi.org/10.1099/mic.0.000836)

2019

Document Version:
Peer reviewed version (aka post-print)

[Link to publication](#)

Citation for published version (APA):
Fröjd, M. J., & Flärdh, K. (2019). Extrusion of extracellular membrane vesicles from hyphal tips of streptomyces venezuelae coupled to cell-wall stress. *Microbiology (United Kingdom)*, 165(12), 1295-1305. Article 000836. <https://doi.org/10.1099/mic.0.000836>

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

6

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12 © Markus Fröjd and Klas Flärdh. The definitive peer reviewed,
13 edited version of this article is published in *Microbiology*,
14 *165* (12):1295–305, 2019, <https://doi.org/10.1099/mic.0.000836>.

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

19

20 **Abstract**

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

40 **Introduction**

41 Apical growth is a defining feature of organisms that grow as hyphae and form mycelia. Both
42 filamentous fungi and mycelial members of the Actinobacteria, like the genus *Streptomyces*,
43 grow by building their cell walls primarily at the hyphal tips. In both cases, growth depends
44 on systems for control of cell polarity and recruitment of the machinery for cell wall assembly
45 to hyphal tips and branch points, in order to establish and maintain the apical growth zones [1,
46 2]. However, the underlying molecular mechanisms involved in apical growth have evolved
47 independently in the two types of organisms. Fungal tip extension relies on polarization of the
48 actin and tubulin cytoskeleton, motor protein-driven transport, and regulation of exocytosis at
49 the tip [2]. Driving forces for tip extension in fungi include both turgor pressure and forces
50 generated by the cytoskeleton and associated motor proteins [3]. The control of apical growth
51 in streptomycetes and their relatives is centered around the orchestration of assembly of the
52 peptidoglycan (PG) cell wall, as is typical for control of growth and cell shape in bacteria in
53 general [4]. Thus, growth by tip extension requires recruitment of the machinery for PG
54 assembly to the hyphal tip. Establishment of polarity and control of apical PG assembly in
55 *Streptomyces coelicolor* is dependent on the coiled-coil protein DivIVA [5–7]. Turgor
56 pressure is thought to be important for tip extension also in streptomycetes [8, 9], but the
57 underlying details and the architecture of PG assembly at hyphal tips in streptomycetes
58 remain poorly understood.

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

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

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

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

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

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

100 **Methods**

101 **Bacterial strains, plasmids, oligonucleotides and growth media.** Bacterial strains and
102 plasmids used in this work are listed in Table S1. Oligonucleotide primers used are listed in
103 Table S2. *Escherichia coli* strains were grown at 37°C in lysogeny broth (LB) or on LB agar
104 [23] with or without 10 g L⁻¹ of NaCl depending on use of hygromycin. *E. coli* DY380 strain
105 was grown at 30°C. Kanamycin 50 µg ml⁻¹, ampicillin 100 µg ml⁻¹, apramycin 50 µg ml⁻¹,
106 hygromycin 50 µg ml⁻¹, tetracycline 15 µg ml⁻¹, and nalidixic acid 25 µg ml⁻¹ were used as
107 appropriate. All *S. venezuelae* strains were grown, unless stated otherwise, at 30°C in
108 maltose-yeast extract-malt extract medium (MYM) [24], made with 50% tap water, 50%
109 Milli-Q water (Millipore) and supplemented with 2 ml L⁻¹ trace element solution [25], or on
110 MYM agar. Hygromycin 50 µg ml⁻¹ was used as appropriate. All liquid cultures were shaken
111 at 200 rpm during culturing. General DNA manipulation and cloning were done according to
112 standard protocols.

