1 **Multiscale heterogeneity in filamentous microbes**

2

3 Boris Zacchetti[1], Han A. B. Wösten[2] and Dennis Claessen[1]*

4

5 [1] Microbial Biotechnology, Institute of Biology, Leiden University, Sylviusweg 72, 2333 BE
6 Leiden, The Netherlands.

7 [2] Microbiology, Department of Biology, Utrecht University, Padualaan 8, 3584 CH Utrecht, The
8 Netherlands.

9 Correspondence should be addressed to D.C. (email:D.Claessen@biology.leidenuniv.nl)

10

11 Keywords: heterogeneity, fungi, actinomycetes, biotechnology, multicellularity

12

13 **Abstract**

14

15 Microbial cells within clonal populations can display different morphologies or carry out
16 different tasks. This heterogeneity is beneficial at the population level and allows microbes to
17 spread risk or separate incompatible activities. Heterogeneity is also evident in filamentous
18 bacteria and fungi, which form mycelial networks consisting of interconnected hyphae. Here,
19 heterogeneity is observed between clonal mycelial particles, between different zones of
20 colonies, between adjacent hyphae and even between adjacent compartments of individual
21 hyphae. In this review, we compare this multiscale heterogeneity in filamentous bacteria and
22 fungi and discuss the underlying mechanisms. These mechanisms might provide targets to
23 improve the exploitability of these organisms as cell factories in the biotech sector.

24

1. Introduction

One of the assumptions in microbiology was that cells in a monoclonal microbial population would be phenotypically indistinguishable when provided with a constant environment. Over the last two decades, however, the development of high-throughput analytical techniques has enabled microbiologists to study large numbers of cells at the individual level (Binder et al., 2017; Brehm-Stecher and Johnson, 2004; Davis and Isberg, 2016) and to unambiguously demonstrate that processes such as metabolism, transcription, translation and protein secretion are heterogeneous in space and time across cells (Ackermann, 2015; Avery, 2006; Smits et al., 2006; van Boxtel et al., 2017; Veening et al., 2008b; Wösten et al., 2013). Examples of these heterogeneities have been documented in a wide range of microorganisms, including some of the best-characterized prokaryotic and eukaryotic model organisms (e.g. *Escherichia coli, Bacillus subtilis* and *Saccharomyces cerevisiae*, amongst others) (Chastanet et al., 2010; Elowitz et al., 2002; Levy et al., 2012; Maamar et al., 2007). While heterogeneity has been mostly addressed in unicellular microbes, it is also evident in multicellular species. In this review, we will focus on heterogeneities in filamentous microorganisms that are employed as cell factories in the industrial sector. We will discuss the consequences (disadvantages and benefits) of these heterogeneities and the mechanisms through which these are established.

2. The consequences of phenotypic heterogeneity

Phenotypic heterogeneity allows microbes to withstand environmental fluctuations and carry out specialized functions at the level of single cells. In their natural habitats, microbes are confronted with rapidly changing environmental conditions. The best-known mechanism to withstand such changes is to modulate gene expression (Jacob and Monod, 1961). Changes in gene expression can lead to profound phenotypic changes, including cellular differentiation. Many microbes, however, ensure that a number of cells within a clonal population already

possess certain defensive traits, even when the corresponding environmental stimulus is not present (Philippi and Seger, 1989). As a consequence of this strategy, commonly referred to as bet-hedging, only a fraction of the cells will pay the cost (e.g. reduced metabolic proficiency) associated with the expression of those genes conferring potentially useful features. Should the environmental conditions become adverse and change in their favor, these cells would already be equipped to withstand the altered conditions and are therefore more likely to survive. This behavior is beneficial to the entire population (Grimbergen et al., 2015).

Bet-hedging and phenotypic heterogeneity have been extensively studied in *Bacillus subtilis*. This Gram-positive bacterium forms endospores when exposed to stress conditions (e.g. starvation or the presence of toxins) (Errington, 2003; Higgins and Dworkin, 2012). These spores are metabolically dormant and highly resistant to extreme temperatures, desiccation and ionizing radiation. When environmental conditions suitable for growth are restored, spores germinate to establish colonies of vegetative cells. A bet-hedging control of sporulation has two main advantages. On the one hand, it assures that not all cells commit themselves to sporulation, which is notoriously a lengthy and irreversible process (Chastanet et al., 2010; Russell et al., 2017). In this way, when only a fraction of the cells sporulates, the non-sporulating ones can quickly reinitiate growth in case the stress condition turns out to be transient, hence preventing the population from becoming outnumbered by competitors. On the other hand, since environmental changes are sometimes too harmful for the sporulation process to complete, the stochastic initiation of sporulation ensures that some cells undergo sporulation even in the absence of adverse conditions (Siebring et al., 2014; Veening et al., 2008c).

Another canonical example of the benefits of microbial individuality is that of bacterial persistence. A fraction of *Escherichia coli* cells forms metabolically dormant persister cells that are able to withstand various environmental insults, such as the prolonged exposure to antibiotics (Balaban et al., 2004), and to resume growth when the original conditions are restored (Fig. 1). Sub-populations of non-growing persisters that survive exposure to antibiotics have also been reported in *Salmonella* (Claudi et al., 2014; Helaine et al., 2014)

and *Mycobacterium* (Manina et al., 2015). The appearance of persisters can also occur when cells are not challenged by antimicrobials (Balaban et al., 2004), although stressful conditions can enhance their abundance within a population (Dörr et al., 2010; Johnson and Levin, 2013; Mulcahy et al., 2010). A similar strategy has been reported in *Saccharomyces cerevisiae*. Within clonal populations of this yeast, certain individuals are characterized by lower growth rates and concomitantly possess higher resistance to heat shock due to the accumulation of the protecting disaccharide trehalose (Levy et al., 2012). Notably, experimentally tuning growth rates of *S. cerevisiae* using chemostat cultures has highlighted that many of the genes activated upon heat stress are also active under conditions of slow growth, which is again substantiated by the observation that cells that grow slowly are more resistant to heat stress (Lu et al., 2009).

Recent studies have shown that bet-hedging strategies culminating in phenotypic heterogeneity also become evident when microbial cells are exposed to fluctuating nutritional regimes (Kotte et al., 2014; Solopova et al., 2014; van Heerden et al., 2014). This is no surprise when considering the rapidly changing nutritional conditions that microbial cells endure in their natural environments. When exposed to mixtures of carbon source, microbes typically consume them in a sequential manner. As a consequence, distinct growth phases are observed that are separated by a lag phase. During this phase, cells are believed to undergo the physiological adaptations needed for the uptake and consumption of the second carbon source. This behavior is known as diauxie (Monod, 1949). The diauxic shift in *Lactococcus lactis* is explained by the fact that only a limited number of cells is able to metabolize the second carbon source. Interestingly, these cells emerge when the preferred carbon source is still present (Solopova et al., 2014). The decision to commit to the metabolism of the less preferred source depends on the metabolic state of the cell prior to the depletion of the preferred carbon source, while the number of cells enacting the shift is inversely proportional to the abundance of the first carbon source. This mechanism provides an alternative explanation for the decades-old concept of metabolic adaptation during diauxic shifts, which may also be relevant for other lag phases observed in microbiology.

109 While bet-hedging is beneficial in unpredictable and fluctuating environments,
110 phenotypical heterogeneity can also be advantageous in non-fluctuating conditions, for
111 instance when different processes have to be carried out simultaneously within a clonal
112 population. This so-called division of labor is characterized by the coexistence of
113 subpopulations of cells specialized in performing complementary tasks (van Gestel et al.,
114 2015a, b; Zhang et al., 2016). Under nutrient-limiting conditions, *B. subtilis* secretes subtilisin
115 E, which degrades proteins into small peptides that are accessible to all community members
116 (Veening et al., 2008a). Single-cell measurements have revealed that only a minority of cells
117 produce and secrete this protease, indicating that only a few members of the clonal population
118 pay the cost associated with its production. It is not yet clear whether this strategy represents
119 a form of pure altruism whereby the producing cells pay the production-associated costs for
120 the benefit of the entire population, or whether it represents a cooperative behavior in that both
121 the producer and the recipient cells mutually benefit from each other. Numerous are the other
122 reported examples where some cells pay the cost for the benefit of the entire population; these
123 include *Salmonella enterica* (Arnoldini et al., 2014; Diard et al., 2013), *Myxococcus xanthus*
124 (Velicer et al., 2000), and certain protozoans (Strassmann et al., 2000). *M. xanthus* represents
125 perhaps the most spectacular example of the commitment of subsets of cells within a given
126 population to a specific function. In the presence of excess nutrients, *M. xanthus* establishes
127 a motile group of cells called a swarm. The swarm explores the environment to forage for
128 nutrients or predate on other bacteria (Reichenbach, 1999). Upon starvation, growth is
129 arrested, and a developmental program is initiated that culminates in the formation of spore-
130 bearing fruiting bodies. Three distinct subpopulations of cells contribute to the formation of
131 fruiting bodies. While only 10% of cells differentiate into spores, roughly 30% form peripheral
132 rods on the outer surface of the fruiting body, while the remaining fraction undergoes
133 programmed cell lysis (Nariya and Inouye, 2008; O'Connor and Zusman, 1991; Wireman and
134 Dworkin, 1977). The fact that such a major fraction of the population undergoes PCD is a
135 remarkable example of social behavior, with the lysing cells providing nutrients and energy for

136 sporulation to complete and in turn ensure the propagation of their genome (Berleman et al., 137 2006; Wireman and Dworkin, 1977).

138 In some microorganisms, division of labor is employed as a strategy to perform 139 incompatible metabolic processes (de Lorenzo et al., 2015; Johnson et al., 2012; Levine et al., 140 2013). The best-known example of this strategy is the spatial segregation of nitrogen fixation 141 and photosynthesis in cyanobacteria (Mitsui et al., 1986) (Fig. 1). The nitrogenase enzyme 142 required for nitrogen fixation is sensitive to oxygen, the product of photosynthesis. For this 143 reason, some cyanobacteria generate specialized cells called heterocysts, which are 144 specialized in nitrogen fixation while lacking the oxygenic photosystem (Adams, 2000). 145 Heterocysts also have a different cell wall composition that contributes to the exclusion of 146 oxygen to protect the nitrogenase enzyme (Kumar et al., 2010). On the other hand, non-147 heterocystous cyanobacteria separate photosynthesis and nitrogen fixation by temporally 148 segregating the two incompatible processes (Berman-Frank et al., 2001).

149

150 3. Mechanisms underlying individuality in unicellular microbes

151

152 When compared to higher multicellular eukaryotes, the regulation of gene expression in 153 microorganisms (especially in prokaryotes) appears to be controlled by only a handful of 154 mechanisms. As a consequence, one would expect any given gene to be expressed at a 155 similar level in isogenic microbial cells exposed to the same environmental conditions. 156 However, it is well accepted that bacterial gene expression is subject to intrinsic noise. In a 157 pioneering study that paved the road for the nascent field of microbial heterogeneity, cells of 158 *E. coli* were engineered to express two distinguishable fluorescent proteins under control of 159 the same promoter. Major differences in the expression of these reporters were detected both 160 within and between cells, indicating that gene expression is subject to intrinsic fluctuations 161 (Elowitz et al., 2002). It is interesting to note that the level of transcription negatively correlates 162 with the heterogeneity in fluorescence emission, indicating that intrinsic noise is more 163 pronounced at low transcriptional levels. Extensive work has shown that a considerable degree

164 of heterogeneity between microbial cells originates from the fact that transcription and
165 translation occur in so called pulses or “bursts” (Blake et al., 2003; Cai et al., 2006; Golding et
166 al., 2005; Ozbudak et al., 2002). Since these stochastic pulses are asynchronous between
167 cells, distinct subpopulations can evolve and coexist. A number of studies on *B. subtilis* have
168 shown how pulsating genetic circuits control processes such as the development of
169 competence, the onset of sporulation, and the response to environmental stresses (Levine et
170 al., 2012; Locke et al., 2011; Süel et al., 2007; Young et al., 2013). Notably, single cell
171 measurements in both prokaryotic and eukaryotic microbes have revealed disparities in the
172 degree of transcriptional noise between different genes within a single cell. These disparities
173 are seemingly not arbitrary, as the transcription of housekeeping genes is generally less noisy
174 than that of genes associated with stress or dispensable metabolic functions (Newman et al.,
175 2006; Silander et al., 2012; Taniguchi et al., 2010).

176 Phenotypic heterogeneity between cells can also originate from transcriptional
177 differences caused by cellular processes. Several studies have shown that physiological
178 factors such as growth rate and cell cycle stage can substantially influence gene expression
179 (Berthoumieux et al., 2013; Slavov and Botstein, 2013). Single-cell studies have recently shed
180 light on the importance of the feedback of growth in causing heterogeneity. For example,
181 fluctuations in the expression of metabolic genes can lead to fluctuations in the growth rate of
182 individual cells, which in turn not only perturb the expression of other metabolic genes, but also
183 of unrelated gene networks (Kiviet et al., 2014; Klumpp and Hwa, 2014; Tan et al., 2009).
184 Deterministic choices can also be a source of cell-to-cell heterogeneity. For example, metal
185 ion scarcity leads to a growth arrest in newborn daughter cells of *S. cerevisiae* (i.e. cells which
186 have not budded yet). As a consequence, two populations of cells emerge: older dividing cells
187 and younger non-dividing cells (Avraham et al., 2013). This is explained by the fact that the
188 vacuole, which is the reservoir for metals, is not propagated to daughter cells, while it is
189 maintained in the mother cells which keep dividing. This strategy results in higher fitness under
190 zinc-limiting conditions than in a mutant strain where vacuole segregation occurs

191 homogeneously. In the latter case, zinc is diluted in fact to an extent that eventually impedes
192 cellular division.

