Microfluidic *Streptomyces* Cultivation for Whole Lifecycle Characterization and Phenotypic Assays Enabled by Nanogap-stabilized Air-Water Interface

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Running Head: Microfluidic platform for *Streptomyces* lifecycle study

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ABSTRACT *Streptomyces* is a model filamentous prokaryote to study multicellular differentiation and a rich reservoir for antibiotics discovery. In their natural conditions, *Streptomyces* grows at the interface of porous soil, air, and water. The morphological development of *Streptomyces* is traditionally performed on agar plates and mostly studied at the population levels. However, the detailed lifecycle of *Streptomyces* has not been well studied due to its complexity and lack of research tools which can mimic their natural conditions in the soil. Here, we developed a simple assembled microfluidic device for cultivation and the entire lifecycle observation of *Streptomyces* development from single-cell level. The microfluidic device composed of a microchannel for loading samples and supplying nutrients, microwell arrays for seeding and growth of single spores, and air-filled chambers aside of the microwells that facilitate growth of aerial hyphae and spores. A unique feature of this device is that each microwell is surrounded by a 1.5 µm gap connected to an air-filled chamber which provide stabilized water-air interface. We used this device to observe the development of single *Streptomyces* spores and found that unlike those in bulk liquid culture, *Streptomyces* can differentiate at water-air interfaces in microscale liquid culture. Finally, we demonstrated that phenotypic A-Factor assay can be performed at defined time point of its lifecycle. This microfluidic device could become a robust tool for studying *Streptomyces* multi-cellular differentiation and interaction at single cell level.

IMPORTANCE We describe a microfluidic device that mimics the natural porous

environment for the growth and development of *Streptomyces*, the model system for bacterial multicellularity. The microfluidic device is used for cultivation and the entire lifecycle observation of *Streptomyces* development from single-cell level, including growth of aerial filaments. The aerial hyphae development of *Streptomyces* at the water-air interface was observed at real time in the microfluidic device. The early growth, opportunistic transformation (in the gap), and merging of aerial hyphae of *Streptomyces* in the microfluidic device were observed for the first time. It will play an important role in finding single-cell heterogeneity to study secondary metabolites related to the complex lifecycle of *Streptomyces*.

KEYWORDS bacterial multicellularity, *Streptomyces* differentiation, aerial filaments, microfluidics, single cell analysis.

INTRODUCTION

Streptomycetes are gram-positive filamentous bacteria that play crucial roles in their habitat because of their broad range of metabolic processes and biotransformation including degradation of chitin and cellulose (1-3). They are the most important natural source of bioactive compounds such as antibiotics and anti-tumor agents, producing two-thirds of the antibiotics of medical and agricultural interest (4-6). In nature, *Streptomyces* grows primarily in soil with porous structures that retain water in the micron-sized

61 cavities and channels. Nutrients, oxygen, water transport and other environmental factors
62 may have profound impact on the their physiology, morphological development and
63 outset of secondary metabolism (7). Although recent research with advanced genomic
64 tools has made great progress in uncovering their genetic potential, a lot of discovered
65 pathways are cryptic, which means they are either silent or poorly expressed for those
66 grown on agar plate or in liquid media in the standard laboratory conditions, presumably
67 due to the inability to recreate the microscale porous structure as well as nutritional and
68 environmental circumstances in their natural soil habitat. In addition, bulk cultivation
69 method only allows spatial control down to millimeter scale, and is often not convenient
70 for nutrient and chemical exchange.

71 Microfluidics has emerged as a new tool to study microbiology because it offers
72 many advantages including micrometer-scale spatial resolution and flexible temporal
73 control of nutrients exchange and chemical gradients (8). Microfluidic tools have been
74 used to study microbiology in many ways such as single-cell isolation and manipulation,
75 bacterial chemotaxis, quorum sensing, and population dynamics (9). Although high-
76 throughput enrichment and sorting of soil-derived *Actinobacteria* in microfluidic droplets
77 have been described (10), the development and differentiation of *Streptomyces* using
78 microfluidic chips are rarely reported until 2016 (11). The challenge in *Streptomyces*
79 cultivation is that their growth and differentiation rely on a stabilized water-air interface.
80 When cultivated on a solid agar, *Streptomyces* have a typical lifecycle including

81 germinate of vegetative hyphae in the solid substrate, forming of hydrophobic aerial
82 hyphae, and development of airborne spores which allow dispersion (12). However, in a
83 standard liquid medium, *Streptomyces* mainly exists as vegetative hyphae that tangle
84 together to form many small pellets and clumps with very few aerial hyphae (13).
85 Therefore, direct miniaturization of standard liquid culture in a microchamber without a
86 stabilized water-air interface is not a generalizable method for cultivation of
87 *Streptomyces*.

88 To overcome these challenges, we describe a microfluidic device integrating liquid
89 containing microwells and air-filled chambers to establish a stabilized water-air interface
90 for cultivation, the whole lifecycle observation of *Streptomyces* differentiation and
91 phenotypic assay. The device can achieve micron-scale spatial resolution, maintain
92 culture conditions over an extended period of time with optional nutrient and chemical
93 exchange, and enable single-cell cultivation and observation; thus, it is a useful tool for
94 exploring *Streptomyces*'s development and behavior under controlled circumstances. The
95 microfluidic platform was validated by culturing two representative *Streptomyces* strains,
96 and a phenotypic assay with A-Factor. A-Factor and analogues are autoregulatory factors
97 involved in secondary metabolism and/or morphological differentiation in Actinomycetes
98 (14). They are essential for the aerial hyphae formation in *Streptomyces* (Fig. S3A).
99 However, previous studies showed that the timing of A-Factor introduction is important
100 for morphogenesis and secondary metabolism (15). The nutrient exchange channel

incorporated in the microfluidic chip allows the A-Factor assay to be performed at defined time points in the lifecycle with mutant organism that is deficient in A-Factor synthesis.

RESULTS AND DISCUSSION

Design of the microfluidic device. We designed a microfluidic device with an array of microwells for the entire lifecycle observation of *Streptomyces* including cultivation and observation. This device incorporates two important design features: i) stable water-air interface enabled by nanogaps; ii) well controlled nutrient and chemical exchange through a microchannel. *Streptomyces* undergoes complex lifecycles involving vegetative hyphae growth in substrates and aerial hyphae growth in the air. We mimicked the natural microenvironment by assembling liquid-containing microwells and air-filled chambers for vegetative growth and aerial growth, respectively. The gap between two assembled glass plates is 1.5 μm, which is slightly larger than the diameter of the hyphae (1 μm). This separation ensures that the aerial hyphae can readily pass through the gap (Fig. 1A). The chamber is 15 μm in height so that the aerial hyphae have sufficient space to keep a natural state. When the microwells are filled with liquid, water-air interfaces are formed between the microwells and the air-filled chambers. The glass plates offer a reliable barrier to minimize evaporation. The plates also exhibit hydrophobic surface after treatment with fluorosilane, and thus the surface tension was relatively high (Fig. 2B).

The interface was stable for continuous growth and observation without deleterious drift or shift.

We regard the liquid surface as a spherical surface so that the capillary pressure ΔP can be derived from the following equations:

$$\Delta P = \frac{2\sigma cos\theta}{r} \quad (1)$$

where, σ is the liquid surface tension (7.28×10^{-2} N/m); θ is the contact angle between the liquid surface and the solid plate; maximum value is 105°; the radius (r) of it equals to half of the gap height (0.75 μm). Thus, the capillary pressure ΔP can be calculated to be 5.03×10^4 Pa, which is large enough to form a stable gas-liquid interface (Fig. 2D, E). *Streptomyces* spores were appropriately diluted and loaded into microwells to achieve single spore isolation in microwells following the Poisson distribution. Spores can germinate, branch to form vegetative hyphae in microwells, and later pass through the gap and differentiate into aerial hyphae in air-filled chambers that eventually develops into mature spores. The lifecycle of *Streptomyces* can last for several days, and thus we infused culture media continuously from the channel to guarantee adequate nutrient supply. The mycelia in the microwells would not be disturbed because of the narrow joint between the channel and the microwells. The entire developmental process could be monitored using an inverted microscope.