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

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

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

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

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

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

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

177 **Results**

178 *Extrusion of vesicles from hyphal tips upon growth arrest*

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

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

196 *Vesicle extrusion is not induced by osmotic stress*

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

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

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

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

230 *Vancomycin treatment leads to vesicle extrusion*

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

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

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

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

262 *Vesicle extrusion and polarisome remodeling*

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

270 *Effect of polarisome proteins on vesicle extrusions*

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

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

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

298 *Vesicle-like expulsions are membranous*

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

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

313 *Nucleoids are not extruded in vesicles upon vancomycin treatment*

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

327 **Discussion**

328 The ability of bacteria to release extracellular membrane vesicles attracts great interest [16,
329 18, 19], but the mechanisms of vesicle biogenesis are often unclear. This is particularly true
330 for Gram-positives, in which vesicles would have to traverse the thick cell wall in order to be
331 secreted. Alternatively, and more well-documented, formation and release of vesicles may be
332 associated with processes leading to loss of cell integrity and cell death [20]. Vesicle

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

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

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

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

369 It should be noted that mycobacteria and corynebacteria use a similar mode of polar growth as
370 streptomycetes, directed by clusters of DivIVA [37–39]. Therefore, the route for vesicle
371 release that we describe here may be valid and important also for biogenesis of the
372 extracellular vesicles that have been observed in for example *Mycobacterium tuberculosis*
373 [18, 40]. Mycobacteria have a complex and multilayered cell envelope. Although vesicles
374 have been observed adhering to cells at many positions along the cell surface, the cell poles
375 where the envelope is assembled appear to be the most feasible places where they could be
376 extruded, perhaps similarly to what we describe here.

377 Extracellular vesicles have previously been reported in *Streptomyces* spp. in conjunction with
378 specialized metabolites. Droplets of exudates on the surface of sporulating lawns of *S.*
379 *coelicolor* and *Streptomyces lividans* contain the antibiotics actinorhodin and
380 undecylprodigiosine, respectively, and were shown to be enriched in membrane vesicles [41,
381 42]. Further, production of lipid-like polyene polyketides linearmycins by *Streptomyces* sp.
382 Mg1 is associated with generation of extracellular membrane vesicles that facilitate the

383 diffusibility of the linear mycins [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 forms upon growth arrest.

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

413 **Author statements**

414 **Funding information.** This work was supported by a project grant from the Swedish
415 Research Council (2015-05452) to KF

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

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

419 **Acknowledgements.** We thank Elisabeth Barane for excellent technical assistance. Susan
420 Schlimpert is gratefully acknowledged for her donation of plasmids. The work was funded by
421 a grant from the Swedish Research Council (2015-05452) to KF and infrastructure support
422 from the Science Faculty at Lund University for the microscopy setup for bacterial live cell
423 imaging.

424

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560

561 **Tables and figure legends**

562

563

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
<i>ΔafsK</i>				
Vancomycin	132	81.8	18.2	79.2
<i>ΔfilP</i>				
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.

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

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

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

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

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

594 *S. venezuelae* wild type (top panel) and ΔafsK (bottom panel) were cultured in a microfluidic
595 perfusion system, whereupon vancomycin ($1 \mu\text{g mL}^{-1}$) was added to the chamber for 30 min,

596 and then washed away. Upon vancomycin addition, hyphal extension is arrested and many
597 cells lose turgor or lyse (arrow). However, some cells can maintain turgor and resume growth
598 from new growth centers upon removal of vancomycin. This survival is often coupled to the
599 expulsion of vesicle-like structures from the hyphal tip compartments (arrowheads). Scale bar,
600 5 μm .