193 The generation of phenotypic heterogeneity and multi-stability have also been the
194 subject for numerous mathematical models. Such models are important for better
195 understanding the principles behind bet-hedging and provide predictive value that can be
196 tested experimentally. Recent models include the stochastic nature of cellular processes and
197 provide a powerful framework for understanding phenotypic switching between different
198 cellular states. We here wish to refer to some excellent papers for readers interested to learn
199 more about this aspect (see e.g. Henson, 2003; Jia et al., 2014; Meister et al., 2014; Wilkinson,
200 2009, and references therein).

201

202 4. Heterogeneity in filamentous organisms

203

204 The features of phenotypic heterogeneity discussed in the previous sections relate to
205 unicellular microorganisms, the cells of which are, at least under most conditions, spatially
206 separated from one-another. In contrast, many multicellular microbes such as filamentous
207 actinomycetes and fungi grow by means of interconnected filaments that only physically
208 separate into unicellular propagules during the reproductive phase (Claessen et al., 2014).
209 From a morphological perspective, the mode of growth of filamentous actinomycetes is similar
210 to that of filamentous fungi. This is the reason why, despite bearing the structural features of
211 bacteria, actinomycetes were originally believed to be fungi (Goodfellow et al., 1983). In
212 contrast to most unicellular organisms, filamentous fungi and actinomycetes possess a
213 complex life cycle characterized by distinct developmental stages and the co-existence of
214 different specialized cells. Both kinds of microorganisms propagate via spores, dormant cells
215 equipped to withstand harsh environmental conditions (Barka et al., 2016; Walker and White,
216 2005). Spores germinate under favorable conditions, leading to germ tubes that elongate to
217 form thread-like cells called hyphae. Hyphae of filamentous fungi and actinomycetes have a
218 diameter of about 2-10 μm and 0.5-2 μm, respectively. They elongate at their tip (or apex),

219 while new hyphae emerge subapically by branching (Flärdh, 2010; Riquelme, 2013). The
220 combination of apical growth and branching yields an interwoven cellular network called a
221 mycelium. The growing vegetative mycelium (also called substrate mycelium) colonizes the
222 environment by radiating leading hyphae from peripheral regions of the colony. The
223 encountered polymeric substrates are degraded by means of secreted hydrolytic enzymes and
224 the degradation products are internalized by the cells to serve as nutrients (Barka et al., 2016).
225 When nutrients become scarce, colonies of filamentous microbes develop into complex
226 multicellular consortia of different cell types (Chater, 1998; Krijgsheld et al., 2013a; Kues and
227 Liu, 2000). For instance, while the peripheral regions of *Streptomyces* colonies proceed with
228 vegetative growth, more central and non-growing parts of the colony undergo an ordered
229 process of chemical and morphological differentiation (Borkovich and Ebbole, 2010; Manteca
230 et al., 2005a). Such chemical differentiation is responsible for the production of various
231 secondary metabolites, many of which are exploited for commercial use (Barka et al., 2016;
232 Bérdy, 2005; Hopwood, 2007) (*see below*). Coinciding with this chemical differentiation,
233 specialized aerial hyphae emerge on the colony surface. The aerial hyphae of actinomycetes
234 differentiate into chains of unigenomic spores, while those of fungi form more elaborate
235 asexual (e.g. condidiophores) or sexual reproductive structures (e.g. mushrooms).

236 When grown in close proximity to certain yeasts (e.g *S. cerevisiae*) or when faced with
237 conditions of nutrient scarcity, some streptomycetes (i.e. the best-studied and industrially the
238 most relevant representatives of the actinomycetes) form so-called “exploring” cells. These
239 cells can travel over nutrient-void abiotic surfaces and promote the spreading of colonies over
240 large surfaces (Jones et al., 2017). Despite being morphologically similar to aerial hyphae in
241 that they do not branch, exploratory hyphae miss the hydrophobic coating which is distinctive
242 of aerial structures, and hence represent a new cellular type with features of both vegetative
243 and aerial hyphae. This functional differentiation is reminiscent of a bet-hedging strategy, with
244 explorer cells allowing dispersal in those cases in which sporulation might be too costly or take
245 too long to complete (Jones and Elliot, 2017).

246 Although the distinction in structure and function between vegetative and reproductive
247 hyphae has been known for many decades (Chater, 1998), we are now beginning to
248 understand that heterogeneity is also evident in mycelial aggregates growing in liquid
249 environments, between zones of mycelia, between adjacent hyphae within a colony zone and
250 even between compartments of a single hypha. In the following sections we will first describe
251 methodologies used to study heterogeneity in filamentous microbial populations before
252 discussing intra- and inter-hyphal heterogeneity in the vegetative mycelium of streptomycetes
253 and filamentous fungi on solid substrates. We will then discuss heterogeneity in liquid-grown
254 mycelia and its effect on production performances of commercially valuable products.
255
256 *4.1 Techniques for studying phenotypic heterogeneity in filamentous microbes*
257
258 The analysis of heterogeneity relies on techniques that enable the qualitative and quantitative
259 assessment of physiological traits at the single cell level. Microfluidics and flow-cytometry
260 approaches have contributed strongly to the study of phenotypic heterogeneity in unicellular
261 microbes (Ackermann, 2015; Avery, 2006; Davis and Isberg, 2016). Microfluidics systems use
262 miniaturized growth chambers that allow growth of various cell types in a finely controlled
263 microenvironment (Delvigne et al., 2017). Because of this feature, differences observed
264 between cells can exclusively be attributed to intrinsic cellular heterogeneity and not to varying
265 environmental conditions. Most microfluidics devices can easily be accommodated in various
266 type of microscopes which, in turn, allows to finely track growth of single cells, but also to use
267 fluorescent reporters (i.e. fluorescent proteins and dyes). Different concepts have been
268 developed in recent years, encompassing a large range of sizes and designs (Grunberger et
269 al., 2014; Hol and Dekker, 2014; Reece et al., 2016; Wu and Dekker, 2016). The use of
270 microfluidics for studying filamentous organisms is limited (Grünberger et al., 2013), which
271 relates to the fact that mycelia typically form large multicellular structures formed by hyphae
272 growing and branching in three dimensions. As a result, the mycelium easily grows out of the

273 crafted chambers. Microfluidic approaches would be feasible only by confining growth to two
274 dimensions, which could however dramatically affect the physiology of the mycelium.
275 While microfluidic approaches are valuable for studying the behaviour of individual
276 cells, flow cytometry allows for the rapid analysis of large numbers of cells. Multiple parameters
277 are analysed, including cell size, granularity, and fluorescence. As in the case of microfluidics,
278 suspended cells or cell aggregates (mainly encountered in filamentous microbes) sense a
279 constant environment in well-mixed submerged cultures, which allows to directly designate the
280 observed heterogeneity as an intrinsic property of the system under analysis. Notably,
281 conventional flow cytometers are not suitable for the analysis of mycelial particles, due to the
282 large size of these structures. However, a number of cytometric apparatuses are nowadays
283 available that were specifically developed for large objects and have been successfully used
284 to study differences between mycelial particles within populations of filamentous microbes (de
285 Bekker et al., 2011b; Petrus et al., 2014; van Veluw et al., 2012). These approaches are
286 however limited to the discrimination of heterogeneity *between* distinct particles and lack the
287 resolution to study heterogeneity *within* individual particles. To study heterogeneities at a lower
288 scale (e.g. between distinct filaments in individual particles), fluorescence microscopy-based
289 approaches are most commonly used. Alternatively, laser capture microdissection (LCM) can
290 be used to collect individual mycelial sections or even individual filaments, which can be
291 subsequently analysed in a comprehensive manner using -omics or next generation
292 sequencing techniques (de Bekker et al., 2011a; de Bekker et al., 2011b).
293 One other technique to study heterogeneity in filamentous microbes is nanoscale
294 secondary ion mass spectrometry (nanoSIMS). nanoSIMS provides information on the
295 molecular and isotopic compositions of various types of biological samples with a high spatial
296 resolution (He et al., 2017; Nunez et al., 2017). This technique has recently been used to
297 detect differences in carbon assimilation between adjacent cells of the non-branching
298 actinomycete *Microthrix parvicella* (Sheik et al., 2016). NanoSIMS can thus be used to
299 characterize metabolic differences between cellular compartments along hyphae.
300

301 *4.2 Heterogeneity in the vegetative mycelium of filamentous organisms on solid substrates*

302

303 The vegetative mycelium of fungi and streptomycetes simultaneously performs a large number
304 of different tasks. Besides producing and secreting enzymes for nutrient assimilation, mycelia
305 transport nutrients and chemically differentiate to produce a plethora of secondary metabolites
306 (Barka et al., 2016; Borkovich and Ebbole, 2010; Hopwood, 2007). Given that many of these
307 metabolites are of great value to industry, much attention has traditionally been focused on the
308 optimization of production performances in filamentous microbes. However, research in this
309 direction has often been performed using "blind" screening procedures rather than strain
310 optimization strategies based on a deep knowledge of the producing organism (Papagianni,
311 2004). What has for instance been largely ignored so far is where the production of all these
312 compounds occurs within the mycelium, and how approaches to increase productivity correlate
313 with changes in the localization of production.

314 The vegetative mycelium of several *Streptomyces* species is heterogeneous with
315 respect to cellular morphology and physiology. More specifically, the vegetative growth of
316 streptomycetes has been found to encompass two phases during which different cell types are
317 formed (Manteca et al., 2005a, b). The young mycelium that is established after spore
318 germination is highly compartmentalized. The approximately 1-µm-wide compartments are
319 thought to be separated by membrane structures and/or thin peptidoglycan-containing septa
320 (Yagüe et al., 2013; Yagüe et al., 2016). This first compartmentalized mycelium, called MI
321 mycelium, undergoes an ordered process of dismantling, which is followed by a second growth
322 phase during which a multinucleated mycelium is established (MII). The cellular compartments
323 in this mycelium are significantly larger than those formed in the MI mycelium (Manteca et al.,
324 2005b). Following growth, the MII mycelium undergoes a new round of dismantling, while the
325 remaining viable hyphae form reproductive aerial hyphae that grow into the air (Manteca et al.,
326 2007; Manteca et al., 2005a). Both death rounds are the effect of a regulated cell suicide
327 process, which bears close analogies to that of apoptosis in eukaryotic cells. This resemblance
328 is illustrated by indicators such as the disruption of the cell wall and the cell membrane, the

329 degradation of DNA and the release of the cytoplasmic content into the extracellular medium
330 (Manteca et al., 2006). A proteomic characterization of the first apoptotic process in *S.*
331 *coelicolor* has highlighted that the majority of the *S. coelicolor* proteins involved in the first
332 apoptotic process localize at the cell wall, which thus seems to represent the first target to be
333 dismantled during the PCD process (Manteca et al., 2010). Other proteins participating in cell
334 dismantling are enzymes involved in the metabolism of fatty acids, various hydrolases,
335 catabolic enzymes, and proteases. The activity of these enzymes is accompanied by an
336 increase in membrane permeability and the subsequent leakage of cytosolic components into
337 the extracellular medium. While the process of cellular dismantling has been observed and
338 described in several streptomycetes, virtually nothing is known about its regulation and how it
339 spatially and temporarily correlates with other processes, such as antibiotic production.

340 Heterogeneity in the vegetative mycelium of filamentous fungi grown on solid
341 substrates occurs between zones of a colony, between neighboring hyphae within a zone and
342 between compartments of a single hypha (Fig. 2). The first reports on inter-zonal heterogeneity
343 focused on protein secretion within the vegetative mycelium of *Aspergillus niger* and
344 *Phanerochaete chrysosporium.* Secretion of the starch-degrading enzyme glucoamylase was
345 found to be spatially confined to the peripheral zone of *A. niger* (Wösten et al., 1991), while
346 lignin peroxidases were found to be released within the central zone of colonies of
347 *P. chrysosporium* (Moukha et al., 1993a; Moukha et al., 1993b). Later studies revealed that
348 each zone of an *A. niger* colony has its own secretome composition (Krijgsheld et al., 2012).
349 For instance, 6 and 10 proteins are at least 4-fold more and less abundant, respectively, in the
350 outer zone when compared to an intermediate zone. Interestingly, zonal differences in
351 expression in *A. niger* colonies can be explained by both medium-dependent and medium-
352 independent mechanisms (Levin et al., 2007). The concentration and nature of the carbon
353 source determines about half of the variation in gene expression, whereas the other half is
354 attributed to differentiation processes in the vegetative mycelium (Levin et al., 2007). The
355 nature of these differentiation processes is not yet known.

356 Growth at the outer zone of a fungal colony is supported by nutrients in the substrate
357 while the carbon source is exhausted in the central parts of the colony (clearly, the same holds
358 for the mycelia of bacterial species). Here, the hyphae switch from growth on exogenous to
359 endogenous carbon (Pollack et al., 2008). This is accompanied by vacuolization, reduced
360 growth rate, and a decrease of the hyphal diameter. Vacuolar degradation produces sufficient
361 endogenous carbon to support the formation of so-called secondary hyphae. In contrast to
362 streptomycetes, the endogenous carbon source is not released extracellularly and then
363 internalized by other hyphae, but it is transported to the tips of the newly formed filaments. This
364 mechanism secures the nutrients for the fungus rather than enabling competing microbes to
365 absorb them from the environment. Yet, the autolysis of hyphae with the release of nutrients
366 in the medium may also take place in starving zones of colonies (Perez-Leblic et al., 1982).
367 Future studies are needed to reveal which strategy of nutrient recycling is the most dominant
368 in the fungal mycelium.

