

1 **Isolation of persisters enabled by  $\beta$ -lactam-induced filamentation reveals their**  
2 **single-cell awakening characteristics**

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## 21 **Abstract**

22 When exposed to lethal doses of antibiotics, bacterial populations are most often not completely  
23 eradicated. A small number of phenotypic variants, defined as ‘persisters’, are refractory to antibiotics  
24 and survive treatment. Despite their involvement in relapsing infections caused by major pathogens,  
25 processes determining phenotypic switches from and to the persister state largely remain elusive. This  
26 is mainly due to the low frequency of persisters in a population and the lack of reliable persistence  
27 markers, both hampering studies of persistence at the single-cell level. Problematically, existing  
28 methods to enrich for persisters result in samples with very low persister densities and/or a too high  
29 abundance of other cell types. Here we present a novel and highly effective persister isolation method  
30 involving cephalexin, an antibiotic that induces extensive filamentation of susceptible cells. We show  
31 that antibiotic-tolerant cells can easily be separated by size after a short cephalexin treatment, and  
32 that the isolated cells are genuine persisters. We used our isolation method to monitor persister  
33 outgrowth at the single-cell level in a microfluidic device, thereby conclusively demonstrating that  
34 awakening is a stochastic phenomenon. We anticipate that our novel approach can have far-reaching  
35 consequences in the persistence field, by allowing single-cell studies at a much higher throughput than  
36 previously reported.

## 37 **Introduction**

38 Persisters are phenotypically distinct variants in a microbial population that survive a lethal antibiotic  
39 dose and are able to regrow after treatment ceases [1,2]. Given this population heterogeneity,  
40 interrogation of the persister physiology should rely on single-cell studies to properly capture their  
41 defining traits. However, these studies require considerable and fast enrichment of persisters as they  
42 are usually present at low frequencies and known to be in a metastable state. Problematically, apart  
43 from their antibiotic tolerance, no reliable marker currently exists to distinguish persisters from  
44 normal, susceptible cells. The state-of-the-art method to enrich for persisters involves lysis of  
45 susceptible cells by ampicillin, followed by sedimentation of intact persister cells [3]. Due to the poor  
46 separation efficiency during sedimentation, this method fails to efficiently remove cell debris and  
47 results in a persister density that is most often too low for microscopic studies. Furthermore, prolonged  
48 exposure of the culture to antibiotics or dead cell material could potentially affect persister formation  
49 [4–6]. The latter problem was addressed by Cañas-Duarte et al., who optimized a method to rapidly  
50 lyse susceptible cells using a chemo-enzymatic lysis solution [7]. Problematically, they did not validate  
51 antibiotic tolerance of their isolated cells, nor did they report the purity and density of the resulting  
52 sample. Other approaches using GFP expression, RpoS::mCherry expression, or the RNA-binding  
53 Thioflavin T as fluorescent markers for persistence, make too strong assumptions on the physiological  
54 state of persisters and therefore generate samples that are highly contaminated with normal,  
55 susceptible cells [8–10]. Attempts to enrich persisters using chemical pretreatment [6] or strains that  
56 are engineered to accumulate toxins [11] potentially generate artefacts that confound insights in  
57 naturally occurring persistence.

58 In this study, we established a novel, highly efficient persister isolation method that largely addresses  
59 the challenges posed by single-cell persistence studies. We show that persisters can be effectively  
60 isolated by filtration after  $\beta$ -lactam-induced filamentation. Cells isolated in this way are *bona fide*  
61 persisters as they survive during antibiotic treatment, regrow after treatment, and exhibit tolerance

62 towards antibiotics with different targets. We then used our isolation method to resolve a key  
63 outstanding question in the persistence field. Single-cell recovery of persisters after treatment was  
64 monitored in a microfluidic ‘mother machine’ device. These data show that persister awakening occurs  
65 at a constant rate, reflecting stochasticity. Our novel approach might prove useful for future single-cell  
66 studies of persistence.