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

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

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

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

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

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

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

568 **Figures and tables**

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571

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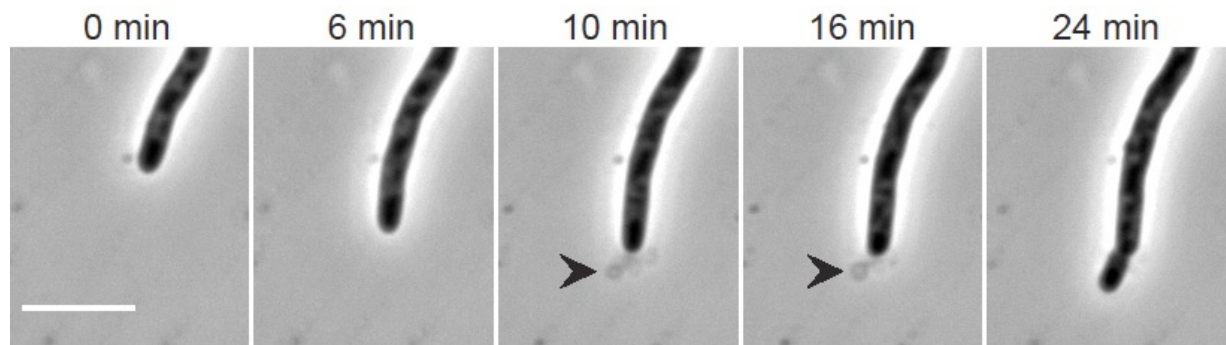
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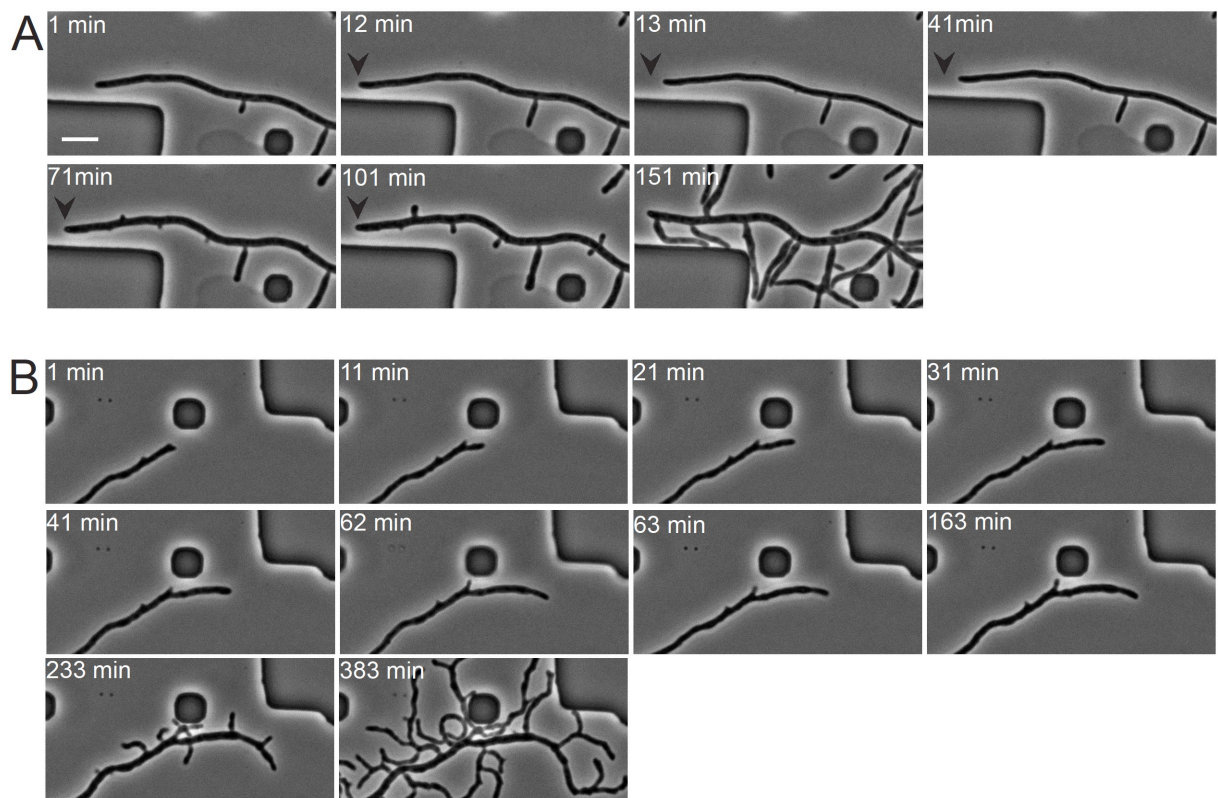