369 Enzyme secretion was initially believed to only occur in growing fungal hyphae
370 (Wessels, 1993), whereas it is nowadays clear that it can also occur in non-growing zones of
371 a colony (Krijgsheld et al., 2013b; Levin et al., 2007). How proteins are released into the culture
372 medium by non-growing hyphae is not yet understood knowing that pores in the hyphal cell
373 walls are too small to enable proteins to freely diffuse (Wessels, 1988, 1993). In the case of
374 growing hyphae, such pores are not needed since proteins to be released in the culture
375 medium can co-migrate with the newly synthesized cell wall polysaccharides that are extruded
376 at the tips of growing hyphae and pushed from the inner to the outer part of the cell wall by the
377 turgor pressure and the addition of new cell wall material. Notably, although both growing and
378 non-growing colony zones can secrete proteins in the culture medium, not every zone does
379 so. The sub-peripheral zone of *A. niger* colonies is able to sporulate when environmental
380 conditions are favorable to enable this differentiation process. This zone does not secrete
381 proteins even when sporulation does not take place (Krijgsheld et al., 2013b). A strain of *A.*
382 *niger* in which the sporulation gene *flbA* is deleted is no longer able to asexually reproduce

383 and secretes proteins throughout the whole mycelium (Krijgsheld et al., 2013b). The *flbA*
384 deletion strain also shows a more complex secretome consisting of a number of proteins that
385 are not secreted by the wild-type strain. Together, these observations indicate that sporulation
386 inhibits protein secretion in fungal colonies. From a functional perspective, this appears as
387 coherent behavior. Once hyphae engage in sporulation, it would be inefficient to invest energy
388 in the secretion of enzymes involved in vegetative growth. To further study the phenomenon
389 of sporulation inhibited protein secretion, the impact of deletion of *fluG* in *A. niger* was studied
390 (Wang et al., 2015). This gene is at the start of the sporulation program in *Aspergillus nidulans.*
391 Yet, the *fluG* mutant strain of *A. niger* was shown not to be affected in sporulation. However,
392 in contrast to wild-type *A. niger*, the deletion strain shows breakdown of starch under the whole
393 colony. From these and other data it was concluded that FluG is a repressor of secretion in the
394 sporulation zone.

395 Immuno-localization showed that not every hypha within the outer zone of the *A. niger*
396 colony secretes glucoamylase (Wösten et al., 1991). Indeed, two types of hyphae were shown
397 to exist in this zone; hyphae that highly and hyphae that lowly express the glucoamylase gene
398 (Vinck et al., 2005). This heterogeneity in expression was also observed for other genes
399 encoding hydrolytic enzymes (Vinck et al., 2011). In fact, those hyphae that highly express one
400 of the hydrolase genes were also found to highly express the other hydrolase-encoding genes.
401 In addition, they possess a higher transcriptional and translational activity when compared to
402 hyphae that show lower expression. Nevertheless, both types of hyphae show a similar growth
403 speed, indicating that for secretion to take place a higher transcriptional and translational
404 activity is needed. Our recent findings show that the hyphae showing lower transcriptional and
405 translational activity are also more resistant to heat stress (M Tegelaar, R Bleichrodt and HAB
406 Wösten, unpublished data). Thus, hyphae seem to show a division of labor strategy at the
407 periphery of *Aspergillus* colonies.

408 Division of labor is also evident between hyphal compartments of *A. niger* (Tegelaar
409 and Wösten, 2017). In this fungus, apical compartments are self-sustaining in growth. This

410 was concluded from the finding that the growth rate in these compartments remains unaffected
411 when they are mechanically detached from the rest of the hypha. Interestingly, the first
412 subapical compartments (up to eight) function as a backup system for growth by forming new
413 branches upon damage of the apical compartment (Tegelaar and Wösten, 2017). This backup
414 system appears crucial in nature considering the fact that fungal colonies continuously explore
415 substrates that may locally be hostile for growth. By forming sub-apical branches that do not
416 grow parallel to the damaged hypha, but rather grow away from it, the organism can avoid a
417 second confrontation with the source of damage (i.e. a competing organism or a nutrient void
418 zone).
419
420 *4.3 Multilevel heterogeneity in liquid environments*
421
422 Fungi and streptomycetes produce respectively about 42% and 32% of the more than 23,000
423 known microbial bio-active compounds (i.e. compounds with antifungal, antibacterial, antiviral,
424 antitumor, cytotoxic and immunosuppressive activity) (Barka et al., 2016; Hopwood, 2007;
425 Lazzarini et al., 2000). They also possess a remarkable capacity to produce and efficiently
426 secrete various hydrolytic enzymes that allow them to degrade almost any naturally occurring
427 polymer (Anné et al., 2012; Hoffmeister and Keller, 2007). The ability of streptomycetes and
428 filamentous fungi to produce this treasure trove of commercially-valuable compounds and
429 enzymes has led to their large-scale industrial exploitation (Hopwood, 2007). In industry,
430 microbes are typically grown in large bioreactors. This choice is dictated by the fact that these
431 systems provide the most reproducible and efficient manner to obtain high growth and
432 production rates, which are achieved through parametric control and the efficient provision of
433 nutrients and oxygen to cells. Notably, the continuous and often vigorous mixing of the culture
434 medium creates a more homogenous environment for the mycelia when compared to growth
435 on solid substrates. Yet, gradients can still exist, especially with the large volumes that are
436 characteristic of industrial fermentation processes. Notably, heterogeneity in process
437 parameters (e.g. pH, temperature, concentration of biomass and nutrients) can result in

438 physiological heterogeneity and the occurrence of culture segregation in a number of
439 microorganisms (Delvigne et al., 2009; Takors, 2012). In addition, fluctuating aeration regimes
440 have been shown to decrease product formation both in streptomycetes and filamentous fungi
441 (Larsson and Enfors, 1998; Yegneswaran et al., 1991).

442 The mode-of-growth of streptomycetes and filamentous fungi in bioreactors is markedly
443 different when compared to solid substrates. Depending on the strain and culture setup, the
444 mycelium of these filamentous microbes can display a range of different morphologies (Braun
445 and Vecht-Lifshitz, 1991; Tresner et al., 1967; van Dissel et al., 2014). Many species, among
446 which the industrial cell factories *S. lividans* and *A. niger,* can form dense mycelial particles
447 called pellets (also micro-colonies for filamentous fungi). These particles can have a diameter
448 larger than 1 mm, with the pellets formed by *Aspergillus* being generally larger than those of
449 streptomycetes (van Veluw et al., 2012; van Veluw et al., 2013). This mode-of-growth
450 promotes physiological heterogeneity due to the differential diffusion of oxygen, nutrients and
451 metabolic (by)products. One of the consequences of growth in dense pellets is that hyphae in
452 the central part of these particles are typically starved due to the limited availability of oxygen
453 and nutrients (Bizukojc and Gonciarz, 2015; Clark, 1962; Driouch et al., 2012; Gerlach et al.,
454 1998; Wittier et al., 1986). The impact of nutrient and oxygen limitation on pelleted growth is
455 also evident in other multicellular communities (e.g. biofilms) formed by single or multiple
456 species (Kragh et al., 2016; von Ohle et al., 2010). The interplay between environmentally-
457 determined heterogeneity and actively regulated development is however still obscure. In this
458 context, it is interesting to mention that cells residing within a biofilm structure have been found
459 to be more heterogeneous as opposed to planktonic cells. In experiments with *Pseudomonas*
460 *aeruginosa*, phenotypical variation was found to arise when cells were cultured in the form of
461 biofilms. Although the factors inducing this heterogeneity are unknown, a recombination-
462 dependent system was found to provide the source of genotypic variation leading to the
463 observed phenotypes. Furthermore, cells that had gained mutations after residing in biofilms
464 displayed more variation in swimming capability and enhanced resistance to a number of

465 environmental insults including oxidative stress and exposure to antimicrobials (Boles et al.,
466 2004).

467 In addition to differential responses to environmental cues, deterministic choices may
468 also stimulate heterogeneous growth in liquid environments. Most *Streptomyces* strains do not
469 sporulate in liquid-grown cultures; nevertheless, a certain degree of developmental and
470 physiological heterogeneity is evident throughout the mycelium. As on solid substrates, the
471 mycelial structure changes throughout growth, and is characterized by frequent
472 compartmentalization at early time points (Manteca et al., 2008; Manteca et al., 2005a).
473 Following a round of cellular dismantling, a multinucleated mycelium is established which
474 contains fewer compartments. By this time, the production of antibiotics becomes noticeable.
475 Contrary to growth on solid substrates, neither is this newly established mycelium dismantled,
476 nor does sporulation occur. Gene expression profiling indicated however, that the majority of
477 transcripts identified on solid substrates are also present in liquid-grown cultures, including
478 activators of secondary metabolism and development (Yagüe et al., 2014). Together, these
479 findings indicate that heterogeneity is common in liquid-grown streptomycetes.

480 Another form of heterogeneity was discovered in pellet-forming streptomycetes and
481 fungi by analyzing large numbers of pellets with a flow cytometry approach. This revealed that
482 cultures of both filamentous fungi and streptomycetes contain at least two normally distributed
483 populations of pellets that differ in size (de Bekker et al., 2011b; van Veluw et al., 2012; van
484 Veluw et al., 2013) (Fig. 2). This heterogeneity is observed in a range of strains and growth
485 media, suggesting that it is inherent to the mode-of-growth of these organisms. Interestingly,
486 gene expression in micro-colonies can also be described as a bimodal distribution. For
487 instance, two populations of *A. niger* micro-colonies exist in submerged cultures; one highly
488 and one lowly expressing the glucoamylase gene (de Bekker et al., 2011b). In *Streptomyces*
489 *coelicolor*, 37 proteins were found to be significantly different in abundance between the
490 populations of large and small pellets. While 17 of these proteins are significantly
491 overrepresented in large pellets as opposed to the small ones, 20 are significantly
492 underrepresented (van Veluw et al., 2012). Several of the proteins that are over- or

493 underrepresented could be assigned to specific functional classes, with a number of stress-
494 related proteins being overrepresented in the population of large pellets. The protein that is
495 most strongly enhanced (around 30-fold) in the larger pellets is EgtD, a protein involved in the
496 biosynthesis of the rare amino acid ergothioneine. The synthesis of this molecule is rare in
497 microbes, with a higher incidence in actinobacteria (including mycobacteria) and filamentous
498 fungi. The role of ergothioneine in these organisms is still obscure, but it has antioxidant
499 properties, which suggests that it might be involved in a stress-response mechanism. Other
500 stress-related proteins being overrepresented in the population of large pellets include
501 polypeptides encoded by genes in the *osdR* locus, including the gene for the universal stress
502 protein (USP) (SCO0200) (van Veluw et al., 2012). Recent studies revealed that *osdR* controls
503 development and oxidative stress, and is functionally similar to DosR, the oxygen-sensitive
504 dormancy response regulator in *Mycobacterium tuberculosis* (Urem et al., 2016). It is
505 interesting to mention that the classes of genes being differently expressed in *Streptomyces*
506 pellets are known to be subject to transcriptional noise in other microbes (*see above*).
507
508 **5. Mechanisms underlying heterogeneity in filamentous microbes**
509
510 *5.1 Inter-hyphal and inter-compartmental heterogeneity*
511
512 The hyphae of streptomycetes and the higher fungi (i.e. ascomycetes and basidiomycetes) are
513 compartmentalized by cross-walls (also called septa). In streptomycetes, some of these cross-
514 walls have channels, which potentially would allow streaming of cytoplasmic content, although
515 this has never been demonstrated directly (Bleichrodt et al., 2012; Celler et al., 2016;
516 Jakimowicz and van Wezel, 2012; Yagüe et al., 2016). In addition to cross-walls, recent work
517 has shown that extended membranous structures are able to spatially and functionally
518 organize the vegetative mycelium of streptomycetes (Celler et al., 2016; Yagüe et al., 2016)
519 (Fig. 3). These cross-membranes are responsible for the formation of the alternating pattern
520 of viable and dead hyphae in the early MI mycelium and also block the diffusion of cytoplasmic

521 proteins in 29% of the cases. Cross-membranes might thus maintain heterogeneity between
522 compartments of the same cell by preventing molecules to mix by diffusion or streaming.

523 Septa of filamentous fungi consist of invaginations of the cell wall that are ligned with
524 plasma membrane. Septa have a central pore of 50–500 nm (Moore and Mcalear, 1962;
525 Shatkin and Tatum, 1959) that allows streaming of cytosol and even organelles, thus enabling
526 cytoplasmic mixing throughout the mycelium. Yet, the pores of *Aspergillus* can be reversibly
527 opened and closed by peroxisome-derived organelles called Woronin bodies (Bleichrodt et al.,
528 2012) (Fig. 3). The absence of Woronin bodies prevents septal closure, thereby abolishing the
529 possibility to maintain long-term heterogeneity in cytosolic composition between neighboring
530 compartments and/or hyphae (Bleichrodt et al., 2012). It should be noted that even an open
531 septum can maintain differences in cytosolic composition due to differential gene expression.
532 In this case, however, heterogeneity can be only maintained in a minutes time-frame. Yet, this
533 may be sufficient for some developmental processes to be initiated (Bleichrodt et al., 2015a;
534 Bleichrodt et al., 2015b). Together, an arrest or reduction in cytoplasmic streaming between
535 adjacent compartments can maintain long term heterogeneity in RNA and protein composition.
536 Notably, the plugging of septa via Woronin bodies has no effect on inter-compartmental
537 transport of glucose (Bleichrodt et al., 2015b). This is explained by the fact that *Aspergillus*
538 uses permeases to enable the selective transport of metabolites. In this scenario, inter-
539 compartmental and inter-hyphal heterogeneous distributions are only obtained for those
540 components that cannot cross the selective plasma membrane of septa (e.g. large proteins,
541 ribosomes, organelles, and metabolites that lack a permease in the plasma membrane lining
542 the septal cross wall).