Lifecycle observation of *Streptomyces coelicolor* on-chip culture in liquid medium. *S. coelicolor* is a model organism of *Streptomyces*, and the complete genome of

the type strain *S. coelicolor* M145 has been sequenced; it is used in many studies of *Streptomyces* growth and development (2). We cultivated *S. coelicolor* in liquid minimal medium on the microfluidic device, and observed its entire lifespan (Fig. S1, Movie S1). After 9 h of dormancy, the spore emerged from one germ tube, which prolonged and formed branches. Each branch showed apical growth, indicating that the group of cells grew at an exponential phase in the microwell. The hyphae could spread randomly in liquid medium because there was no solid substrate confinement. The hyphae gradually approached the water-air interface, broke the surface tension, and grew into the air-filled chamber at 28 h (growth almost perpendicular to the edge of the microwell). The aerial hyphae progressively elongated and formed branches in all directions. There were curls and spirals at the end of the hyphae. Meanwhile, the vegetative hyphae developed many layers, and eventually almost filled the entire microwell. The vegetative hyphae and the aerial hyphae stopped growing after 60 hours (Movie S1).

When cultivated in the flask-scale liquid medium, aerial hyphae formation and sporulation are blocked in most *Streptomyces* strains (16), but when cultured in bioreactors, some strains may be able to sporulate due to stress conditions such as strong agitation (17). It has been suggested that nutrient depletion and the reuse of materials led to the hyphae differentiation in liquid medium (18), and programmed cell death also trigger the differentiation process in liquid and solid media (13). Although the specific signals are unclear, *N*-acetylglucosamine produced by the decomposition of

peptidoglycan may be one of the signals (19). However, small-scale single-cell development has not been carefully illustrated, and differentiation might be limited. In this study, we cultivated *S. coelicolor* in a microfluidic device and found that vegetative hyphae did not lyse; rather, they continually grew even after the emergence of aerial hyphae. The culture media were supplied continuously in the microfluidic device such that the nutrition is not exhausted, indicating that the differentiation phenomenon may not be necessarily correlated with nutrient depletion.

Differentiation of *S. coelicolor* on-chip culture in liquid YEME medium. *S. coelicolor* can form aerial hyphae and spores in standing liquid cultures with minimal media but not with complete media (20). Here, we inoculated single spores in microwells with nutrient-rich YEME medium and cultivated the samples several days to test whether they can differentiate (Fig. 3). The results showed that *S. coelicolor* still has a complete lifecycle in liquid YEME medium including vegetative hyphae in microwells (Fig. 3A), aerial hyphae outside the well (Fig. 3C), and mature spores (Fig. 3D). Scanning electron microscopy analysis revealed that the hyphae in the microwells had a relatively smooth surface (Fig. 3B). Hyphae in the chamber had a layer of well-organized hydrophobic proteins (21) (Fig. 3C) with compartments between each spore (Fig. 3D). These results are consistent with the development of *S. coelicolor* grown on solid plates and previous reports on the microscopic feature of hydrophobic proteins (22).

Accordingly, *S. coelicolor* has entire lifecycles in the liquid environment regardless

of the nutrient status. An earlier study showed that the expression of most genes is comparable between liquid and solid cultures, including genes involved in the hydrophobic cover formation and even a few genes regulating the early stages of sporulation (23). Genes involved in the final stages of hydrophobic cover/spore maturation are up-regulated in solid cultures compared with liquid cultures. These findings suggest that *S. coelicolor* can differentiate in both solid and liquid cultures. Transcripts and proteins are ready before aerial hyphae formation. Once *S. coelicolor* senses the existence of air, they begin to grow aerial hyphae and develop into mature spores. In standing liquid cultures, there may be a physical constraint that hinders the formation of aerial hyphae: The nutrient-rich media contains more complex ingredients, which is likely to attach to the hyphae surface and reduce the hydrophobicity of the hyphae, making it difficult for the aerial hyphae to erect.

Interestingly, we observed the merging of aerial hyphae when *S. coelicolor* was cultivated in the microfluidic chip (Fig. 4, Movie S2). This universal phenomenon in Streptomycetes is called hyphal anastomosis (or hyphal fusion), which was firstly confirmed in *S. scabies* (24), and is considered to be very important for intra-hyphal communication, nutrients and water translocation, and general homeostasis within a colony (25). There is hypha-to-hypha fusion, two hyphal tips grow towards each other until contact and fuse (Fig. 4A), as well as hypha-to-peg or hypha-to-side fusion, a hyphal tip approaches the side of another existing hypha and directly fuse with it (Fig.

4B, C) occurred in this study.

Comparison of *S. griseus* wild type and mutant. To evaluate whether the differentiation in microfluidic liquid culture approach with *Streptomyces* can be generalized, another model organism *S. griseus* was cultivated in the microfluidic chip to observe its differentiation in liquid cultures. We cultured *S. griseus* in liquid MM medium and YEME medium, respectively, and observed its three lifespan stages through optical microscopy and electron microscopy (Fig. S2). The results confirmed that *S. griseus* can pass through its whole lifecycle in both liquid cultures.

The differentiation mechanism of *S. griseus* on the solid plate has been well-studied. The entire process begins with the expression of *afsA* that controls the synthesis of A-Factor, a type of γ-butyrolactones known as microbial hormones. A-Factor is essential in the regulatory pathways of sporulation (26), it can bind to A-Factor receptor protein ArpA and relieves the suppression of ArpA to *adpA*. AdpA can then stimulate a series of responses involving morphological development and secondary metabolism (Fig. S3A). Genes involved in the formation of aerial hyphae and spore, including *ssgA* (27) , *adsA* (26), *amfR* (28), as well as extracellular proteases (29, 30) and protease inhibitor encoding genes (31) are regulated by AdpA. When *afsA* is knocked down, the mutant cannot form aerial hyphae on solid YEME plate. We constructed a *S. griseus* Δ*afsA* mutant via genetical engineering to compare the mechanism of differentiation between solid and liquid cultures (Fig. S3).

We inoculated the mutant on solid YEME medium and cultivated it for several days. Compared with wild-type, the mutant strain can neither develop aerial hyphae nor pigmented spores (Fig. 5B). When cultured on a microfluidic device, the hyphae were mainly in microwells with very few hyphae outside microwells. These were very short even after being cultivated for several days. They could not form spores (Fig. 5D). Scanning electron microscopy showed that the hyphae surface of *S. griseus* Δ*afsA* mutant was relatively smooth (Fig. 5D). Thus, we inferred that these short hyphae are still vegetative hyphae. While they emerged out of the microwell, they could not develop further. The phenotype of *S. griseus* Δ*afsA* mutant grown in liquid culture was similar to that grown on the solid plate. These results demonstrate the consistency in observation of differentiation behavior of *S. griseus* wild type and mutant strains between microfluidic and bulk cultures.

Feeding of A-Factor analogue and recovery of the mutant phenotype. The A-Factor analogue *β*-keto SCB2 was chemically synthesized as described above and added to the culture medium to examine whether the mutant could recover a wild-type phenotype. Previous studies showed that A-Factor is chemically unstable with a half-life of several hours. The production of A-Factor is growth-dependent and accumulated with the growth of hyphae (Fig. 5A). It reaches peak concentration of 25–30 ng/mL and rapidly decreases thereafter.(15) According to the development of *S. griseus* and the instability of A-Factor, we fed *β*-keto SCB2 to the Δ*afsA* mutant at several time points in

241 the lifecycle. When *β*-keto SCB2 was added at 20 h and 30 h after inoculation, the mutant
242 could form aerial hyphae and spores (Fig. 5E, F).

243 Scanning electron microscopy further confirmed that there were hydrophobic proteins
244 on the surfaces of hyphae and spores observed from *S. griseus* Δ*afsA* mutant on device
245 with *β*-keto SCB2 supplied at 30 h after initial cultivation. (Fig. S4). When *β*-keto SCB2
246 was added at 40 h after inoculation, the mutant could no longer form aerial hyphae (Fig.
247 5G). These results are consistent with previous studies showing that timing is critical for
248 A-Factor's switching function (Fig. 5A) (15). There is a decision phase at the middle of
249 the exponential growth, which is an A-Factor-sensitive period. The exogenous addition of
250 A-Factor after this time can no longer influence morphological differentiation (15). We
251 conclude that the microfluidic chip is compatible with A-Factor assay by introducing the
252 test compound at defined time point, and demonstrated the timing-sensitive effect of A-
253 Factor on morphological differentiation of mutant deficient in A-Factor synthesis, which
254 is in good agreement with the classical literature reports.