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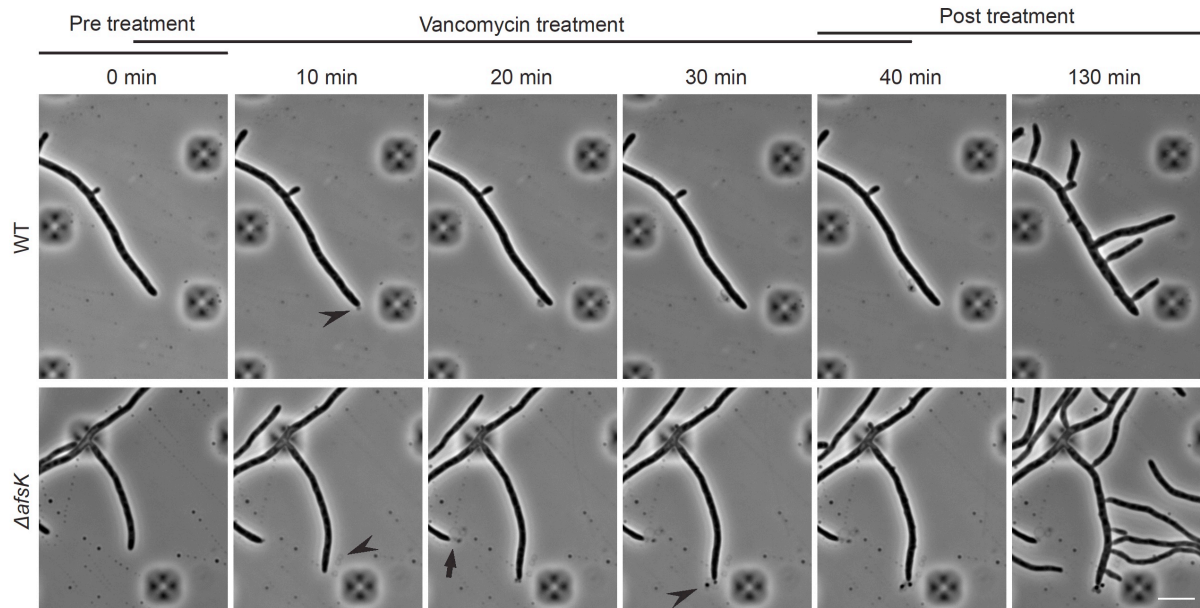
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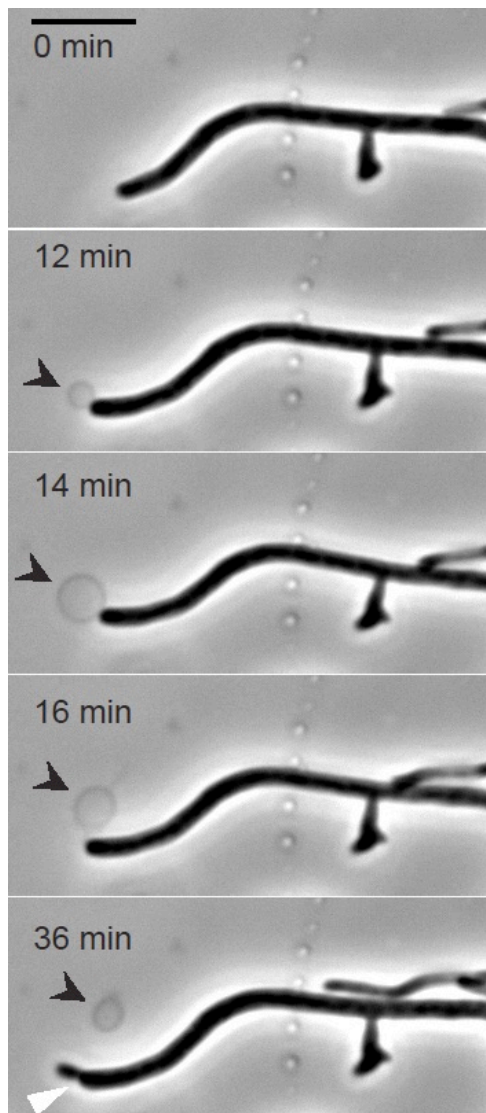