543

544 *5.2 Inter-pellet heterogeneity*

545

546 The aggregation of distinct particles is a driving factor for generating size heterogeneity
547 between pellets. Aggregation in streptomycetes is mediated by extracellular glycans on the
548 surface of germlings and young mycelia (Zacchetti et al., 2016). These glycans are produced

549 under control of the *cslA/glxA* operon and the *mat* cluster (Chaplin et al., 2015; de Jong et al.,
550 2009; Petrus et al., 2016; van Dissel et al., 2015; Xu et al., 2008). The structure of the glycan
551 produced by CslA and GlxA is still unknown, while the polymer produced by the Mat proteins
552 is poly-β-(1,6)-N-acetylglucosamine (PNAG) (van Dissel et al., 2018). Abolishing the formation
553 of these glycans yields particles whose size is no longer bimodally distributed and that are
554 hence more homogeneous in size. Also, in filamentous fungi aggregation is a critical factor in
555 generating size heterogeneity. In this case, aggregation is a two-step process. The first phase
556 involves the aggregation of ungerminated spores and is followed by a second aggregation
557 phase that occurs between germlings (Grimm et al., 2004). Mutants of *A. niger* affected in the
558 formation of spore-associated pigments yield more homogeneously-distributed pellets (van
559 Veluw et al., 2013). The underlying mechanism is not known but one wonders whether
560 filamentous microbes make use of size heterogeneity to optimally adapt to the environment.
561 Micro-colonies of different size might experience environmental stimuli differently and may thus
562 differently react to these cues.
563
564 **6. Parallels and differences between unicellular and multicellular systems**
565
566 The cellular architecture of filamentous microbes generates layers of complexity that are rarely
567 observed in unicellular species and that result in the multiscale heterogeneity discussed in this
568 review. As a result of this complexity, some of the well-described aspects of phenotypic
569 heterogeneity have not yet been characterized in filamentous microbes. Mechanisms
570 analogous to those reported in unicellular systems, such as intrinsic noise in transcription and
571 translation, are inherent to the behaviour of their machinery, and therefore a likely source of
572 heterogeneity in any biological system, including filamentous microbes. Uniquely for
573 filamentous microorganisms is the syncytial nature of mycelia. The distribution of DNA (i.e.
574 nuclei in fungi or chromosomes in bacteria) may not only differ between compartments, but
575 also within compartments. This, in turn, would result in some regions possessing more copies
576 of a given gene. Such dosage effects are known to bear a profound effect on decision making

577 in a number of cellular systems (Chai et al., 2011; Narula et al., 2015; Slager et al., 2014;
578 Soler-Bistue et al., 2015; Veening et al., 2006). Additionally, the positioning of nuclei can also
579 result in differential gene expression within single compartments. For instance, paired nuclei
580 in compartments of the mushroom-forming fungus *Schizophyllum commune* can migrate away
581 from each other, resulting in changes in gene expression (Schuurs et al., 1998). Thus, while
582 some of the mechanisms involved in generating heterogeneity could be similar between
583 filamentous and unicellular microbes, some factors (e.g. the presence of inter-compartmental
584 streaming and multinucleate compartments) are probably unique for filamentous
585 microorganisms.

586 Differences in the mechanisms through which heterogeneities arise might also differ
587 between filamentous bacteria and filamentous fungi. Not only is gene regulation different
588 between bacteria and fungi, also their sizes differ. The cellular volume of a fungal filament is
589 roughly 100 times larger than that of a streptomycete given the 10-fold larger diameter of a
590 fungal hypha. This may affect the concentration of various intracellular species. As a
591 consequence, noise dynamics might differ in these systems. However, no quantitative data of
592 abundance of molecules exist that cause heterogeneity in filamentous microbes, thus
593 hindering a direct comparison.

594 One of the most remarkable aspects of microbial phenotypic heterogeneity is its beneficial
595 role in increasing population fitness in the face of changing environmental conditions (see
596 section 2). It is currently unknown whether this is also true for filamentous microbes. Mycelial
597 heterogeneity may be beneficial in terrestrial soils, where spatial and temporal variations exist
598 in for instance the availability of nutrients and oxygen, temperature, pH, and the amount of
599 growth-inhibiting compounds (Stoyan et al., 2000). Considering the saprophytic lifestyle of
600 most filamentous microbes and the close proximity of hyphae within a colony, one would
601 predict a benefit for segregating functions across the colony. This would be particularly useful
602 for acquiring nutrients or secreting costly compounds that ultimately become available to all
603 surrounding hyphae. In this scenario, inter-hyphal heterogeneities in the secretion of enzymes
604 (as those observed in *A. niger*) might very well reflect a division of labour strategy, in that only

a subset of hyphae commit to the production of extracellular hydrolases, thereby liberating nutrients that can be taken up by both producing and non-producing hyphae. Another example where heterogeneity could provide fitness benefits to the colony is in the production of antibiotics. However, this awaits further experimental evidence.

7. Conclusions and future perspectives

Striking parallels exist between filamentous fungi and actinomycetes with respect to morphology, heterogeneity and the architecture of mycelia. Despite the increasing number of studies, we have only started to dissect the mechanisms underlying heterogeneity in these organisms. While cytoplasmic streaming in the fungal mycelium has been known for many decades, it has only recently been reported in streptomycetes (Celler et al., 2016). Selective blocking of this process, either via Woronin bodies in fungi or membranous structures in actinomycetes, leads to physiological differences between adjacent compartments and zones of the colony. One of the outstanding questions to address is how the external and internal signals are processed and translated into changes in cytoplasmic streaming and phenotypic heterogeneity. We believe that the developments in the field of microscopy will enable us to obtain unprecedented insight into the molecular functioning of these compartment-separating structures within hyphae.

In this review we have described the different forms of heterogeneity that have been reported in filamentous fungi and streptomycetes. Interestingly, apart from inter-colony, inter-zonal, inter-hyphal and inter-compartmental heterogeneity one may expect the existence of intra-compartmental heterogeneity. Such heterogeneity may be promoted by increasing the compartmental length and reducing the number of nuclei (fungi) or chromosomes (filamentous bacteria). Alternatively, RNAs and pathways that determine the fate of RNA could be spatially and temporally localized in subcellular compartments. More knowledge about the dynamics of

631 nucleic acids in filaments is thus of utmost importance to better our understanding of
632 heterogeneity.

633 While it is evident that heterogeneity is beneficial to filamentous microbes in natural
634 environments, this feature is undesirable in industry for two reasons. First, heterogeneity
635 decreases controllability of the fermentation process, and secondly, several lines of evidence
636 indicate that morphology and specific productivity appear to be tightly coupled. For instance,
637 production performance can be increased by reducing morphological heterogeneity (size
638 distribution of pellets) in *Streptomyces* cultures (van Dissel et al., 2015; van Wezel et al., 2006;
639 Wang et al., 2017; Wardell et al., 2002). Generally speaking, smaller micro-colonies are
640 preferable for the production of enzymes, while bigger ones are better suited for the production
641 of antibiotics (van Dissel et al., 2014). Promoting increased septation in *S. lividans* results in a
642 reduced pellet size and in turn in increased enzyme secretion (van Wezel et al., 2006).
643 Interfering with mycelial aggregation also results in smaller mycelial particles and increased
644 protein secretion (van Dissel et al., 2015). While some of these phenotypes have solely been
645 explained as the result of the increased growth rates of smaller particles, part of the increased
646 production may be due to the reduced size heterogeneity. Likewise, increased homogeneity
647 could also stimulate antibiotic production, given that mutants of *Saccharopolyspora erythraea*
648 that on average form larger pellets than the parental strain also produce more erythromycin
649 (Wardell et al., 2002). At the same time, we cannot exclude that hyphae within mycelia of liquid-
650 grown cultures differentiate to fulfil specific functions. In this case, heterogeneous cultures may
651 be more productive. In light of this, it is critical to better understand the molecular mechanisms
652 underlying heterogeneity in filamentous organisms, a quest that might be facilitated in the near
653 future by the increasing power of next-generation sequencing technologies applied at the
654 single cell level and the further advancement of high-end microscopy. Only once these
655 mechanisms will have been unraveled, will we be able to tackle heterogeneity in non-natural
656 settings, with the alluring prospect of enhanced production performances in the biotech sector.
657
658

659 **Acknowledgements**

660 Work in the Claessen lab is supported by a VIDI grant (12957) from the Netherlands

661 Organisation for Scientific Research.

662

663 **References**

664

665 Ackermann, M., 2015. A functional perspective on phenotypic heterogeneity in microorganisms. Nat.
666 Rev. Microbiol. 13 (8), 497-508.
667 Adams, D.G., 2000. Heterocyst formation in cyanobacteria. Curr. Opin. Microbiol. 3 (6), 618-624.
668 Anné, J., Maldonado, B., Van Impe, J., Van Mellaert, L., Bernaerts, K., 2012. Recombinant protein
669 production and streptomycetes. J. Biotechnol. 158 (4), 159-167.
670 Arnoldini, M., Vizcarra, I.A., Peña-Miller, R., Stocker, N., Diard, M., Vogel, V., Beardmore, R.E., Hardt,
671 W.D., Ackermann, M., 2014. Bistable expression of virulence genes in *Salmonella* leads to the
672 formation of an antibiotic-tolerant subpopulation. PLoS Biol. 12 (8), e1001928.
673 Avery, S.V., 2006. Microbial cell individuality and the underlying sources of heterogeneity. Nat. Rev.
674 Microbiol. 4 (8), 577-587.
675 Avraham, N., Soifer, I., Carmi, M., Barkai, N., 2013. Increasing population growth by asymmetric
676 segregation of a limiting resource during cell division. Mol. Syst. Biol. 9, 656.
677 Balaban, N.Q., Merrin, J., Chait, R., Kowalik, L., Leibler, S., 2004. Bacterial persistence as a phenotypic
678 switch. Science 305 (5690), 1622-1625.
679 Barka, E.A., Vatsa, P., Sanchez, L., Gaveau-Vaillant, N., Jacquard, C., Klenk, H.P., Clément, C., Ouhdouch,
680 Y., van Wezel, G.P., 2016. Taxonomy, physiology, and natural products of *Actinobacteria*.
681 Microbiol. Mol. Biol. Rev. 80 (1), 1-43.
682 Bérdy, J., 2005. Bioactive microbial metabolites. J. Antibiot. (Tokyo) 58 (1), 1-26.
683 Berleman, J.E., Chumley, T., Cheung, P., Kirby, J.R., 2006. Rippling is a predatory behavior in
684 *Myxococcus xanthus*. J. Bacteriol. 188 (16), 5888-5895.
685 Berman-Frank, I., Lundgren, P., Chen, Y.B., Küpper, H., Kolber, Z., Bergman, B., Falkowski, P., 2001.
686 Segregation of nitrogen fixation and oxygenic photosynthesis in the marine cyanobacterium
687 *Trichodesmium*. Science 294 (5546), 1534-1537.
688 Berthoumieux, S., de Jong, H., Baptist, G., Pinel, C., Ranquet, C., Ropers, D., Geiselmann, J., 2013.
689 Shared control of gene expression in bacteria by transcription factors and global physiology of
690 the cell. Mol. Syst. Biol. 9, 634.
691 Binder, D., Drepper, T., Jaeger, K.E., Delvigne, F., Wiechert, W., Kohlheyer, D., Grünberger, A., 2017.
692 Homogenizing bacterial cell factories: analysis and engineering of phenotypic heterogeneity.
693 Metab. Eng. 42, 145-156.
694 Bizukojc, M., Gonciarz, J., 2015. Influence of oxygen on lovastatin biosynthesis by *Aspergillus terreus*
695 ATCC 20542 quantitatively studied on the level of individual pellets. Bioprocess Biosyst. Eng.
696 38 (7), 1251-1266.
697 Blake, W.J., Kærn, M., Cantor, C.R., Collins, J.J., 2003. Noise in eukaryotic gene expression. Nature 422
698 (6932), 633-637.
699 Bleichrodt, R.J., Hulsman, M., Wösten, H.A.B., Reinders, M.J.T., 2015a. Switching from a unicellular to
700 multicellular organization in an *Aspergillus niger* hypha. mBio 6 (2), e00111.
701 Bleichrodt, R.J., van Veluw, G.J., Recter, B., Maruyama, J., Kitamoto, K., Wösten, H.A.B., 2012. Hyphal
702 heterogeneity in *Aspergillus oryzae* is the result of dynamic closure of septa by Woronin
703 bodies. Mol. Microbiol. 86 (6), 1334-1344.