255 **Mechanism of water-air interface differentiation and possible applications of the**
256 **microfluidic device.** To confirm whether the differentiation of *Streptomyces* is different
257 between that grown on solid plates and that grown in liquid media, we conducted an A-
258 Factor-regulated experiment in *S. griseus* using the microfluidic-based device. Timing
259 studies on the microfluidic device and scanning electron microscopy showed that the
260 differentiation mechanism of *S. griseus* in the liquid environment is the same as that on

261 solid plates. We also found that sporulation is not correlated with lysis of vegetative
262 hyphae suggesting that genes encoding extracellular proteases and protease inhibitors
263 may not be necessary for differentiation.

264 Differentiation mechanisms of *Streptomyces* are essential for the study of secondary
265 metabolism. Using this microfluidic device, we successfully restored the wild-type
266 phenotype of *S. griseus* Δ*afsA* mutant by addition of the A-Factor analogue *β*-keto SCB2.
267 Mechanisms by which other chemical molecules affect the morphological differentiation
268 and secondary metabolism of *Streptomyces* can also be studied through this device with
269 significantly reduced consumption of compound of interest by virtue of miniaturization.
270 Previous studies on the differentiation of *Streptomyces* in liquid media mainly focused on
271 the analysis of pellets and clumps formation (32). Some strains require the formation of
272 pellets to produce secondary metabolites, such as *S. coelicolor* (undecylprodigiosin and
273 actinorhodin) (13) and *S. olidensis* (retamycin) (33) while pellets and clumps formation
274 reduce the antibiotic productions in *S. noursei* (nystatin) (34) and *S. fradiae* (tylosin)
275 (35). The microfluidic device we developed will help to establish the developmental
276 model of different *Streptomyces* strains in liquid cultures, which will be beneficial to the
277 optimization of industrial fermentation.

278 In addition, a previous study found that *S. coelicolor* was able to produce several
279 secondary metabolites during its germination: albaflavenone (antibacterial activity
280 against *Bacillus subtilis*), the polyketide germicidin A, and chalcone (inhibits

281 germination) (36). As the whole lifecycle of *Streptomyces* can be observed by the
282 microfluidic device, functions of secondary metabolites produced by *Streptomyces* strains
283 in their early stages of growth can also be further studied.
284
285 **CONCLUSION**
286 We developed a microfluidic device for cultivation and the entire lifecycle
287 observation of *Streptomyces*, a dominant paradigm in bacterial multicellularity evolution.
288 Compared with traditional methods, this microfluidic device can achieve single-cell long-
289 term dynamic cultivation and observation enabled by nanogap-stabilized air-water
290 interfaces. Two model strains of *Streptomyces* (*S. coelicolor* and *S. griseus*) were
291 cultivated in the microfluidic device at single-cell/spore resolution. Cellular development
292 and differentiation of the entire *Streptomyces* lifecycle was monitored with microscopy
293 and further characterized with scanning electron microscopy on the microfluidic device
294 for the first time. We also studied *Streptomyces*' development under different nutrient
295 conditions or chemical stimuli, and we may also use it to investigate the cell-cell
296 interaction between *Streptomyces* and pathogenic bacteria.
297 We found that aerial mycelia may grow at a very early stage, and such formation may
298 not be necessarily correlated with nutrient depletion. Moreover, we constructed a *S.*
299 *griseus* Δ*afsA* mutant (exogenously supplemented A-Factor analog to the mutant) and
300 compared the differentiation mechanism between solid and microscale liquid

environments. We inferred that *Streptomyces* still has the entire lifecycle in a microscale liquid environment, and the differentiation mechanism is the same as that on solid plates. These new observations shed light on the understanding of *Streptomyces*' multicellular development and differentiation. Although the current biological data are findings and conclusion derived from visual inspections of images, other scientific analysis and representation also can be achieved by further image analysis, for example, growth of the different morphological states (Fig. 3) could be characterized and compared by derived growth rates from image data of filamenting organisms (11).

Overall, we anticipate that our new method provides a better platform for the study of *Streptomyces*' development in the natural porous and moist soil environment. The microwell arrays with stabilized air-water interfaces can mimic ecological niches and help us identify single-cell heterogeneity. *Streptomyces*' complete lifecycle on the microfluidic device may also awaken cryptic secondary metabolite gene clusters for the secretion of secondary metabolites and lead to the discovery of novel antibiotics for combating global crisis of antimicrobial resistance.

MATERIALS AND METHODS

Bacterial strains and materials. The microbial strains used in this work include *Streptomyces coelicolor* M145, *Streptomyces griseus* IFO 13350, and *S. griseus* Δ*afsA* mutant. These strains were cultured on the Mannitol-Soy agar plate at 28 ℃ for about a

week to allow spore germination. The spores were harvested by sterile cotton swabs and suspended in the sterilized culture medium. The suspension was filtered through a filter tube filled with cotton wool to remove aerial hyphae. The OD_{600} of the spore suspension was adjusted to 0.15 to ensure that most of the microwells had a single spore. Liquid minimum (MM) medium and yeast extract-malt extract (YEME) medium were used for on-chip cultivation.

Fabrication of the device. The microfluidic device was made of two glass plates and fabricated by standard photolithography as well as wet chemical etching techniques (37). The photomasks were designed using AutoCAD (San Rafael, CA) and ordered from MicroCAD photomask Co. Ltd. (Shenzhen, China). The top plate has a 55-μm-deep channel, with 40 microwells symmetrically distributed along the channel with a volume of 0.27 nL individually. The bottom plate consists of an array of nanogaps of 1.5 μm high (Fig. 1A and Fig. 2A). The top plate has two access holes drilled by a diamond drill bit 0.8 mm in diameter. The glass plates were cleaned with ethanol, oxidized in a plasma cleaner, and silanized by 1*H*,1*H*,2*H*,2*H*-perfluorooctyl trichlorosilane.

Device operation and cell cultivation. The glass chip was thoroughly cleaned with ethanol and tightly clamped by clips. The spore suspension was aspirated into a pipette and loaded into the channel leading to the microwells (Fig. 1B). Then suspension in the channel was aspirated from the outlet to remove excess spores to prevent channel block caused by hyphae growth, but medium and spores in microwells could be retained (Fig.

1B). Two syringes were connected to the device by Teflon tubing to infuse culture medium continuously for long-term cultivation (Fig. 1B). The device was placed under an inverted microscope to capture pictures every hour. A CO_2 microscope cage incubator was placed around the microscope to maintain the temperature at 28 ℃ for *Streptomyces* cultivation.

Scanning Electron Microscopy. When cultivation terminated, the device was transferred to a freezer at -20 ℃ for one minute to freeze the sample, so that the hyphae could not move when we opened the device. The device was disassembled quickly, and the top plate was cut into 0.5 × 0.7 cm pieces and fixed in 3% glutaraldehyde overnight at 4 ℃ to maintain the bacteria's physiological status. The sample was then washed with DI water to remove glutaraldehyde, dehydrated in an ethanol series (50%, 70%, 85%, 95%, and 100%), critical point dried, and sputter-coated with platinum under vacuum. The sample was observed under a scanning electron microscope.

Synthesis of A-Factor analogue *β*-keto SCB2. *S. coelicolor* butanolides (SCBs) are *γ*-butyrolactones from *S. coelicolor*, and *β*-keto-SCB2 is a stereoisomer of A-Factor (38, 39). The synthesis of *β*-keto-SCB2 was began with methyl 3-oxocyclobutane-1-carboxylate to methyl 5-oxotetrahydrofuran-3-carboxylate by the Baeyer-Villiger oxidation (40-43). Methyl 5-oxotetrahydrofuran-3-carboxylate was then reduced to 4-(hydroxymethyl)-dihydrofuran-2(3*H*)-one by the addition of $NaBH_4$ followed by protection of hydroxyl group with *tert*-butyldimethylsilyl (TBS) chloride. Octanoyl

chloride was slowly added to react with 4-(((*tert*-butyldimethylsilyl)oxy)methyl)dihydrofuran-2(3*H*)-one to give 4-(((*tert*-butyldimethylsilyl)oxy)methyl)-3-octanoyldihydrofuran-2-(3*H*)-one. The silyl protecting group was then removed with tetrabutylammonium fluoride to afford *β*-keto-SCB2. Mass spectrometric analysis characterized the synthetic products. See SI for detailed synthetic steps.