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607
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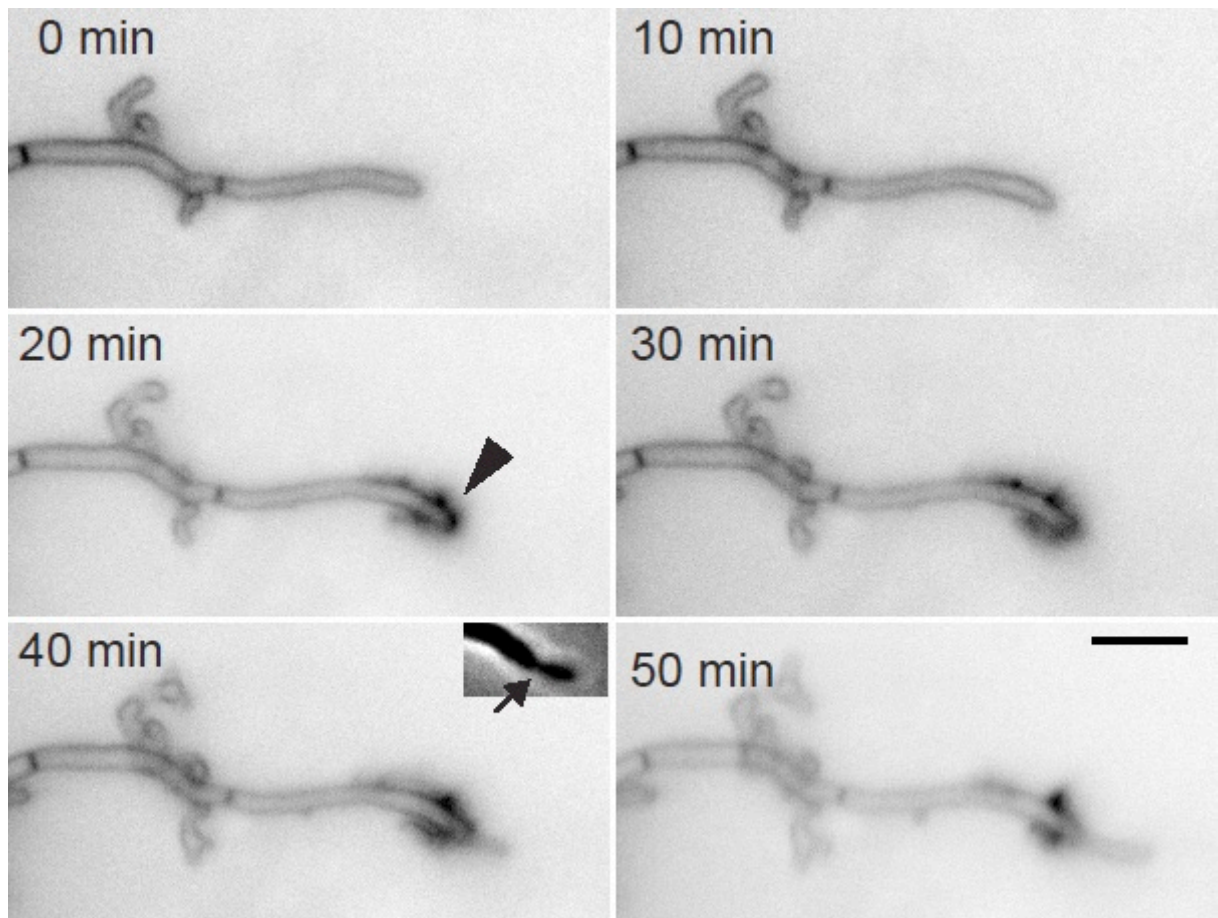