704 Bleichrodt, R.J., Vinck, A., Read, N.D., Wösten, H.A.B., 2015b. Selective transport between
705 heterogeneous hyphal compartments via the plasma membrane lining septal walls of
706 *Aspergillus niger*. Fungal Genet. Biol. 82, 193-200.
707 Boles, B.R., Thoendel, M., Singh, P.K., 2004. Self-generated diversity produces "insurance effects" in
708 biofilm communities. Proc. Natl. Acad. Sci. U. S. A. 101 (47), 16630-16635.
709 Borkovich, K.A., Ebbole, D.J., 2010. Cellular and molecular biology of filamentous fungi. ASM Press.
710 Braun, S., Vecht-Lifshitz, S.E., 1991. Mycelial morphology and metabolite production. Trends
711 Biotechnol. 9 (1), 63-68.
712 Brehm-Stecher, B.F., Johnson, E.A., 2004. Single-cell microbiology: tools, technologies, and
713 applications. Microbiol. Mol. Biol. Rev. 68 (3), 538-559.
714 Cai, L., Friedman, N., Xie, X.S., 2006. Stochastic protein expression in individual cells at the single
715 molecule level. Nature 440 (7082), 358-362.
716 Celler, K., Koning, R.I., Willemse, J., Koster, A.J., van Wezel, G.P., 2016. Cross-membranes orchestrate
717 compartmentalization and morphogenesis in *Streptomyces*. Nat. Commun. 7, 11836.
718 Chai, Y.R., Norman, T., Kolter, R., Losick, R., 2011. Evidence that metabolism and chromosome copy
719 number control mutually exclusive cell fates in *Bacillus subtilis.* EMBO J. 30 (7), 1402-1413.
720 Chaplin, A.K., Petrus, M.L.C., Mangiameli, G., Hough, M.A., Svistunenko, D.A., Nicholls, P., Claessen, D.,
721 Vijgenboom, E., Worrall, J.A.R., 2015. GlxA is a new structural member of the radical copper
722 oxidase family and is required for glycan deposition at hyphal tips and morphogenesis of
723 *Streptomyces lividans*. Biochem. J. 469 (3), 433-444.
724 Chastanet, A., Vitkup, D., Yuan, G.C., Norman, T.M., Liu, J.S., Losick, R.M., 2010. Broadly heterogeneous
725 activation of the master regulator for sporulation in *Bacillus subtilis*. Proc. Natl. Acad. Sci. U. S.
726 A. 107 (18), 8486-8491.
727 Chater, K.F., 1998. Taking a genetic scalpel to the *Streptomyces* colony. Microbiol-sgm 144, 1465-1478.
728 Claessen, D., Rozen, D.E., Kuipers, O.P., Søgaard-Andersen, L., van Wezel, G.P., 2014. Bacterial
729 solutions to multicellularity: a tale of biofilms, filaments and fruiting bodies. Nat. Rev.
730 Microbiol. 12 (2), 115-124.
731 Clark, D.S., 1962. Submerged citric acid fermentation of ferrocyanide-treated beet molasses:
732 morphology of pellets of *Aspergillus niger*. Can. J. Microbiol. 8, 133-136.
733 Claudi, B., Spröte, P., Chirkova, A., Personnic, N., Zankl, J., Schürmann, N., Schmidt, A., Bumann, D.,
734 2014. Phenotypic variation of *Salmonella* in host tissues delays eradication by antimicrobial
735 chemotherapy. Cell 158 (4), 722-733.
736 Davis, K.M., Isberg, R.R., 2016. Defining heterogeneity within bacterial populations via single cell
737 approaches. Bioessays 38 (8), 782-790.
738 de Bekker, C., Bruning, O., Jonker, M.J., Breit, T.M., Wösten, H.A.B., 2011a. Single cell transcriptomics
739 of neighboring hyphae of *Aspergillus niger*. Genome Biol. 12 (8), R71.
740 de Bekker, C., van Veluw, G.J., Vinck, A., Wiebenga, L.A., Wösten, H.A.B., 2011b. Heterogeneity of
741 *Aspergillus niger* microcolonies in liquid shaken cultures. Appl. Environ. Microbiol. 77 (4), 1263-
742 1267.
743 de Jong, W., Wösten, H.A.B., Dijkhuizen, L., Claessen, D., 2009. Attachment of *Streptomyces coelicolor*
744 is mediated by amyloidal fimbriae that are anchored to the cell surface via cellulose. Mol.
745 Microbiol. 73 (6), 1128-1140.
746 de Lorenzo, V., Sekowska, A., Danchin, A., 2015. Chemical reactivity drives spatiotemporal organisation
747 of bacterial metabolism. FEMS Microbiol. Rev. 39 (1), 96-119.
748 Delvigne, F., Baert, J., Sassi, H., Fickers, P., Grunberger, A., Dusny, C., 2017. Taking control over
749 microbial populations: Current approaches for exploiting biological noise in bioprocesses.
750 Biotechnol. J. 12 (7).
751 Delvigne, F., Boxus, M., Ingels, S., Thonart, P., 2009. Bioreactor mixing efficiency modulates the activity
752 of a prpoS::GFP reporter gene in *E. coli.* Microb. Cell Fact. 8, 15.
753 Diard, M., Garcia, V., Maier, L., Remus-Emsermann, M.N., Regoes, R.R., Ackermann, M., Hardt, W.D.,
754 2013. Stabilization of cooperative virulence by the expression of an avirulent phenotype.
755 Nature 494 (7437), 353-356.

756 Dörr, T., Vulić, M., Lewis, K., 2010. Ciprofloxacin causes persister formation by inducing the TisB toxin
757 in *Escherichia coli*. PLoS Biol. 8 (2), e1000317.
758 Driouch, H., Hänsch, R., Wucherpfennig, T., Krull, R., Wittmann, C., 2012. Improved enzyme production
759 by bio-pellets of *Aspergillus niger*: targeted morphology engineering using titanate
760 microparticles. Biotechnol. Bioeng. 109 (2), 462-471.
761 Elowitz, M.B., Levine, A.J., Siggia, E.D., Swain, P.S., 2002. Stochastic gene expression in a single cell.
762 Science 297 (5584), 1183-1186.
763 Errington, J., 2003. Regulation of endospore formation in *Bacillus subtilis*. Nat. Rev. Microbiol. 1 (2),
764 117-126.
765 Flärdh, K., 2010. Cell polarity and the control of apical growth in *Streptomyces*. Curr. Opin. Microbiol.
766 13 (6), 758-765.
767 Gerlach, S.R., Siedenberg, D., Gerlach, D., Schügerl, K., Giuseppin, M.L.F., Hunik, J., 1998. Influence of
768 reactor systems on the morphology of *Aspergillus awamori*. Application of neural network and
769 cluster analysis for characterization of fungal morphology. Process Biochem. 33 (6), 601-615.
770 Golding, I., Paulsson, J., Zawilski, S.M., Cox, E.C., 2005. Real-time kinetics of gene activity in individual
771 bacteria. Cell 123 (6), 1025-1036.
772 Goodfellow, M., Mordarski, M., Williams, S.T., 1983. The biology of the actinomycetes. Academic Press,
773 London.
774 Grimbergen, A.J., Siebring, J., Solopova, A., Kuipers, O.P., 2015. Microbial bet-hedging: the power of
775 being different. Curr. Opin. Microbiol. 25, 67-72.
776 Grimm, L.H., Kelly, S., Hengstler, J., Göbel, A., Krull, R., Hempel, D.C., 2004. Kinetic studies on the
777 aggregation of *Aspergillus niger* conidia. Biotechnol. Bioeng. 87 (2), 213-218.
778 Grünberger, A., Schöler, K., Probst, C., Wiechert, W., Noack, S., Kohlheyer, D., 2013. Simple
779 microfluidics for complex organisms: A microfluidic chip system for growth and morphogenesis
780 studies of filamentous fungi. 17th International Conference on Miniaturized Systems for
781 Chemistry and Life Sciences, Microtas 2013. 2, 1273-1275.
782 Grunberger, A., Wiechert, W., Kohlheyer, D., 2014. Single-cell microfluidics: opportunity for bioprocess
783 development. Curr. Opin. Biotechnol. 29, 15-23.
784 He, C., Fong, L.G., Young, S.G., Jiang, H., 2017. NanoSIMS imaging: an approach for visualizing and
785 quantifying lipids in cells and tissues. J. Investig. Med. 65 (3), 669-672.
786 Helaine, S., Cheverton, A.M., Watson, K.G., Faure, L.M., Matthews, S.A., Holden, D.W., 2014.
787 Internalization of *Salmonella* by macrophages induces formation of nonreplicating persisters.
788 Science 343 (6167), 204-208.
789 Henson, M.A., 2003. Dynamic modeling of microbial cell populations. Curr. Opin. Biotechnol. 14 (5),
790 460-467.
791 Higgins, D., Dworkin, J., 2012. Recent progress in *Bacillus subtilis* sporulation. FEMS Microbiol. Rev. 36
792 (1), 131-148.
793 Hoffmeister, D., Keller, N.P., 2007. Natural products of filamentous fungi: enzymes, genes, and their
794 regulation. Nat. Prod. Rep. 24 (2), 393-416.
795 Hol, F.J.H., Dekker, C., 2014. Zooming in to see the bigger picture: Microfluidic and nanofabrication
796 tools to study bacteria. Science 346 (6208), 438-+.
797 Hopwood, D.A., 2007. *Streptomyces* in nature and medicine: the antibiotic makers. Oxford University
798 Press, USA, New York.
799 Jacob, F., Monod, J., 1961. Genetic regulatory mechanisms in the synthesis of proteins. J. Mol. Biol. 3,
800 318-356.
801 Jakimowicz, D., van Wezel, G.P., 2012. Cell division and DNA segregation in *Streptomyces*: how to build
802 a septum in the middle of nowhere? Mol. Microbiol. 85 (3), 393-404.
803 Jia, C., Qian, M., Kang, Y., Jiang, D., 2014. Modeling stochastic phenotype switching and bet-hedging in
804 bacteria: stochastic nonlinear dynamics and critical state identification. Quant. Biol. 2 (3), 110-
805 125.
806 Johnson, D.R., Goldschmidt, F., Lilja, E.E., Ackermann, M., 2012. Metabolic specialization and the
807 assembly of microbial communities. ISME J. 6 (11), 1985-1991.

808 Johnson, P.J.T., Levin, B.R., 2013. Pharmacodynamics, population dynamics, and the evolution of
809 persistence in *Staphylococcus aureus*. PLoS Genet. 9 (1), e1003123.
810 Jones, S.E., Elliot, M.A., 2017. *Streptomyces* exploration: competition, volatile communication and new
811 bacterial behaviours. Trends Microbiol. 25 (7), 522-531.
812 Jones, S.E., Ho, L., Rees, C.A., Hill, J.E., Nodwell, J.R., Elliot, M.A., 2017. *Streptomyces* exploration is
813 triggered by fungal interactions and volatile signals. eLife 6, e21738.
814 Kiviet, D.J., Nghe, P., Walker, N., Boulineau, S., Sunderlikova, V., Tans, S.J., 2014. Stochasticity of
815 metabolism and growth at the single-cell level. Nature 514 (7522), 376-379.
816 Klumpp, S., Hwa, T., 2014. Bacterial growth: global effects on gene expression, growth feedback and
817 proteome partition. Curr. Opin. Biotechnol. 28, 96-102.
818 Kotte, O., Volkmer, B., Radzikowski, J.L., Heinemann, M., 2014. Phenotypic bistability in *Escherichia*
819 *coli*'s central carbon metabolism. Mol. Syst. Biol. 10, 736.
820 Kragh, K.N., Hutchison, J.B., Melaugh, G., Rodesney, C., Roberts, A.E., Irie, Y., Jensen, P.Ø., Diggle, S.P.,
821 Allen, R.J., Gordon, V., Bjarnsholt, T., 2016. Role of multicellular aggregates in biofilm
822 formation. mBio 7 (2), e00237.
823 Krijgsheld, P., Altelaar, A.F., Post, H., Ringrose, J.H., Müller, W.H., Heck, A.J.R., Wösten, H.A.B., 2012.
824 Spatially resolving the secretome within the mycelium of the cell factory *Aspergillus niger*. J.
825 Proteome Res. 11 (5), 2807-2818.
826 Krijgsheld, P., Bleichrodt, R., van Veluw, G.J., Wang, F., Muller, W.H., Dijksterhuis, J., Wösten, H.A.B.,
827 2013a. Development in *Aspergillus*. Studies in Mycology (74), 1-29.
828 Krijgsheld, P., Nitsche, B.M., Post, H., Levin, A.M., Müller, W.H., Heck, A.J.R., Ram, A.F.J., Altelaar, A.F.,
829 Wösten, H.A.B., 2013b. Deletion of *flbA* results in increased secretome complexity and
830 reduced secretion heterogeneity in colonies of *Aspergillus niger*. J. Proteome Res. 12 (4), 1808-
831 1819.
832 Kues, U., Liu, Y., 2000. Fruiting body production in basidiomycetes. Appl. Microbiol. Biotechnol. 54 (2),
833 141-152.
834 Kumar, K., Mella-Herrera, R.A., Golden, J.W., 2010. Cyanobacterial heterocysts. Cold Spring Harb.
835 Perspect. Biol. 2 (4), a000315.
836 Larsson, G., Enfors, S.O., 1998. Studies of insufficient mixing in bioreactors: effects of limiting oxygen
837 concentrations and short term oxygen starvation on *Penicillium chrysogenum*. Bioprocess Eng.
838 3 (3), 123-127.
839 Lazzarini, A., Cavaletti, L., Toppo, G., Marinelli, F., 2000. Rare genera of actinomycetes as potential
840 producers of new antibiotics. Antonie Van Leeuwenhoek 78 (3-4), 399-405.
841 Levin, A.M., de Vries, R.P., Conesa, A., de Bekker, C., Talon, M., Menke, H.H., van Peij, N.N.M.E.,
842 Wösten, H.A.B., 2007. Spatial differentiation in the vegetative mycelium of *Aspergillus niger*.
843 Eukaryot. Cell 6 (12), 2311-2322.
844 Levine, J.H., Fontes, M.E., Dworkin, J., Elowitz, M.B., 2012. Pulsed feedback defers cellular
845 differentiation. PLoS Biol. 10 (1), e1001252.
846 Levine, J.H., Lin, Y., Elowitz, M.B., 2013. Functional roles of pulsing in genetic circuits. Science 342
847 (6163), 1193-1200.
848 Levy, S.F., Ziv, N., Siegal, M.L., 2012. Bet hedging in yeast by heterogeneous, age-correlated expression
849 of a stress protectant. PLoS Biol. 10 (5), e1001325.
850 Locke, J.C., Young, J.W., Fontes, M., Hernandez Jimenez, M.J., Elowitz, M.B., 2011. Stochastic pulse
851 regulation in bacterial stress response. Science 334 (6054), 366-369.
852 Lu, C., Brauer, M.J., Botstein, D., 2009. Slow growth induces heat-shock resistance in normal and
853 respiratory-deficient yeast. Mol. Biol. Cell 20 (3), 891-903.
854 Maamar, H., Raj, A., Dubnau, D., 2007. Noise in gene expression determines cell fate in *Bacillus subtilis*.
855 Science 317 (5837), 526-529.
856 Manina, G., Dhar, N., McKinney, J.D., 2015. Stress and host immunity amplify *Mycobacterium*
857 *tuberculosis* phenotypic heterogeneity and induce nongrowing metabolically active forms. Cell
858 Host Microbe 17 (1), 32-46.