## 67 **Results and discussion**

### 68 **Cephalexin treatment followed by filtration enables highly efficient isolation of persister cells**

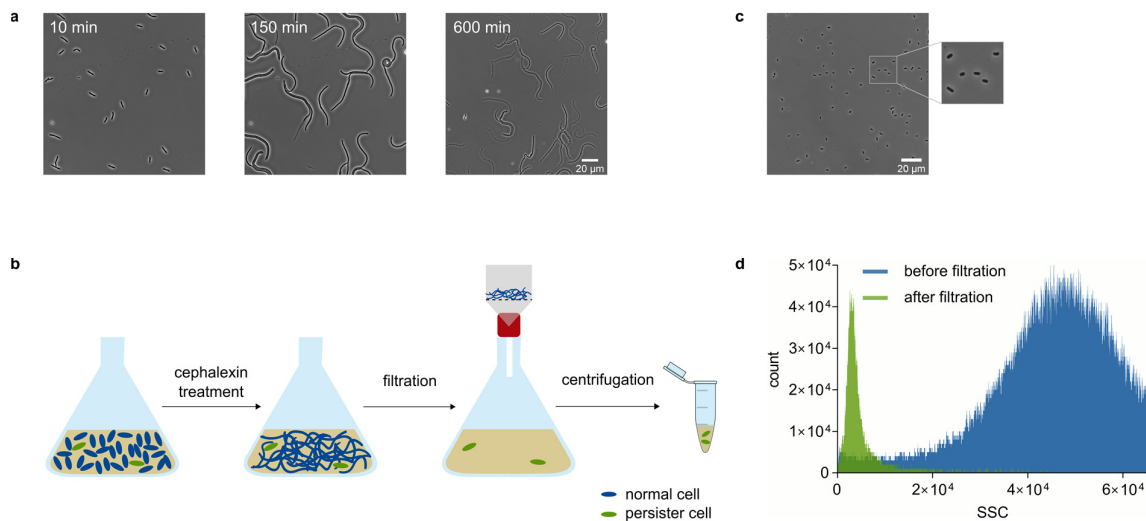
69 Similar to the ampicillin-lysis method of Keren et al. [3], our method distinguishes persisters from  
70 normal cells based on their antibiotic tolerance, the core feature that universally characterizes all  
71 persisters and does not make any assumptions on their physiological state or underlying mechanisms.  
72 Our approach is specifically aimed at limiting the amount of cell debris in the resulting sample, as well  
73 as shortening the antibiotic exposure time. To this end, we benefit from the killing characteristics of  
74 cephalexin, a  $\beta$ -lactam that does not immediately induce lysis, but first induces severe filamentation  
75 of susceptible cells before lysis is initiated ([Figure 1a](#); [Suppl. Movie 1](#)). Cephalexin targets penicillin-  
76 binding protein (PBP) 3, also known as FtsI, a transpeptidase that is essential for peptidoglycan  
77 synthesis during cell division [12]. Drug-tolerant persisters are not affected by cephalexin and  
78 therefore do not filament in its presence, enabling their isolation from a culture by filtration ([Figure](#)  
79 [1b](#)).

80  $\beta$ -lactams only exhibit effective activity at low cell densities [13], implying that cultures should be in  
81 early exponential phase when cephalexin treatment starts. The biphasic killing pattern resulting from  
82 a long-term treatment with cephalexin confirms the presence of persisters in this low-density culture  
83 ([Suppl. Figure S1a](#)). By comparing the number of persisters isolated with our filtration method to the  
84 total number of persisters at the plateau of the time-kill curve ([Suppl. Figure S1a](#)), we estimated that  
85 isolation occurs with an average efficiency of 28 %. The remaining persisters are presumably lost during  
86 filtration, as filamented cells cause clogging of the filter.

87 Notably, the fact that filamentation occurs at a much shorter timescale than lysis considerably reduces  
88 the antibiotic exposure time as compared to the ampicillin lysis method. This was confirmed by  
89 performing our filtration protocol at different time points during a longer-term cephalixin treatment  
90 ([Suppl. Figure S2a](#)). These data show that the number of isolated cells does not change significantly for  
91 cephalixin treatments longer than one hour ( $p=0.17$ ), implying that a one-hour treatment is sufficient  
92 to obtain the persisters of the culture by filtration. Any treatment shorter than one hour results in  
93 contamination with susceptible cells, while longer treatments successively generate more debris of  
94 dead cells in the sample ([Suppl. Figure S2b](#)). Indeed, an optimal treatment time of one hour results in  
95 a final sample that contains short, antibiotic-tolerant persisters and very little cell debris ([Figure 1c](#)).  
96 The purity of the resulting samples was also confirmed by the side scatter distributions of samples  
97 before and after filtration ([Figure 1d](#)).