619

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

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

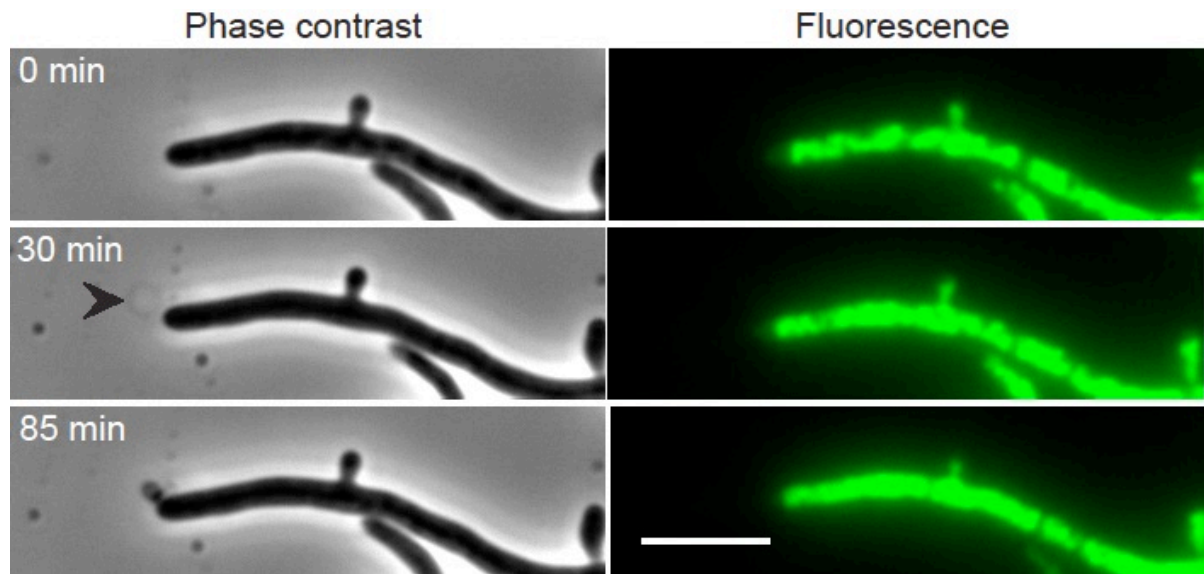
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629
 630 **Figure 5: Extrusions released from a *scy* deletion mutant of *Streptomyces venezuelae* are**
 631 **membranous.**

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

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

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S. venezuelae hupA⁺/hupA-sfGFP was cultured in a microfluidic perfusion system, whereupon

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vancomycin was added to the chamber. Upon vancomycin addition (minute 0), growth arrests

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and some hyphal tip compartments manage to survive and are able to regrow from new

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growth centers once vancomycin has been removed (minute 30, arrowhead). Coupled to this

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survival is the expulsion of vesicle-like structures from the hyphal tips. The expulsions are

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heterogeneous in phase contrast signal. No expulsions appear to contain the DNA-associated

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HupA-sfGFP protein. Phase-contrast channel is shown on the left and fluorescent channel

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shown on the right. Times are relative time after addition of vancomycin. Scale bar, 5 μ m.

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Supplementary materials

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

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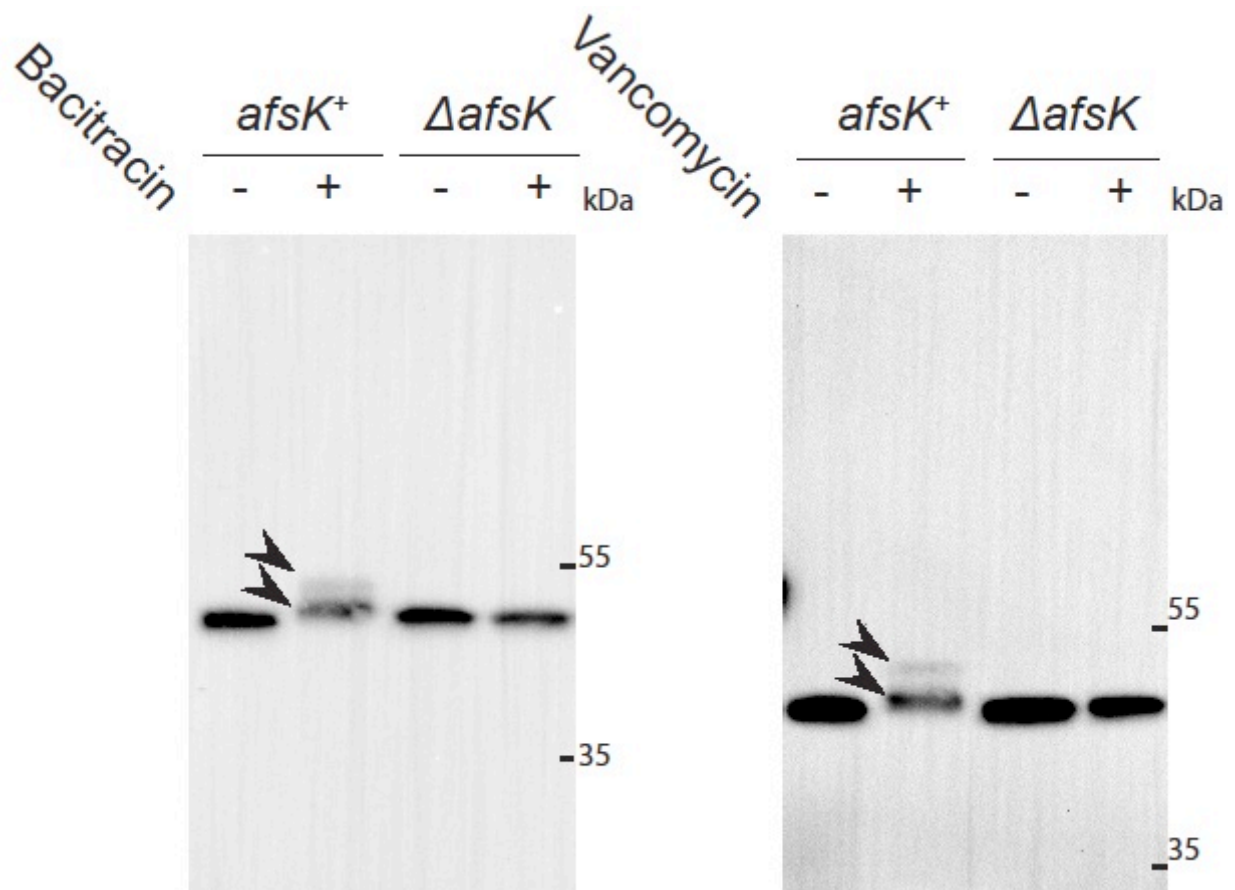
Keywords: hyphae, *Streptomyces*, cell wall, peptidoglycan, cell membrane, cell polarity