859 Manteca, A., Alvarez, R., Salazar, N., Yagüe, P., Sanchez, J., 2008. Mycelium differentiation and
860 antibiotic production in submerged cultures of *Streptomyces coelicolor*. Appl. Environ.
861 Microbiol. 74 (12), 3877-3886.
862 Manteca, A., Claessen, D., Lopez-Iglesias, C., Sanchez, J., 2007. Aerial hyphae in surface cultures of
863 *Streptomyces lividans* and *Streptomyces coelicolor* originate from viable segments surviving an
864 early programmed cell death event. FEMS Microbiol. Lett. 274 (1), 118-125.
865 Manteca, A., Fernandez, M., Sanchez, J., 2005a. A death round affecting a young compartmentalized
866 mycelium precedes aerial mycelium dismantling in confluent surface cultures of *Streptomyces*
867 *antibioticus*. Microbiology 151, 3689-3697.
868 Manteca, A., Fernandez, M., Sanchez, J., 2005b. Mycelium development in *Streptomyces antibioticus*
869 ATCC11891 occurs in an orderly pattern which determines multiphase growth curves. BMC
870 Microbiol. 5, 51.
871 Manteca, A., Fernandez, M., Sanchez, J., 2006. Cytological and biochemical evidence for an early cell
872 dismantling event in surface cultures of *Streptomyces antibioticus*. Res. Microbiol. 157 (2), 143-
873 152.
874 Manteca, A., Sanchez, J., Jung, H.R., Schwämmle, V., Jensen, O.N., 2010. Quantitative proteomic
875 analysis of *Streptomyces coelicolor* development demonstrates that onset of secondary
876 metabolism coincides with hyphae differentiation. Mol. Cell. Proteomics 9 (7), 1423-1436.
877 Meister, A., Du, C., Li, Y.H., Wong, W.H., 2014. Modeling stochastic noise in gene regulatory systems.
878 Quant. Biol. 2 (1), 1-29.
879 Mitsui, A., Kumazawa, S., Takahashi, A., Ikemoto, H., Cao, S., Arai, T., 1986. Strategy by which nitrogen-
880 fixing unicellular cyanobacteria grow photoautotrophically. Nature 323 (6090), 720-722.
881 Monod, J., 1949. The growth of bacterial cultures. Annu. Rev. Microbiol. 3, 371-394.
882 Moore, R.T., Mcalear, J.H., 1962. Fine structure of mycota. 7. Observations on septa of ascomycetes
883 and basidiomycetes. Am. J. Bot. 49 (1), 86-&.
884 Moukha, S.M., Wösten, H.A.B., Asther, M., Wessels, J.G.H., 1993a. *In situ* localization of the secretion
885 of lignin peroxidases in colonies of *Phanerochaete chrysosporium* using a sandwiched mode of
886 culture. J. Gen. Microbiol. 139, 969-978.
887 Moukha, S.M., Wösten, H.A.B., Mylius, E.J., Asther, M., Wessels, J.G.H., 1993b. Spatial and temporal
888 accumulation of mRNAs encoding two common lignin peroxidases in *Phanerochaete*
889 *chrysosporium*. J. Bacteriol. 175 (11), 3672-3678.
890 Mulcahy, L.R., Burns, J.L., Lory, S., Lewis, K., 2010. Emergence of *Pseudomonas aeruginosa* strains
891 producing high levels of persister cells in patients with cystic fibrosis. J. Bacteriol. 192 (23),
892 6191-6199.
893 Nariya, H., Inouye, M., 2008. MazF, an mRNA interferase, mediates programmed cell death during
894 multicellular *Myxococcus* development. Cell 132 (1), 55-66.
895 Narula, J., Kuchina, A., Lee, D.Y.D., Fujita, M., Suel, G.M., Igoshin, O.A., 2015. Chromosomal
896 Arrangement of Phosphorelay Genes Couples Sporulation and DNA Replication. Cell 162 (2),
897 328-337.
898 Newman, J.R., Ghaemmaghami, S., Ihmels, J., Breslow, D.K., Noble, M., DeRisi, J.L., Weissman, J.S.,
899 2006. Single-cell proteomic analysis of *S. cerevisiae* reveals the architecture of biological noise.
900 Nature 441 (7095), 840-846.
901 Nunez, J., Renslow, R., Cliff, J.B., 3rd, Anderton, C.R., 2017. NanoSIMS for biological applications:
902 Current practices and analyses. Biointerphases 13 (3), 03B301.
903 O'Connor, K.A., Zusman, D.R., 1991. Development in *Myxococcus xanthu*s involves differentiation into
904 two cell types, peripheral rods and spores. J. Bacteriol. 173 (11), 3318-3333.
905 Ozbudak, E.M., Thattai, M., Kurtser, I., Grossman, A.D., van Oudenaarden, A., 2002. Regulation of noise
906 in the expression of a single gene. Nat. Genet. 31 (1), 69-73.
907 Papagianni, M., 2004. Fungal morphology and metabolite production in submerged mycelial
908 processes. Biotech. Adv. 22 (3), 189-259.
909 Perez-Leblic, M.I., Reyes, F., Martinez, M.J., Lahoz, R., 1982. Cell wall degradation in the autolysis of
910 filamentous fungi. Mycopathologia 80 (3), 147-155.

911 Petrus, M.L.C., van Veluw, G.J., Wösten, H.A.B., Claessen, D., 2014. Sorting of *Streptomyces* cell pellets
912 using a complex parametric analyzer and sorter. J. Vis. Exp. 84, e51178.
913 Petrus, M.L.C., Vijgenboom, E., Chaplin, A.K., Worrall, J.A., van Wezel, G.P., Claessen, D., 2016. The
914 DyP-type peroxidase DtpA is a Tat-substrate required for GlxA maturation and morphogenesis
915 in *Streptomyces*. Open Biol. 6 (1), 150149.
916 Philippi, T., Seger, J., 1989. Hedging one's evolutionary bets, revisited. Trends Ecol. Evol. 4 (2), 41-44.
917 Pollack, J.K., Li, Z.J., Marten, M.R., 2008. Fungal mycelia show lag time before re-growth on
918 endogenous carbon. Biotechnol. Bioeng. 100 (3), 458-465.
919 Reece, A., Xia, B.Z., Jiang, Z.L., Noren, B., McBride, R., Oakey, J., 2016. Microfluidic techniques for high
920 throughput single cell analysis. Curr. Opin. Biotechnol. 40, 90-96.
921 Reichenbach, H., 1999. The ecology of the myxobacteria. Environ. Microbiol. 1 (1), 15-21.
922 Riquelme, M., 2013. Tip growth in filamentous fungi: a road trip to the apex. Annu. Rev. Microbiol. 67,
923 587-609.
924 Russell, J.R., Cabeen, M.T., Wiggins, P.A., Paulsson, J., Losick, R., 2017. Noise in a phosphorelay drives
925 stochastic entry into sporulation in *Bacillus subtilis*. EMBO J. 36 (19), 2856-2869.
926 Schuurs, T.A., Dalstra, H.J.P., Scheer, J.M.J., Wessels, J.G.H., 1998. Positioning of nuclei in the secondary
927 mycelium of Schizophyllum commune in relation to differential gene expression. Fungal Genet.
928 Biol. 23 (2), 150-161.
929 Shatkin, A.J., Tatum, E.L., 1959. Electron microscopy of *Neurospora crassa* mycelia. J. Biophys.
930 Biochem. Cytol. 6 (3), 423-&.
931 Sheik, A.R., Muller, E.E., Audinot, J.N., Lebrun, L.A., Grysan, P., Guignard, C., Wilmes, P., 2016. *In situ*
932 phenotypic heterogeneity among single cells of the filamentous bacterium *Candidatus*
933 Microthrix parvicella. ISME J. 10 (5), 1274-1279.
934 Siebring, J., Elema, M.J.H., Drubi Vega, F., Kovács, Á.T., Haccou, P., Kuipers, O.P., 2014. Repeated
935 triggering of sporulation in *Bacillus subtilis* selects against a protein that affects the timing of
936 cell division. ISME J. 8 (1), 77-87.
937 Silander, O.K., Nikolic, N., Zaslaver, A., Bren, A., Kikoin, I., Alon, U., Ackermann, M., 2012. A genome-
938 wide analysis of promoter-mediated phenotypic noise in *Escherichia coli*. PLoS Genet. 8 (1),
939 e1002443.
940 Slager, J., Kjos, M., Attaiech, L., Veening, J.W., 2014. Antibiotic-induced replication stress triggers
941 bacterial competence by increasing gene dosage near the origin. Cell 157 (2), 395-406.
942 Slavov, N., Botstein, D., 2013. Decoupling nutrient signaling from growth rate causes aerobic glycolysis
943 and deregulation of cell size and gene expression. Mol. Biol. Cell 24 (2), 157-168.
944 Smits, W.K., Kuipers, O.P., Veening, J.W., 2006. Phenotypic variation in bacteria: the role of feedback
945 regulation. Nat. Rev. Microbiol. 4 (4), 259-271.
946 Soler-Bistue, A., Mondotte, J.A., Bland, M.J., Val, M.E., Saleh, M.C., Mazel, D., 2015. Genomic location
947 of the major ribosomal protein gene locus determines *Vibrio cholerae* global growth and
948 infectivity. PLoS Genet. 11 (4).
949 Solopova, A., van Gestel, J., Weissing, F.J., Bachmann, H., Teusink, B., Kok, J., Kuipers, O.P., 2014. Bet-
950 hedging during bacterial diauxic shift. Proc. Natl. Acad. Sci. U. S. A. 111 (20), 7427-7432.
951 Stoyan, H., De-Polli, H., Bohm, S., Robertson, G.P., Paul, E.A., 2000. Spatial heterogeneity of soil
952 respiration and related properties at the plant scale. Plant Soil 222 (1-2), 203-214.
953 Strassmann, J.E., Zhu, Y., Queller, D.C., 2000. Altruism and social cheating in the social amoeba
954 *Dictyostelium discoideum*. Nature 408 (6815), 965-967.
955 Süel, G.M., Kulkarni, R.P., Dworkin, J., Garcia-Ojalvo, J., Elowitz, M.B., 2007. Tunability and noise
956 dependence in differentiation dynamics. Science 315 (5819), 1716-1719.
957 Takors, R., 2012. Scale-up of microbial processes: impacts, tools and open questions. J. Biotechnol. 160
958 (1-2), 3-9.
959 Tan, C., Marguet, P., You, L., 2009. Emergent bistability by a growth-modulating positive feedback
960 circuit. Nat. Chem. Biol. 5 (11), 842-848.