98 **Cells isolated by cephalixin treatment and filtration are antibiotic-tolerant and regrow after**  
99 **treatment**

100 Next, we sought to validate that cells isolated by cephalixin treatment and filtration show the key  
101 properties of persisters, being their ability to survive a longer-term antibiotic treatment and to  
102 reinitiate growth after treatment. We tested the first feature by treating a sample of isolated cells with  
103 cephalixin, both in liquid medium and on an agarose pad supplemented with rich medium (Mueller-  
104 Hinton broth; MHB). In liquid culture, cephalixin causes the number of isolated cells to decline slowly  
105 ([Figure 2a](#)), with a rate that does not significantly differ from the killing rate of persisters ( $p = 0.399$ ;  
106 [Suppl. Figure S1a](#); [Suppl. Figure S1b](#)). We hypothesize that this rate of killing, which is much lower than  
107 for susceptible cells, reflects the awakening rate of persisters in the presence of cephalixin [14]. Most  
108 cells on an MHB+agarose pad remain unaffected ([Figure 2b](#); [Suppl. Movie 2](#)). A few isolated cells show  
109 filamentation and lysis, which can presumably be attributed to persister awakening.



110

111 **Figure 1. Cephalixin treatment followed by filtration enables highly efficient isolation of persister cells.** (a) Susceptible,  
112 exponential phase cells filament severely during treatment with cephalixin (50 μg/ml) before lysis occurs. (b) Experimental  
113 setup of our persister isolation method. A culture in exponential phase is treated with cephalixin for one hour to induce  
114 filamentation of susceptible cells. Next, the culture is vacuum filtered (pore size of 5 μm) to separate short, antibiotic-tolerant  
115 persisters from filamented, susceptible cells. After filtration, the culture is centrifuged to remove cephalixin and to increase  
116 the density of the resulting sample. (c) Microscopic visualization of a sample after cephalixin treatment and filtration  
117 demonstrates that it mainly consists of short cells that did not respond to the cephalixin treatment. (d) Side scatter  
118 distributions of a sample before and after filtration confirm that filtration enriches for cells with the lowest side scatter values,  
119 presumably corresponding to the persisters of the culture.

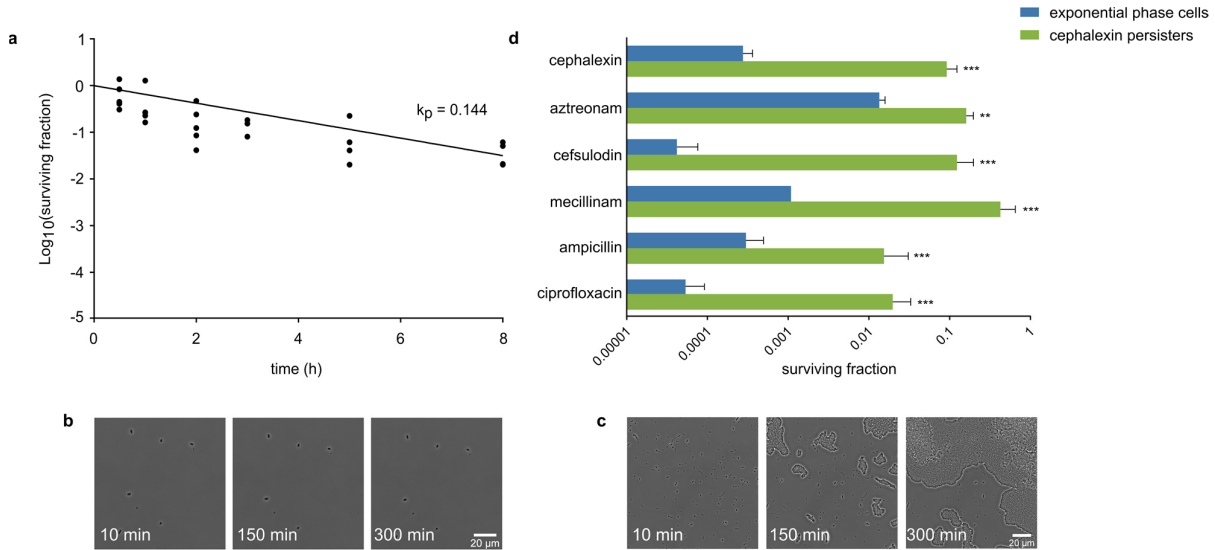
120 Importantly, these microscopic observations additionally demonstrate that the isolated cells cannot  
121 grow in the presence of cephalixin, implying that they are not genetically resistant. We then also  
122 validated that cells isolated by filtration are able to reinitiate growth, by seeding them onto an agarose  
123 pad supplemented with rich medium (Figure 2c; Suppl. Movie 3). 30-40 % of the cells started dividing  
124 within one hour, confirming their culturability after treatment. Growth of other cells was mostly  
125 masked by colonies originating from these early-dividing cells.