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

Strain or plasmid	Genotype and/or description	Source or reference
Strains		
<i>S. venezuelae</i>		
NRRL B-65442	Wild-type <i>S. venezuelae</i> strain	[1]
NA1225	$\Delta filP::frt$	[2]
NA1255	$\Delta scy::frt$	[2]
NA1279	$attB_{\phi BT1}::pSS204$ (contains <i>divIVA-mCherry</i>)	[2]
LUV029	$\Delta afsK::apr$	This work
<i>E. coli</i>		
DH5 α	Cloning strain	[3]
DY380	DH10B $\lambda cl857 \Delta cro-bio < > tet$	[4]
ET12567/pUZ8002	$dam-13::Tn9 dcm-6 hsdM$, carries RK2 derivative with defective <i>oriT</i> , for plasmid mobilization	[5]
Plasmids		
3-E07	Cosmid containing <i>afsK</i>	
pSS48	pIJ10770- <i>hupA-sfGFP</i>	Susan Schlimpert, JIC, Norwich, UK

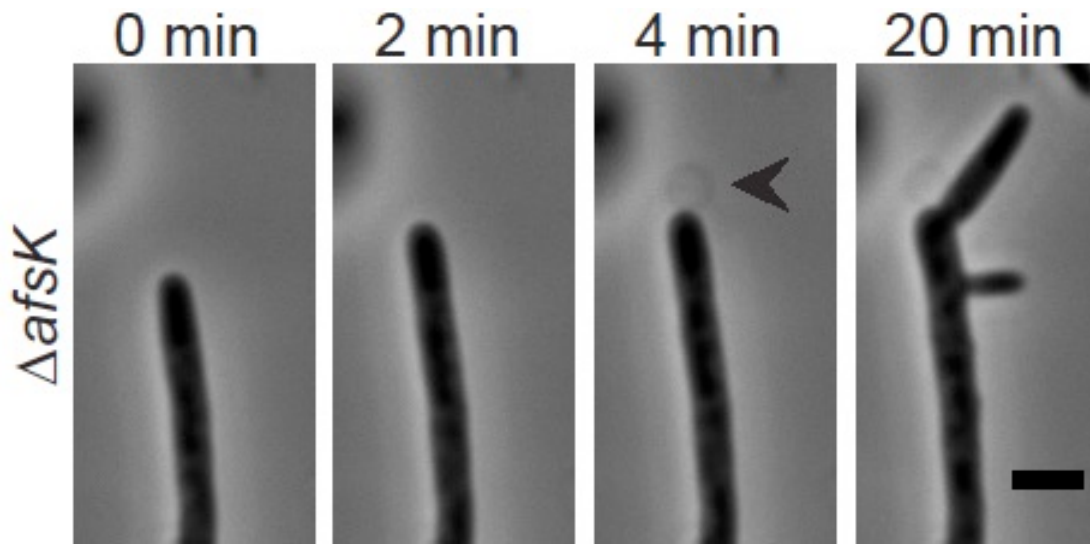
Supplementary table 2. Oligonucleotide primers used in this work.