SUPPLEMENTAL MATERIAL

Supplemental material contains:

Supplementary figures on lifecycle of *S. griseus* (FIG S1-S4), and the synthesis process of A-Factor analogue *β*-keto SCB2, PDF file, 1.54 MB.

Movie S1 shows lifecycle of *S. coelicolor*, AVI file, 3.81 MB.

Movie S2 shows hyphal anastomosis of *S. coelicolor*, AVI file, 0.99 MB.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (Nos. 31970091, 21822408 and 91951103), the program of China Ocean Mineral Resources R&D Association (No. DY135-B-02), the National High Technology Research and Development Program of China (No. 2018YFC0310703), and Senior User Project of RV

KEXUE from Center for Ocean Mega-Science, Chinese Academy of Sciences

(KEXUE2019GZ05).

REFERENCES

1. Chater K. 2016. Recent advances in understanding *Streptomyces*. F1000Research 5.
2. Bentley SD, Chater KF, Cerdeño-Tárraga AM, Challis GL, Thomson NR, James KD, Harris DE, Quail MA, Kieser H, Harper D, Bateman A, Brown S, Chandra G, Chen CW, Collins M, Cronin A, Fraser A, Goble A, Hidalgo J, Hornsby T, Howarth S, Huang CH, Kieser T, Larke L, Murphy L, Oliver K, O'Neil S, Rabbinowitsch E, Rajandream MA, Rutherford K, Rutter S, Seeger K, Saunders D, Sharp S, Squares R, Squares S, Taylor K, Warren T, Wietzorrek A, Woodward J, Barrell BG, Parkhill J, Hopwood DA. 2002. Complete genome sequence of the model actinomycete *Streptomyces coelicolor* A3(2). Nature 417:141-147.
3. Heueis N, Vockenhuber M-P, Suess B. 2014. Small non-coding RNAs in streptomycetes. RNA Biology 11:464-469.
4. Baltz RH. 1998. Genetic manipulation of antibioticproducing *Streptomyces*. Trends Microbiol 6:76-83.
5. Bérdy J. 2005. Bioactive Microbial Metabolites. The Journal of Antibiotics 58:1-26.
6. Lucas X, Senger C, Erxleben A, Gruning BA, Doring K, Mosch J, Flemming S, Gunther S. 2013. StreptomeDB: a resource for natural compounds isolated from *Streptomyces* species. Nucleic Acids Res 41:D1130-D1136.
7. Hesketh A, Chen WJ, Ryding J, Chang S, Bibb M. 2007. The global role of ppGpp synthesis in morphological differentiation and antibiotic production in *Streptomyces coelicolor* A3(2). Genome Biology 8:R161.
8. Whitesides GM. 2006. The origins and the future of microfluidics. Nature 442:368-373.
9. van der Meer JR, Belkin S. 2010. Where microbiology meets microengineering: design and applications of reporter bacteria. Nat Rev Microbiol 8:511-522.
10. Zang E, Brandes S, Tovar M, Martin K, Mech F, Horbert P, Henkel T, Figge MT, Roth M. 2013. Real-time image processing for label-free enrichment of *Actinobacteria* cultivated in picolitre droplets. Lab on a Chip 13:3707-3713.
11. Grünberger A, Schöler K, Probst C, Kornfeld G, Hardiman T, Wiechert W, Kohlheyer D, Noack S. 2017. Real-time monitoring of fungal growth and

morphogenesis at single-cell resolution. Engineering in Life Sciences 17:86-92.

12. Flärdh K, Buttner MJ. 2009. *Streptomyces* morphogenetics: dissecting differentiation in a filamentous bacterium. Nat Rev Microbiol 7:36-49.

13. Manteca A, Alvarez R, Salazar N, Yagüe P, Sanchez J. 2008. Mycelium Differentiation and Antibiotic Production in Submerged Cultures of *Streptomyces coelicolor*. Appl Environ Microbiol 74:3877-3886.

14. Horinouchi S, Beppu T. 1990. Autoregulatory Factors of Secondary Metabolism and Morphogenesis in Actinomycetes. Crit Rev Biotechnol 10:191-204.

15. Horinouchi S. 2002. A microbial hormone, A-factor, as a master switch for morphological differentiation and secondary metabolism in *Streptomyces griseus*. Frontiers in Bioscience 7:2045-2057.

16. Manteca A, Jung HR, Schwammle V, Jensen ON, Sanchez J. 2010. Quantitative Proteome Analysis of *Streptomyces coelicolor* Nonsporulating Liquid Cultures Demonstrates a Complex Differentiation Process Comparable to That Occurring in Sporulating Solid Cultures. Journal of Proteome Research 9:4801-4811.

17. Rioseras B, Lopez-Garcia MT, Yague P, Sanchez J, Manteca A. 2014. Mycelium differentiation and development of *Streptomyces coelicolor* in lab-scale bioreactors: Programmed cell death, differentiation, and lysis are closely linked to undecylprodigiosin and actinorhodin production. Bioresource Technol 151:191-198.

18. Wildermuth H. 1970. Development and Organization of the Aerial Mycelium in *Streptomyces coelicolor*. Microbiology 60:43-50.

19. Rigali S, Titgemeyer F, Barends S, Mulder S, Thomae AW, Hopwood DA, van Wezel GP. 2008. Feast or famine: the global regulator DasR links nutrient stress to antibiotic production by *Streptomyces*. Embo Rep 9:670-675.

20. van Keulen G, Jonkers HM, Claessen D, Dijkhuizen L, Wösten HAB. 2003. Differentiation and Anaerobiosis in Standing Liquid Cultures of *Streptomyces coelicolor*. J Bacteriol 185:1455-1458.

21. Claessen D, Rink R, de Jong W, Siebring J, de Vreugd P, Boersma FGH, Dijkhuizen L, Wosten HAB. 2003. A novel class of secreted hydrophobic proteins is involved in aerial hyphae formation in *Streptomyces coelicolor* by forming amyloid-like fibrils. Gene Dev 17:1714-1726.

22. Elliot MA, Karoonuthaisiri N, Huang J, Bibb MJ, Cohen SN, Kao CM, Buttner MJ. 2003. The chaplins: a family of hydrophobic cell-surface proteins involved in aerial mycelium formation in *Streptomyces coelicolor*. Gene Dev 17:1727-1740.

23. Yagüe P, Rodríguez-García A, López-García MT, Rioseras B, Martín JF, Sánchez J, Manteca A. 2014. Transcriptomic Analysis of Liquid Non-Sporulating *Streptomyces coelicolor* Cultures Demonstrates the Existence of a Complex Differentiation Comparable to That Occurring in Solid Sporulating Cultures. PLOS

ONE 9:e86296.

24. Gregory KF. 1956. Hyphal anastomosis and cytological aspects of *Streptomyces scabies*. Can J Microbiol 2:649-655.

25. Glass NL, Rasmussen C, Roca MG, Read ND. 2004. Hyphal homing, fusion and mycelial interconnectedness. Trends Microbiol 12:135-141.

26. Horinouchi S, Beppu T. 2007. Hormonal control by A-factor of morphological development and secondary metabolism in *Streptomyces*. Proceedings of the Japan Academy, Series B 83:277-295.

27. Yamazaki H, Ohnishi Y, Horinouchi S. 2003. Transcriptional switch on of *ssgA* by A-Factor, which is essential for spore septum formation in *Streptomyces griseus*. J Bacteriol 185:1273-1283.

28. Ueda K, Hsheh C-W, Tosaki T, Shinkawa H, Beppu T, Horinouchi S. 1998. Characterization of an A-Factor-Responsive Repressor for *amfR* Essential for Onset of Aerial Mycelium Formation in *Streptomyces griseus*. J Bacteriol 180:5085-5093.

29. Kato J-y, Suzuki A, Yamazaki H, Ohnishi Y, Horinouchi S. 2002. Control by A-Factor of a Metalloendopeptidase Gene Involved in Aerial Mycelium Formation in *Streptomyces griseus*. J Bacteriol 184:6016-6025.