961 Taniguchi, Y., Choi, P.J., Li, G.W., Chen, H., Babu, M., Hearn, J., Emili, A., Xie, X.S., 2010. Quantifying *E.*
962 *coli* proteome and transcriptome with single-molecule sensitivity in single cells. Science 329
963 (5991), 533-538.
964 Tegelaar, M., Wösten, H.A.B., 2017. Functional distinction of hyphal compartments. Sci. Rep. 7 (1),
965 6039.
966 Tresner, H.D., Hayes, J.A., Backus, E.J., 1967. Morphology of submerged growth of streptomycetes as
967 a taxonomic aid. I. Morphological development of *Streptomyces aureofaciens* in agitated liquid
968 media. Appl. Microbiol. 15 (5), 1185-1191.
969 Urem, M., van Rossum, T., Bucca, G., Moolenaar, G.F., Laing, E., Światek-Polatyńska, M.A., Willemse,
970 J., Tenconi, E., Rigali, S., Goosen, N., Smith, C.P., van Wezel, G.P., 2016. OsdR of *Streptomyces*
971 *coelicolor* and the dormancy regulator DevR of *Mycobacterium tuberculosis* control
972 overlapping regulons. mSystems 1 (3), 00014-16.
973 van Boxtel, C., van Heerden, J.H., Nordholt, N., Schmidt, P., Bruggeman, F.J., 2017. Taking chances and
974 making mistakes: non-genetic phenotypic heterogeneity and its consequences for surviving in
975 dynamic environments. J. R. Soc. Interface 14 (132), 20170141.
976 van Dissel, D., Claessen, D., Roth, M., van Wezel, G.P., 2015. A novel locus for mycelial aggregation
977 forms a gateway to improved *Streptomyces* cell factories. Microb. Cell Fact. 14 (1), 44.
978 van Dissel, D., Claessen, D., van Wezel, G.P., 2014. Morphogenesis of streptomyces in submerged
979 cultures. Adv. Appl. Microbiol. 89, 1-45.
980 van Dissel, D., Willemse, J., Zacchetti, B., Claessen, D., Pier, G., van Wezel, G.P., 2018. Production of
981 poly-β-1,6-N-acetylglucosamine by MatAB is required for hyphal aggregation and hydrophilic
982 surface adhesion by *Streptomyces*. Microbial Cell. 5 (6), 269-279.
983 van Gestel, J., Vlamakis, H., Kolter, R., 2015a. Division of labor in biofilms: the ecology of cell
984 differentiation. Microbiol. Spectr. 3 (2), MB-0002-2014.
985 van Gestel, J., Vlamakis, H., Kolter, R., 2015b. From cell differentiation to cell collectives: *Bacillus*
986 *subtilis* uses division of labor to migrate. PLoS Biol. 13 (4), e1002141.
987 van Heerden, J.H., Wortel, M.T., Bruggeman, F.J., Heijnen, J.J., Bollen, Y.J., Planqué, R., Hulshof, J.,
988 O'Toole, T.G., Wahl, S.A., Teusink, B., 2014. Lost in transition: start-up of glycolysis yields
989 subpopulations of nongrowing cells. Science 343 (6174), 1245114.
990 van Veluw, G.J., Petrus, M.L.C., Gubbens, J., de Graaf, R., de Jong, I.P., van Wezel, G.P., Wösten, H.A.B.,
991 Claessen, D., 2012. Analysis of two distinct mycelial populations in liquid-grown *Streptomyces*
992 cultures using a flow cytometry-based proteomics approach. Appl. Microbiol. Biotechnol. 96
993 (5), 1301-1312.
994 van Veluw, G.J., Teertstra, W.R., de Bekker, C., Vinck, A., van Beek, N., Muller, W.H., Arentshorst, M.,
995 van der Mei, H.C., Ram, A.F.J., Dijksterhuis, J., Wösten, H.A.B., 2013. Heterogeneity in liquid
996 shaken cultures of *Aspergillus niger* inoculated with melanised conidia or conidia of pigmented
997 mutants. Studies in Mycology 74, 47-57.
998 van Wezel, G.P., Krabben, P., Traag, B.A., Keijser, B.J.F., Kerste, R., Vijgenboom, E., Heijnen, J.J., Kraal,
999 B., 2006. Unlocking *Streptomyces* spp. for use as sustainable industrial production platforms
1000 by morphological engineering. Appl. Environ. Microbiol. 72 (8), 5283-5288.
1001 Veening, J.W., Igoshin, O.A., Eijlander, R.T., Nijland, R., Hamoen, L.W., Kuipers, O.P., 2008a. Transient
1002 heterogeneity in extracellular protease production by *Bacillus subtilis*. Mol. Syst. Biol. 4, 184.
1003 Veening, J.W., Smits, W.K., Hamoen, L.W., Kuipers, O.P., 2006. Single cell analysis of gene expression
1004 patterns of competence development and initiation of sporulation in *Bacillus subtilis* grown on
1005 chemically defined media. J. Appl. Microbiol. 101 (3), 531-541.
1006 Veening, J.W., Smits, W.K., Kuipers, O.P., 2008b. Bistability, epigenetics, and bet-hedging in bacteria.
1007 Annu. Rev. Microbiol. 62, 193-210.
1008 Veening, J.W., Stewart, E.J., Berngruber, T.W., Taddei, F., Kuipers, O.P., Hamoen, L.W., 2008c. Bet-
1009 hedging and epigenetic inheritance in bacterial cell development. Proc. Natl. Acad. Sci. U. S. A.
1010 105 (11), 4393-4398.
1011 Velicer, G.J., Kroos, L., Lenski, R.E., 2000. Developmental cheating in the social bacterium *Myxococcus*
1012 *xanthus.* Nature 404 (6778), 598-601.

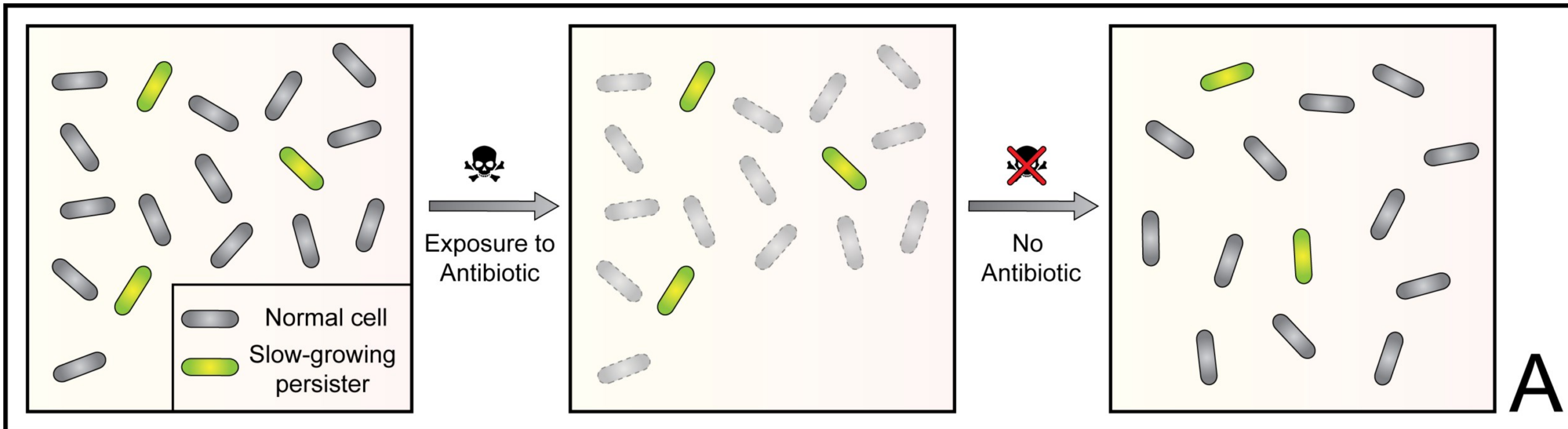
1013 Vinck, A., de Bekker, C., Ossin, A., Ohm, R.A., de Vries, R.P., Wösten, H.A.B., 2011. Heterogenic
1014 expression of genes encoding secreted proteins at the periphery of *Aspergillus niger* colonies.
1015 Environ. Microbiol. 13 (1), 216-225.
1016 Vinck, A., Terlou, M., Pestman, W.R., Martens, E.P., Ram, A.F.J., van den Hondel, C.A.M.J.J., Wösten,
1017 H.A.B., 2005. Hyphal differentiation in the exploring mycelium of *Aspergillus niger*. Mol.
1018 Microbiol. 58 (3), 693-699.
1019 von Ohle, C., Gieseke, A., Nistico, L., Decker, E.M., DeBeer, D., Stoodley, P., 2010. Real-time
1020 microsensor measurement of local metabolic activities in ex vivo dental biofilms exposed to
1021 sucrose and treated with chlorhexidine. Appl. Environ. Microbiol. 76 (7), 2326-2334.
1022 Walker, G.N., White, N.A., 2005. Fungi: biology and applications, 3rd ed. Wiley.
1023 Wang, F.F., Krijgsheld, P., Hulsman, M., de Bekker, C., Müller, W.H., Reinders, M., de Vries, R.P.,
1024 Wösten, H.A.B., 2015. FluG affects secretion in colonies of *Aspergillus niger*. Antonie van
1025 Leeuwenhoek 107 (1), 225-240.
1026 Wang, H., Zhao, G., Ding, X., 2017. Morphology engineering of *Streptomyces coelicolor* M145 by sub-
1027 inhibitory concentrations of antibiotics. Sci. Rep. 7 (1), 13226.
1028 Wardell, J.N., Stocks, S.M., Thomas, C.R., Bushell, M.E., 2002. Decreasing the hyphal branching rate of
1029 *Saccharopolyspora erythraea* NRRL 2338 leads to increased resistance to breakage and
1030 increased antibiotic production. Biotechnol. Bioeng. 78 (2), 141-146.
1031 Wessels, J.G.H., 1988. A steady-state model for apical wall growth in fungi. Acta Bot. Neerl. 37 (1), 3-
1032 16.
1033 Wessels, J.G.H., 1993. Wall growth, protein excretion and morphogenesis in fungi. New Phytol. 123
1034 (3), 397-413.
1035 Wilkinson, D.J., 2009. Stochastic modelling for quantitative description of heterogeneous biological
1036 systems. Nat. Rev. Genet. 10 (2), 122-133.
1037 Wireman, J.W., Dworkin, M., 1977. Developmentally induced autolysis during fruiting body formation
1038 by *Myxococcus xanthus*. J. Bacteriol. 129 (2), 798-802.
1039 Wittier, R., Baumgartl, H., Lübbers, D.W., Schügerl, K., 1986. Investigations of oxygen transfer into
1040 *Penicillium chrysogenum* pellets by microprobe measurements. Biotechnol. Bioeng. 28 (7),
1041 1024-1036.
1042 Wösten, H.A.B., Moukha, S.M., Sietsma, J.H., Wessels, J.G.H., 1991. Localization of growth and
1043 secretion of proteins in *Aspergillus niger*. J. Gen. Microbiol. 137 (8), 2017-2023.
1044 Wösten, H.A.B., van Veluw, G.J., de Bekker, C., Krijgsheld, P., 2013. Heterogeneity in the mycelium:
1045 implications for the use of fungi as cell factories. Biotechnol. Lett. 35 (8), 1155-1164.
1046 Wu, F., Dekker, C., 2016. Nanofabricated structures and microfluidic devices for bacteria: from
1047 techniques to biology. Chem. Soc. Rev. 45 (2), 268-280.
1048 Xu, H., Chater, K.F., Deng, Z., Tao, M., 2008. A cellulose synthase-like protein involved in hyphal tip
1049 growth and morphological differentiation in *Streptomyces*. J. Bacteriol. 190 (14), 4971-4978.
1050 Yagüe, P., López-García, M.T., Rioseras, B., Sánchez, J., Manteca, A., 2013. Pre-sporulation stages of
1051 *Streptomyces* differentiation: state-of-the-art and future perspectives. FEMS Microbiol. Lett.
1052 342 (2), 79-88.
1053 Yagüe, P., Rodríguez-García, A., López-García, M.T., Rioseras, B., Martin, J.F., Sánchez, J., Manteca, A.,
1054 2014. Transcriptomic analysis of liquid non-sporulating *Streptomyces coelicolor* cultures
1055 demonstrates the existence of a complex differentiation comparable to that occurring in solid
1056 sporulating cultures. PLoS One 9 (1), e86296.
1057 Yagüe, P., Willemse, J., Koning, R.I., Rioseras, B., Lopez-García, M.T., Gonzalez-Quiñonez, N., Lopez-
1058 Iglesias, C., Shliaha, P.V., Rogowska-Wrzesinska, A., Koster, A.J., Jensen, O.N., van Wezel, G.P.,
1059 Manteca, A., 2016. Subcompartmentalization by cross-membranes during early growth of
1060 *Streptomyces* hyphae. Nat. Commun. 7, 12467.
1061 Yegneswaran, P.K., Gray, M.R., Thompson, B.G., 1991. Effect of dissolved oxygen control on growth
1062 and antibiotic production in *Streptomyces clavuligerus* fermentations. Biotechnol. Prog. 7 (3),
1063 246-250.

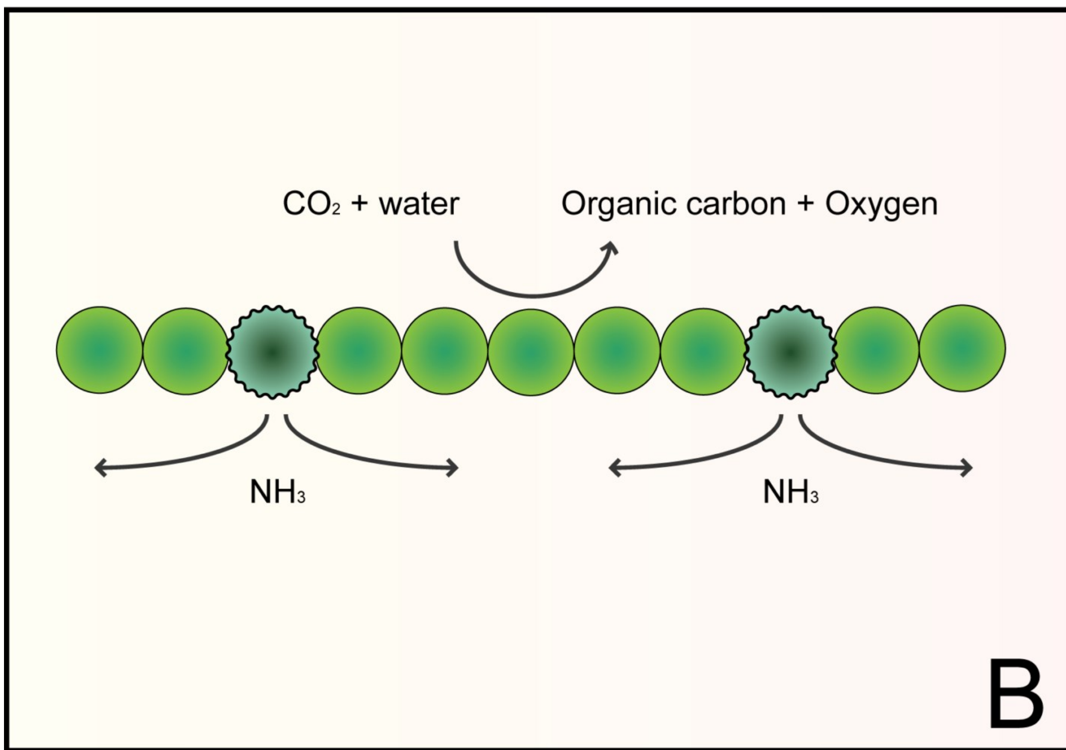
Young, J.W., Locke, J.C.W., Elowitz, M.B., 2013. Rate of environmental change determines stress response specificity. Proc. Natl. Acad. Sci. U. S. A. 110 (10), 4140-4145.
Zacchetti, B., Willemse, J., Recter, B., van Dissel, D., van Wezel, G.P., Wösten, H.A.B., Claessen, D., 2016. Aggregation of germlings is a major contributing factor towards mycelial heterogeneity of *Streptomyces*. Sci. Rep. 6, 27045.
Zhang, Z., Claessen, D., Rozen, D.E., 2016. Understanding microbial divisions of labor. Front. Microbiol. 7, 2070.