Oligonucl	
eotide	Sequence
KF1392	CGCGCGGCGAGGGGTGAGGCATCCGTTCCGGATGGCTCCGGTATGA GGTATTCCGGGGATCCGTCGACC
KF1393	GAACCCGCCGCCAGGAGCTAGGGCAGCGGGTCCTCGGCAGGAGAA GCATGTAGGCTGGAGCTGCTTC
KF1401	CGAGACCCAGACGTACGAC
KF1406	ACCATTCTTCAGGATGGCAAG



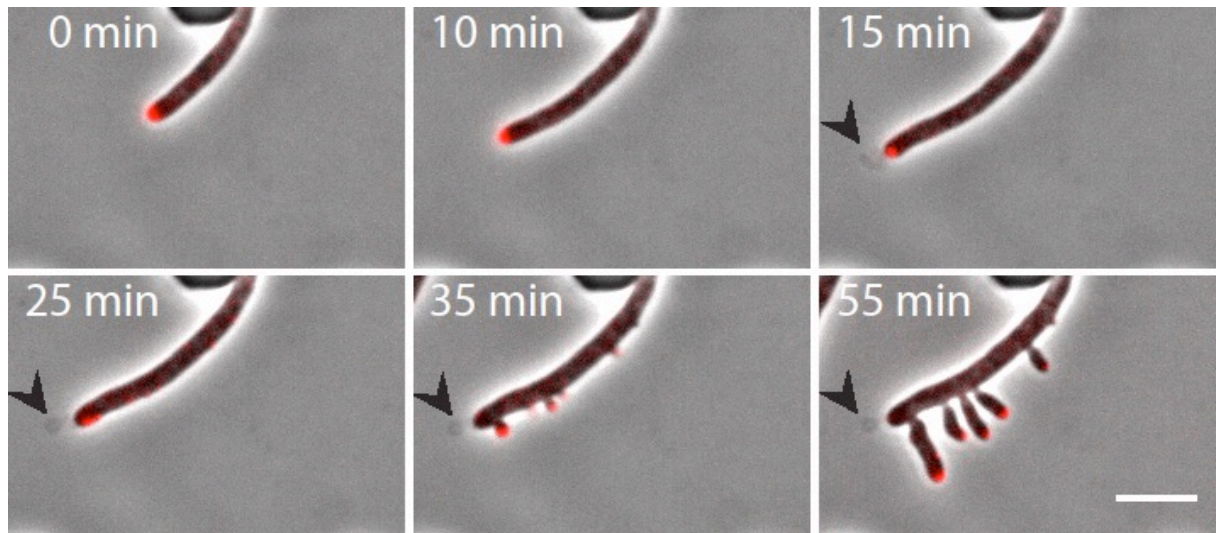
Supplementary figure 1: Addition of bacitracin or vancomycin causes *afsK*-dependent phosphorylation of DivIVA in *Streptomyces venezuelae*

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 μ g total protein from soluble fractions of lysates of cells treated with (+) or without (-) bacitracin or vancomycin. Wild type (*afsK*⁺) and Δ *afsK* (LUV029) strains were used.



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

Montage of time-lapse images showing an example of a spontaneous growth arrest in an *S. venezuelae* $\Delta afsK$ 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 μ m.

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 $\mu\text{g ml}^{-1}$ 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 ΔafsK strain LUV029 cultured in MYM in a microfluidic perfusion system was subjected to a 30 min treatment with MYM supplemented with 1 $\mu\text{g ml}^{-1}$ 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 $\mu\text{g ml}^{-1}$ 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 ΔfilP strain NA1225 cultured in MYM in a microfluidic perfusion system were subjected to a 30 min treatment with MYM supplemented with 1 $\mu\text{g ml}^{-1}$ 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* Δ scy 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 μ m.

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 μ g 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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