30. Tomono A, Tsai Y, Ohnishi Y, Horinouchi S. 2005. Three Chymotrypsin Genes Are Members of the AdpA Regulon in the A-Factor Regulatory Cascade in *Streptomyces griseus*. J Bacteriol 187:6341-6353.

31. Hirano S, Kato J-y, Ohnishi Y, Horinouchi S. 2006. Control of the *Streptomyces* Subtilisin Inhibitor Gene by AdpA in the A-Factor Regulatory Cascade in *Streptomyces griseus*. J Bacteriol 188:6207-6216.

32. van Dissel D, Claessen D, van Wezel GP. 2014. Morphogenesis of *Streptomyces* in Submerged Cultures. Adv Appl Microbiol 89:1-45.

33. Giudici R, Pamboukian CRD, Facciotti MCR. 2004. Morphologically structured model for, antitumoral retamycin production during batch and fed-batch cultivations of *Streptomyces olindensis*. Biotechnol Bioeng 86:414-424.

34. Jonsbu E, McIntyre M, Nielsen J. 2002. The influence of carbon sources and morphology on nystatin production by *Streptomyces noursei*. J Biotechnol 95:133-144.

35. Stratigopoulos G, Gandecha AR, Cundliffe E. 2002. Regulation of tylosin production and morphological differentiation in *Streptomyces fradiae* by TylP, a deduced gamma-butyrolactone receptor. Mol Microbiol 45:735-744.

36. Cihak M, Kamenik Z, Smidova K, Bergman N, Benada O, Kofronova O, Petrickova K, Bobek J. 2017. Secondary Metabolites Produced during the Germination of *Streptomyces coelicolor*. Frontiers in Microbiology 8.

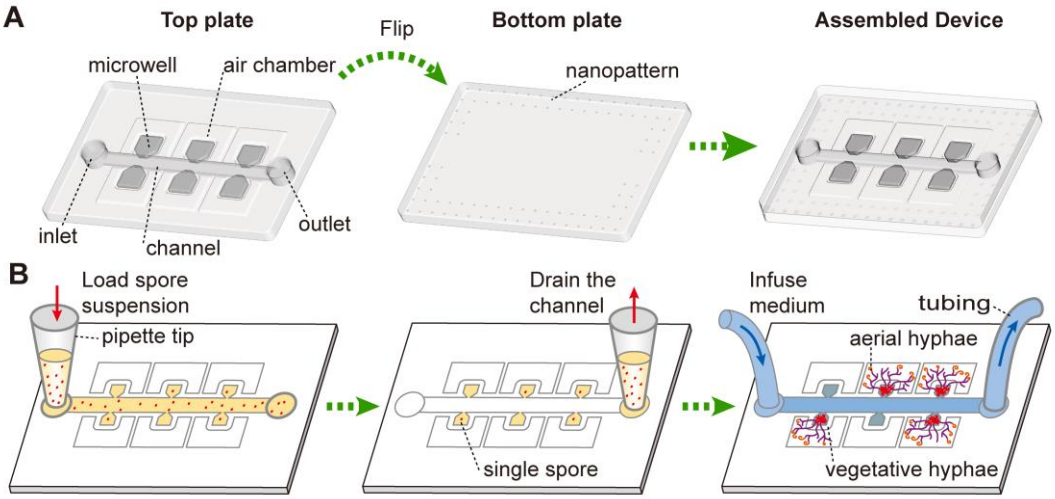
37. He Q, Chen S, Su Y, Fang Q, Chen H. 2008. Fabrication of 1D nanofluidic channels

493 on glass substrate by wet etching and room-temperature bonding. Anal Chim Acta
494 628:1-8.
495 38. Hsiao N-H, Nakayama S, Merlo ME, de Vries M, Bunet R, Kitani S, Nihira T,
496 Takano E. 2009. Analysis of Two Additional Signaling Molecules in *Streptomyces*
497 *coelicolor* and the Development of a Butyrolactone-Specific Reporter System.
498 Chem Biol 16:951-960.
499 39. Zhang Y, Wang M, Tian J, Liu J, Guo Z, Tang W, Chen Y. 2020. Activation of
500 paulomycin production by exogenous γ-butyrolactone signaling molecules in
501 *Streptomyces albidoflavus* J1074. Appl Microbiol Biotechnol 104:1695-1705.
502 40. Mori K, Chiba N. 1990. Synthetic microbial chemistry, XXIII. Synthesis of
503 optically active virginiae butanolides A, B, C, and D and other autoregulators from
504 streptomycetes. Liebigs Annalen der Chemie
505 doi:https://doi.org/10.1002/jlac.199019900105:31-37.
506 41. Parsons PJ, Lacrouts P, Buss AD. 1995. Synthesis of optically active A-factor.
507 Journal of the Chemical Society, Chemical Communications
508 doi:http://dx.doi.org/10.1039/C39950000437:437-438.
509 42. Chavan SP, Pasupathy K, Shivasankar K. 2004. Short and Efficient Synthesis of
510 (±) - A - Factor. Synthetic Commun 34:397-404.
511 43. Sarkale AM, Kumar A, Appayee C. 2018. Organocatalytic Approach for Short
512 Asymmetric Synthesis of (*R*)-Paraconyl Alcohol: Application to the Total
513 Syntheses of IM-2, SCB2, and A-Factor γ-Butyrolactone Autoregulators. The
514 Journal of Organic Chemistry 83:4167-4172.

515

516 **FIGURE LEGENDS**

517



518

519 **FIG 1** Illustration of the microfluidic chip for lifespan observation of *Streptomyces*. (A)

520 Assembly and setup of the device. (B) Spore suspension is loaded into the microwells by

521 a pipette. Concentration of spores is controlled to allow single spore trapping in the

522 microwells based on a Poisson distribution. The channel was drained by pipette to

523 remove spores in the channel. Culture medium was continuously infused into the device.

524 The *Streptomyces* development process was observed by an inverted microscope.

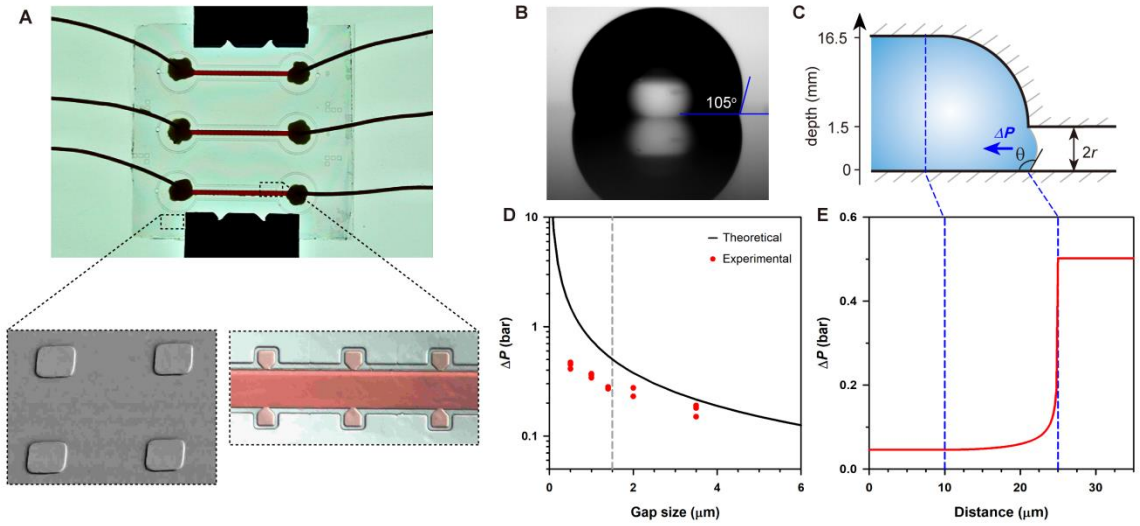
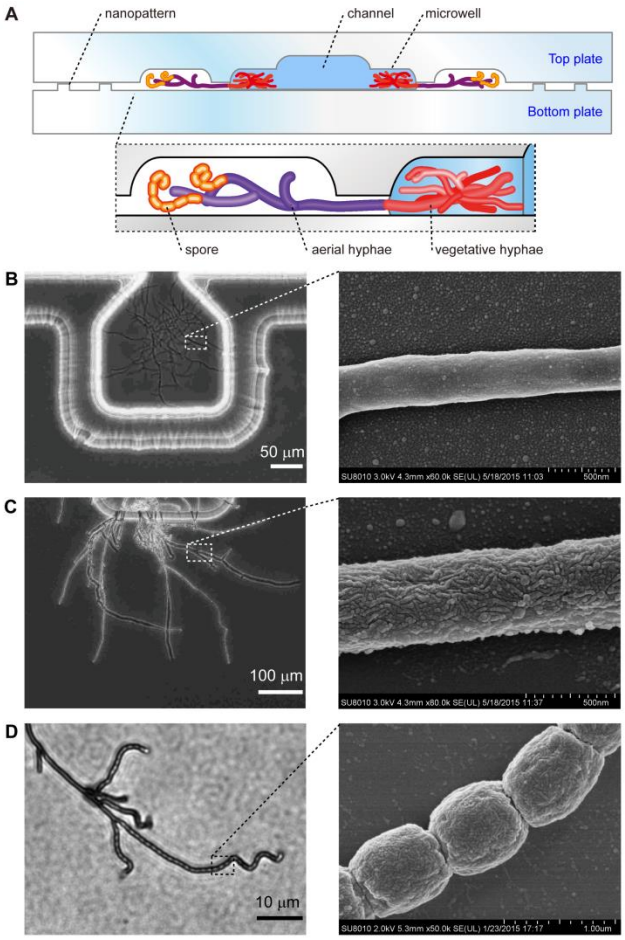