126 Persisters are often assumed to be dormant cells in which antibiotic targets are inactive, resulting in  
127 high tolerance towards various types of antibiotics. To further confirm that cells isolated by cephalixin  
128 treatment and filtration are persisters, we investigated their tolerance towards antibiotics with cellular  
129 targets that differ from PBP3. Cefsulodin is a β-lactam that targets PBP1a and PBP1b, while mecillinam

130 only targets PBP2. Ampicillin has multiple targets, including PBP1a, PBP1b, PBP2, and PBP3 [15]. We  
131 also investigated tolerance towards the  $\beta$ -lactam aztreonam, which has the same target as cephalexin,  
132 and towards the fluoroquinolone ciprofloxacin, which targets DNA topoisomerases. Tolerance was  
133 measured by treating a sample of persisters obtained with our isolation protocol for 5 hours with the  
134 listed antibiotics. The relative fraction of surviving cells after treatment was compared to the surviving  
135 fraction of an exponential phase culture (Figure 2d). For all antibiotics, cells surviving treatment are  
136 significantly enriched in a culture that merely consists of cells isolated by filtration (334-fold for  
137 cephalexin, 12-fold for aztreonam, 2950-fold for cefsulodin, 393-fold for mecillinam, 51-fold for  
138 ampicillin, and 368-fold for ciprofloxacin). Notably, none of the tested antibiotics results in 100 %  
139 survival of the isolated cephalexin persisters. In accordance with our other data (Figure 2a), this can  
140 be partially attributed to killing of persisters as they wake up during treatment. However, these data  
141 presumably also imply that not all persisters are tolerant to all antibiotics, but rather represent a  
142 heterogeneous pool of cells with partially overlapping tolerance to various antibiotics. Together, our  
143 data show that cells isolated by cephalexin treatment and filtration are tolerant towards a longer-term  
144 cephalexin treatment, that they are able to reinitiate growth when treatment ceases, and that they  
145 show a high degree of multidrug tolerance. All these characteristics are key to the persister phenotype  
146 and make us confident that our isolated cells are *bona fide* persister cells.

147 **Single-cell analysis of isolated persisters in the mother machine reveals that persister awakening is**  
148 **a stochastic process**

149 To microscopically examine single persister cells and their regrowth, a major drawback of using  
150 agarose pads is that early-dividing cells quickly overgrow the whole pad. First divisions of potentially  
151 later-dividing, neighbouring cells are thereby obscured, hampering quantitative single-cell analyses of  
152 awakening. To address this problem, we took advantage of the mother machine, a well-established  
153 microfluidic device that allows tracking growth of a large number of individual *E. coli* cells [16].