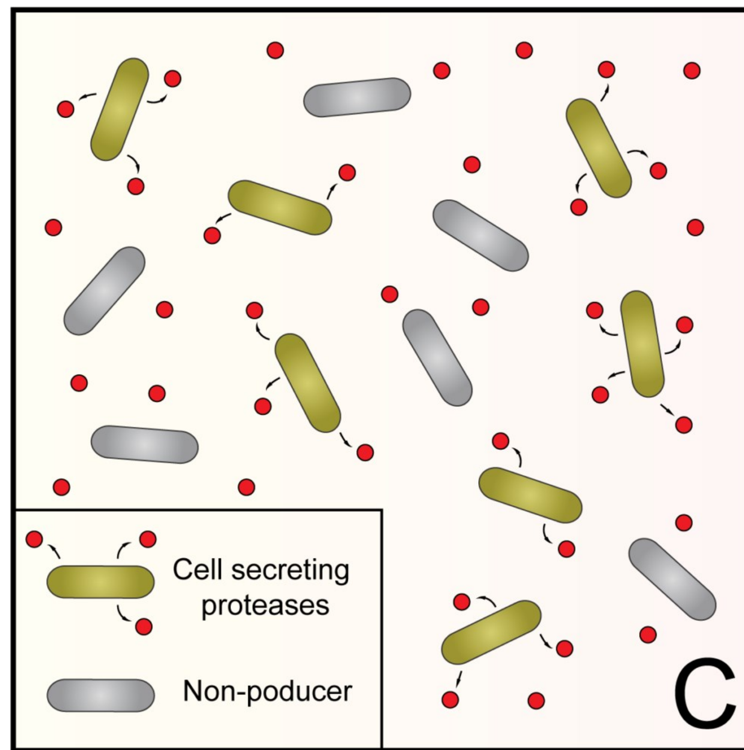
Figure legends

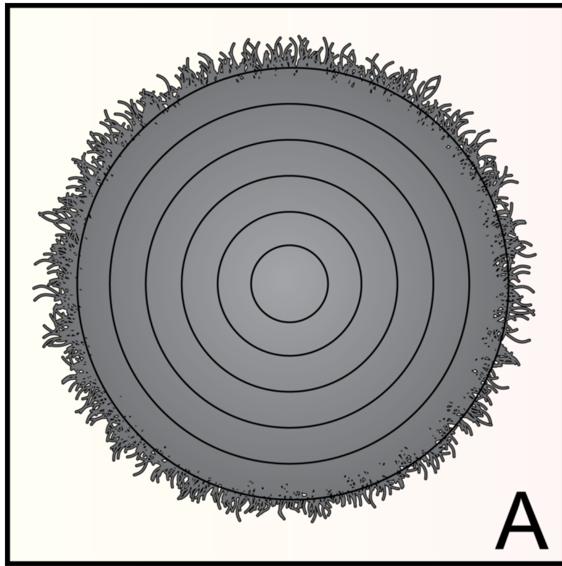
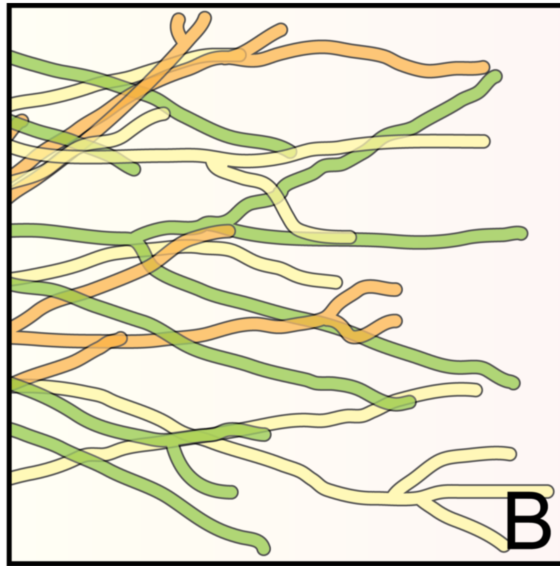
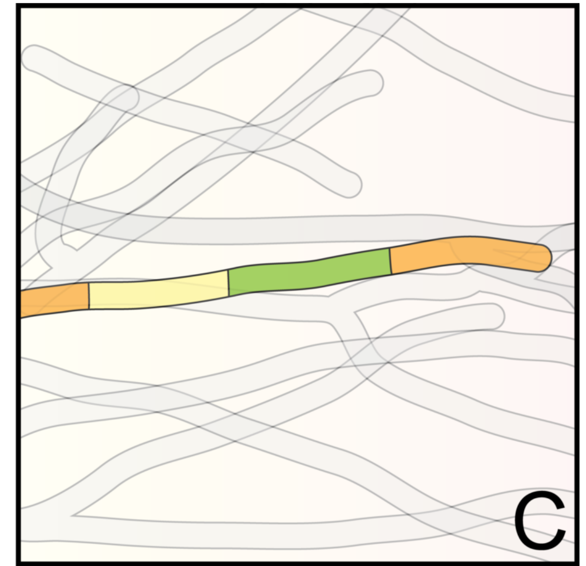
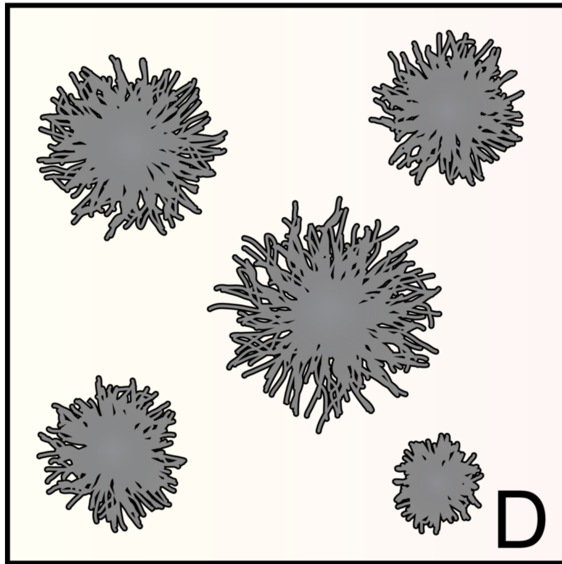
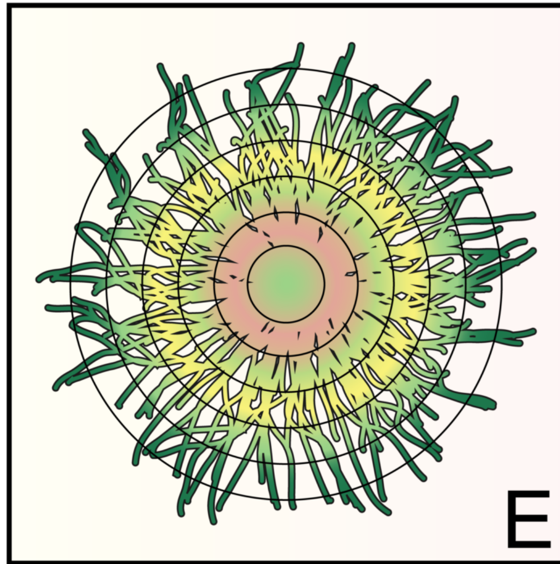
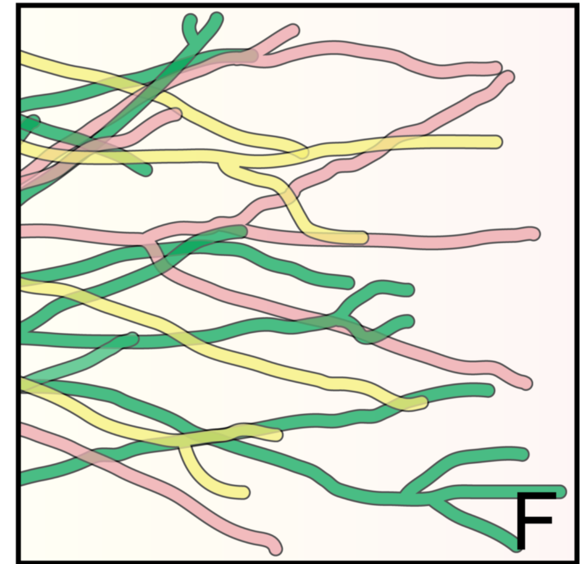
Figure 1: Examples and benefits of microbial heterogeneity. A) Bet-hedging. Microbial clonal populations often contain persisters. Such dormant cells are beneficial in conditions counter-selecting growing cells, for instance due to the presence of an antibiotic. When the normal conditions return, these persisters initiate growth, thereby restoring the population. **B) Division of labor in cyanobacteria.** Oxygenic photosynthesis and nitrogen fixation are chemically incompatible processes, since the oxygen produced through photosynthesis inactivates the nitrogenase enzyme required for nitrogen fixation. Cyanobacteria physically separate these processes by forming specialized nitrogen-fixing cells called heterocysts. The fixed nitrogen will diffuse to the neighboring cells, while heterocysts benefit from the organic carbon produced by cells engaging in photosynthesis. **C) Production of secreted molecules.** The production and secretion of molecules that benefit the entire population is often carried out by only a subset of cells. In this example, some *Bacillus subtilis* cells secrete proteases that provide hydrolyzed nutrients to the whole population under nutrient-limiting conditions. It is not clear whether the non-secreting cells participate in other ways to the overall survival of the population.

1095 **Figure 2. Multiscale heterogeneity in filamentous microorganisms.** Heterogeneity has
1096 been demonstrated at different organizational levels in solid (**A-C**) and liquid-grown (**D-F**)
1097 cultures in filamentous microbes. Inter-zonal heterogeneity (**A**) is characterized by spatial
1098 differences in production (and/or secretion) of molecules in different zones of the colony.
1099 Heterogeneity is also evident between adjacent hyphae in the same region of the colony (**B**),
1100 and even between separate compartments of the same hypha (**C**). In liquid-grown
1101 environments, microcolonies (or pellets) are heterogeneous in size, coinciding with differences
1102 in gene expression and protein secretion (**D**). Heterogeneity is also evident within pellets (**E**),
1103 some of which may be caused by the scarcity of nutrients and oxygen in the central parts of
1104 these structures. Like in solid-grown cultures, intercompartmental heterogeneity is observed
1105 within hyphae in liquid-grown cultures (**F**).
1106
1107 **Figure 3. Compartmentalization is crucial for generating heterogeneity in filamentous**
1108 **organisms.** Filamentous fungi and streptomycetes form hyphae that are compertmentalized
1109 by septa. In addition to septa, streptomycetes also form so-called cross-membranes for
1110 compartmentalization. In fungi, septa can be either open or closed depending on the presence
1111 of so called “Woronin bodies”. Closed septa prevent molecules to freely diffuse between
1112 adjacent compartments. In streptomycetes, extended cross-membrane structures prevent
1113 cytoplasmic streaming, thereby generating heterogeneity within filaments.

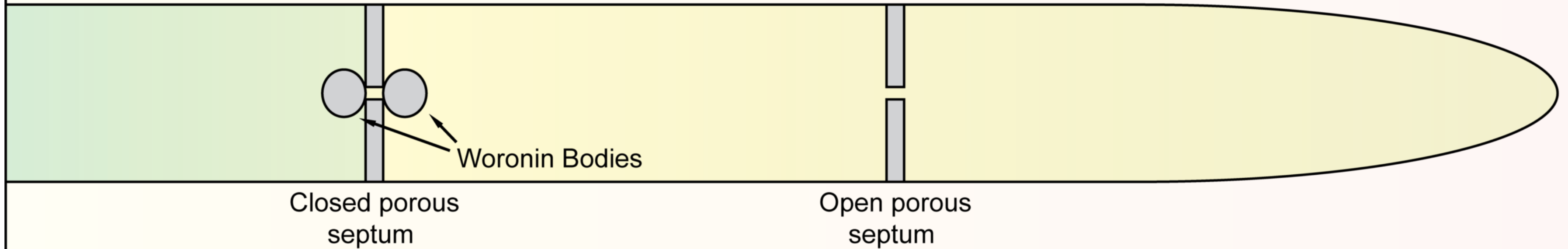

Normal cell
Slow-growing persister
Exposure to Antibiotic
No Antibiotic
A


CO_2 + water
Organic carbon + Oxygen
NH_3
NH_3
B


Cell secreting proteases
Non-poducer
C


A

B

C

D

E

F

Aspergillus hypha



Streptomyces hypha