FIG 2 (A) A picture of the assembled device. 1.5-μm height nanogaps on the bottom plate were observed via scanning electron microscopy. An assembled device filled with red dye and zoom-in view of the channel with red dye. (B) The silanized glass plates of the device have a contact angle of 105° with deionized water. (C) Side view of the water-air interface between the microwell and the gas-filled chamber showing the direction of surface tension of solution at the edge of the microwells. (D) Relationship between surface tension and gap size at the water-air interface, and (E) the surface tension distribution along the microwell.



534

535 **FIG 3** Development of *S. coelicolor* cultivated in a microfluidic device. (A) Schematic of

536 sectional view of the device with *Streptomyces* development. (B-D) The vegetative

537 hyphae (B), aerial hyphae (C), and spores (D) of *S. coelicolor* were observed by optical

538 microscopy (left panel) and electron scanning microscopy (right panel), respectively.

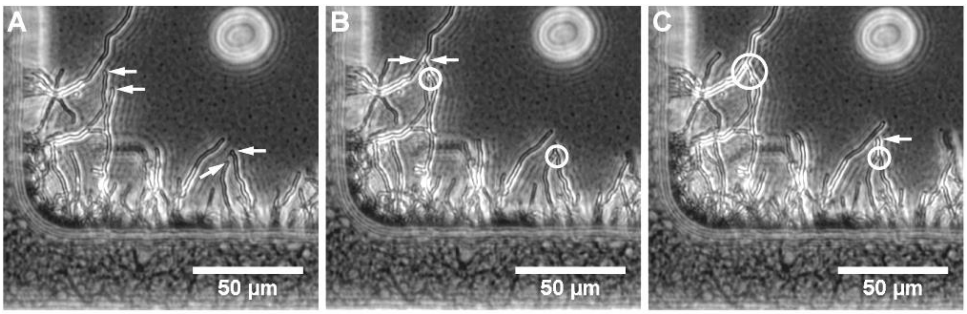
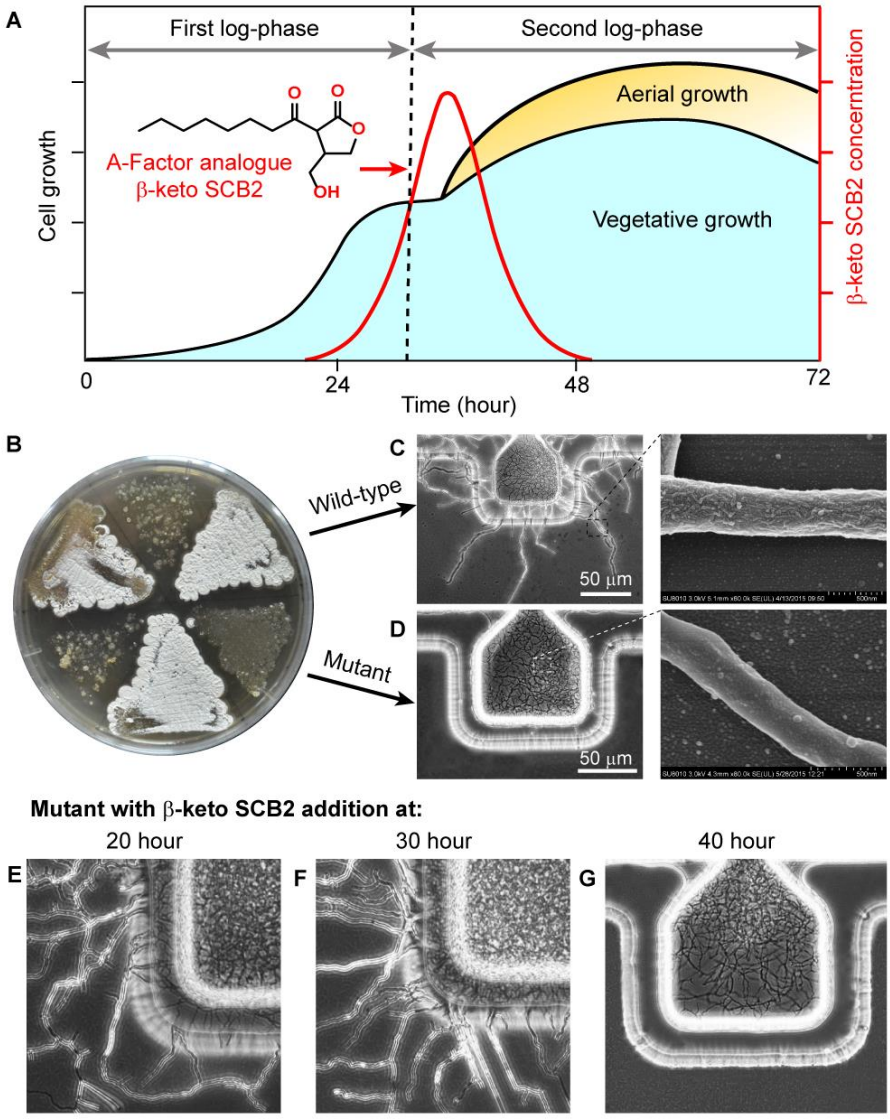


FIG 4 Hyphal anastomosis (fusion) in *S. coelicolor*. Some hyphal tips (arrowed) approaching for fusion (A). (B) shows fused hyphal branches (circled). A fused hyphal tip is growing towards a hyphal peg for subsequently fusion (C).



543

544 **FIG 5** Biosynthesis of A-Factor in a growth-dependent manner (A) and its role in the

545 development of *S. griseus* (B–E). Phenotypes of *S. griseus* wide-type and Δ*afsA* mutant

546 on solid plate (B). Aerial hyphae of *S. griseus* wide-type (C) and Δ*afsA* mutant (D)

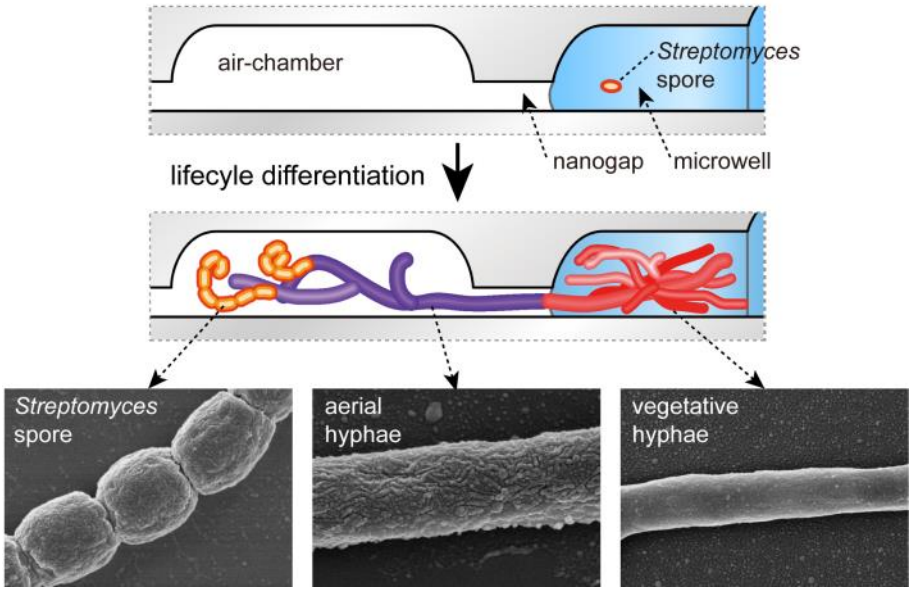
547 cultivated in a microfluidic device were studied via optical microscopy and electron

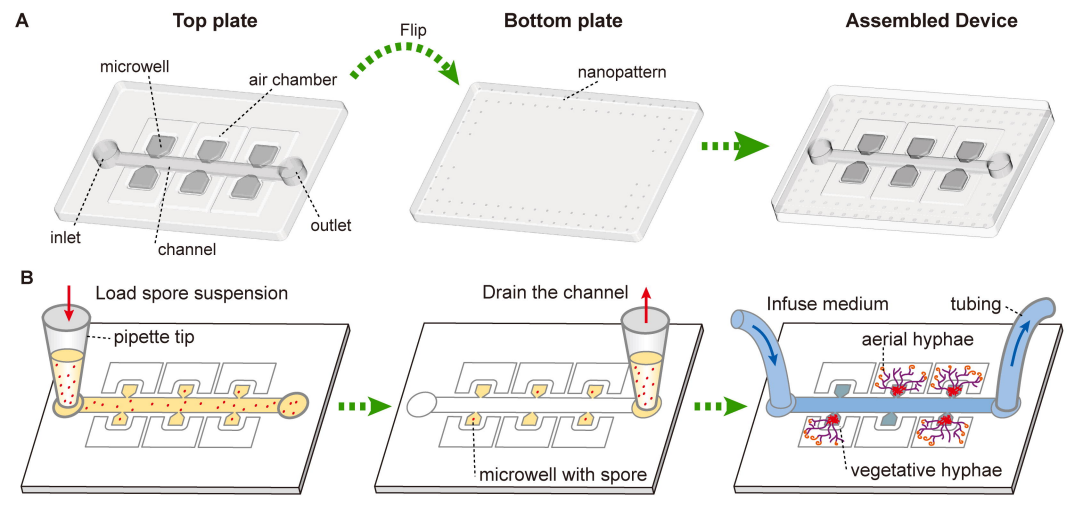
548 scanning microscopy. Feeding of A-Factor analogue and recovery of Δ*afsA* mutant

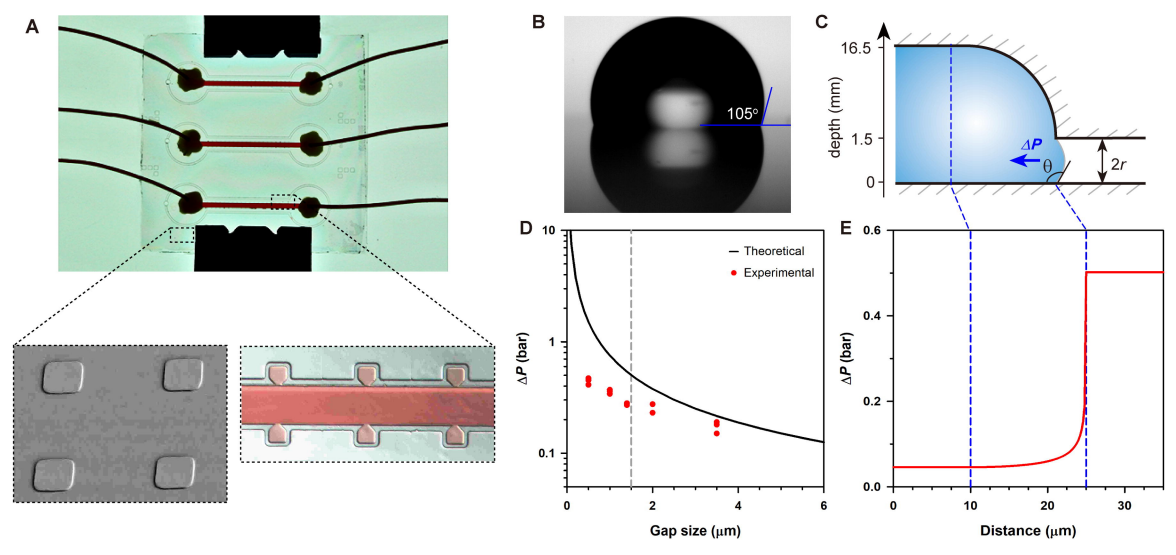
549 phenotype at 20 h (E), 30 h (F), and 40 h (G) after cultivation.

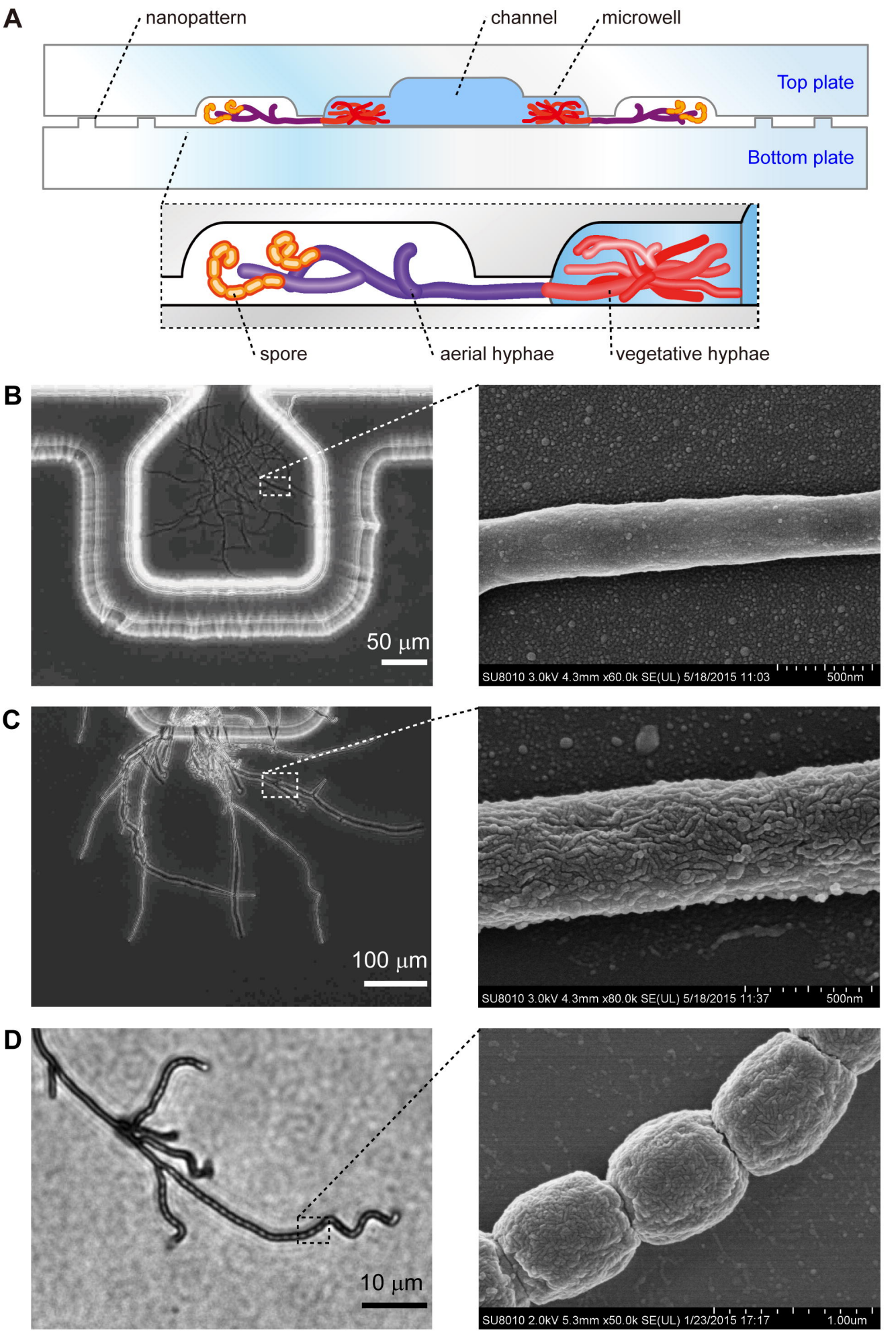
550

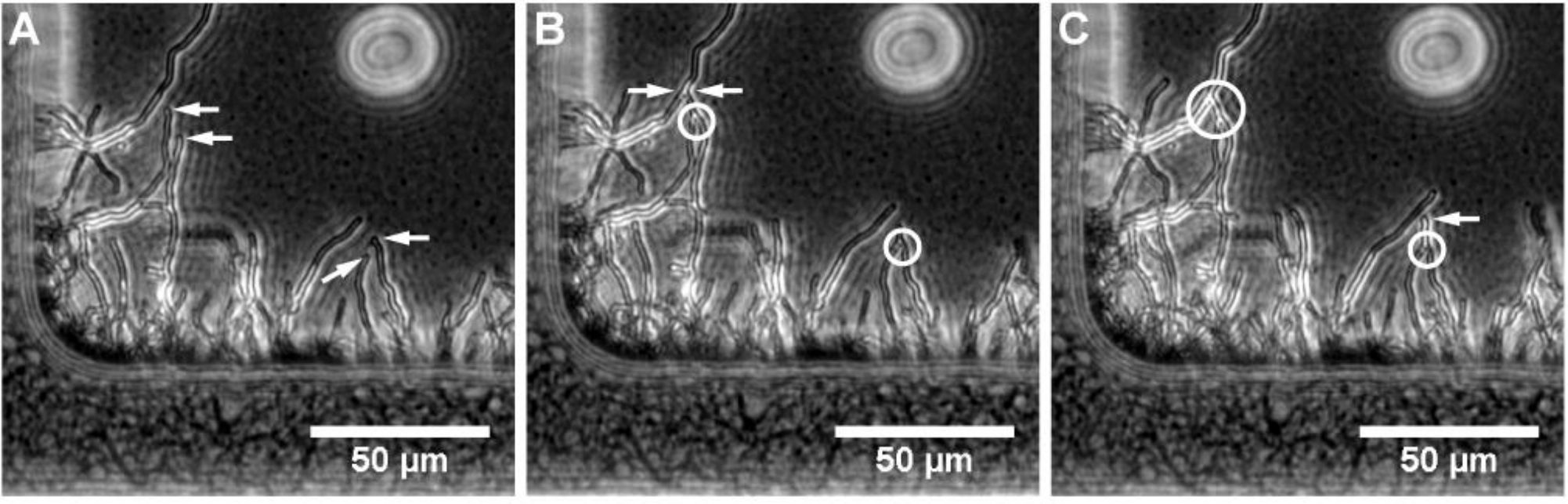
551 **Table of Content Graphic**

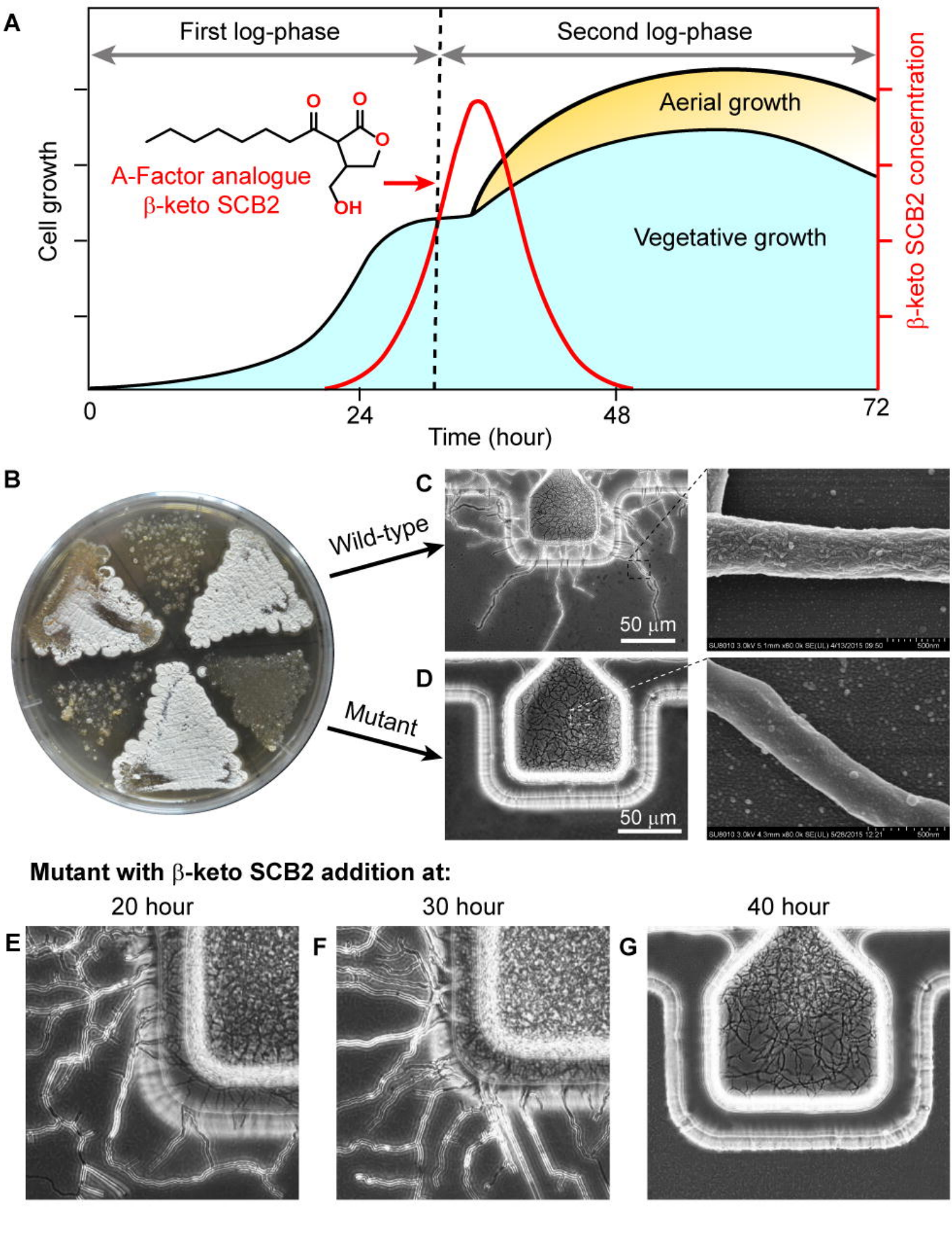
552 


A
Top plate
Flip
Bottom plate
Assembled Device
microwell
air chamber
nanopattern
inlet
channel
outlet
B
Load spore suspension
pipette tip
Drain the channel
Infuse medium
tubing
aerial hyphae
microwell with spore
vegetative hyphae


A
B
105°
C
16.5
1.5
0
depth (mm)
ΔP
θ
2r
D
10
1
0.1
ΔP (bar)
Theoretical
Experimental
0
2
4
6
Gap size (μm)
E
0.6
0.4
0.2
0.0
ΔP (bar)
0
10
20
30
Distance (μm)


A
nanopattern
channel
microwell
Top plate
Bottom plate
spore
aerial hyphae
vegetative hyphae
B
50 μm
SU8010 3.0kV 4.3mm x60.0k SE(UL) 5/18/2015 11:03
500nm
C
100 μm
SU8010 3.0kV 4.3mm x80.0k SE(UL) 5/18/2015 11:37
500nm
D
10 μm
SU8010 2.0kV 5.3mm x50.0k SE(UL) 1/23/2015 17:17
1.00um


A
50 µm
B
50 µm
C
50 µm


A
First log-phase
Second log-phase
Aerial growth
Vegetative growth
A-Factor analogue
β-keto SCB2
O
O
O
OH
Cell growth
β-keto SCB2 concentration
0
24
48
72
Time (hour)
B
C
D
Wild-type
Mutant
50 μm
50 μm
Mutant with β-keto SCB2 addition at:
20 hour
30 hour
40 hour
E
F
G