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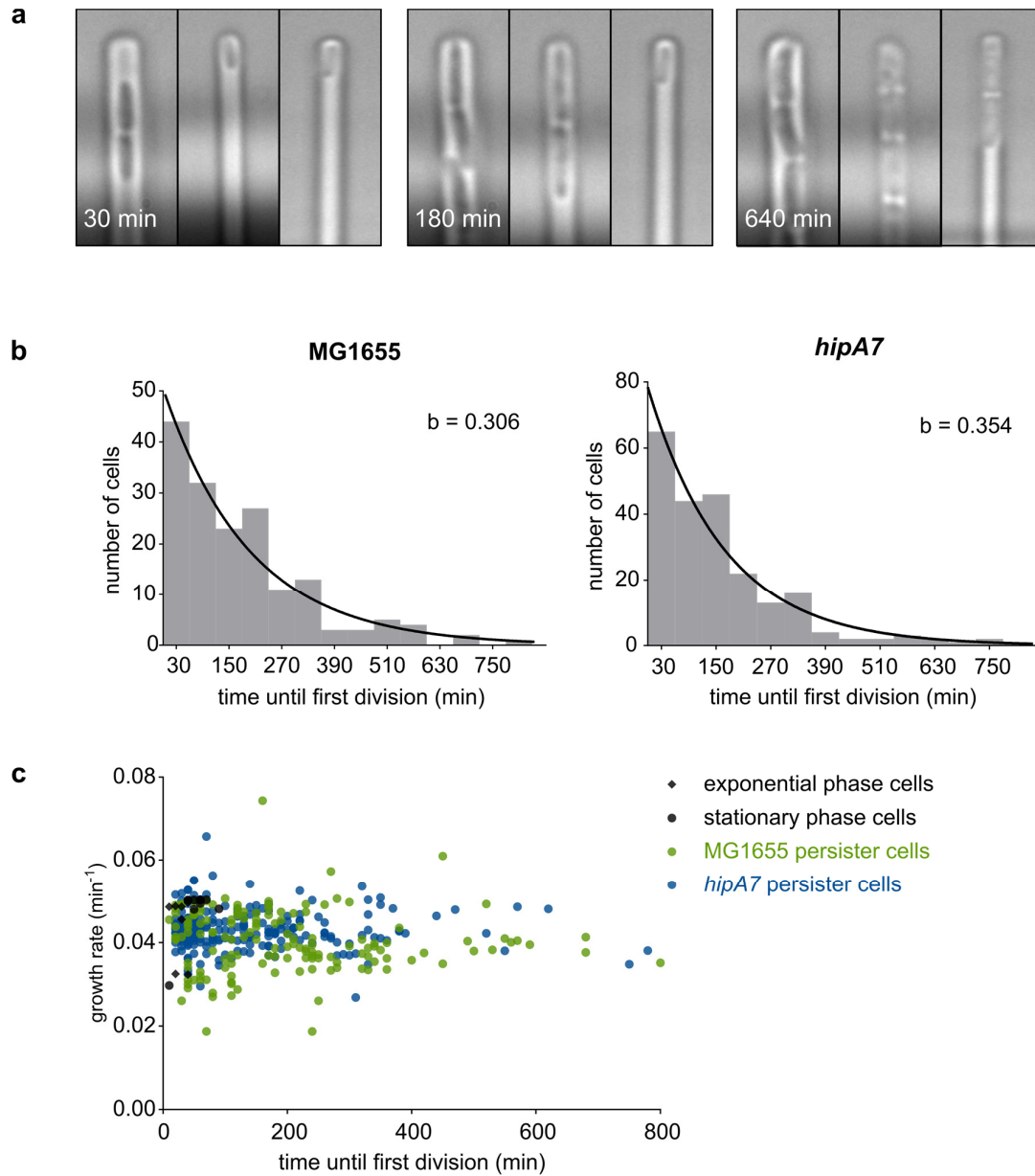
155 **Figure 2. Cells isolated by cephalaxin treatment and filtration are antibiotic-tolerant and regrow after treatment.** (a) Time-  
156 kill curve of isolated cells treated with cephalaxin (50 µg/ml) for 8 hours in liquid medium. A uniphasic exponential curve was  
157 fitted onto the data with a killing rate ( $k_p = 0.144$ ) that is much lower than for susceptible cells ( $k_n = 4.98$ ; [Suppl. Figure S1a](#)).  
158 The killing rate of persisters presumably reflects the rate of persister awakening in the presence of cephalaxin. (b) Treatment  
159 of isolated cells with cephalaxin (50 µg/ml) on an agarose pad supplemented with MHB shows that the majority of the cells  
160 is not affected by the antibiotic. (c) Persisters isolated by filtration start dividing on an agarose pad supplemented with MHB.  
161 (d) Isolated cells display multidrug tolerance, a trait associated with persistence. Fraction of surviving cells after a 5-hour  
162 treatment with cephalaxin (50 µg/ml), aztreonam (0.64 µg/ml), cefsulodin (320 µg/ml), mecillinam (5 µg/ml), ampicillin (40  
163 µg/ml), and ciprofloxacin (0.32 µg/ml), starting from an exponential phase culture or a sample consisting of isolated  
164 cephalaxin persisters. For all tested antibiotics, persisters show a significantly higher tolerance as compared to exponential  
165 phase cells (cephalexin:  $p < 0.0001$ ,  $n = 12$ ; aztreonam:  $p = 0.0004$ ,  $n = 9$ ; cefsulodin:  $p < 0.0001$ ,  $n = 6$ ; mecillinam:  $p < 0.0001$ ,  $n = 9$ ;  
166 ampicillin:  $p < 0.0001$ ,  $n = 9$ ; ciprofloxacin:  $p < 0.0001$ ,  $n = 9$ ).

167 We isolated persisters from a culture using the filtration method described above, inserted these cells  
168 into the channels of the mother machine, and provided them with fresh nutrients ([Figure 3a](#)). Most of  
169 the channels contained either no or only one cell, allowing to track single cells. Based on a few hundred  
170 individual observations, we derived a distribution of single-cell persister awakening times for the wild-  
171 type *E. coli* strain K-12 MG1655 and the well-known high-persistence strain *hipA7* ([Figure 3b](#)). A similar  
172 distribution was obtained for both strains. This distribution shows a surprisingly high cell-to-cell  
173 variability in awakening times, ranging from a few minutes to up to 13 hours. In both cases, an



174 exponential curve fits well to the data, indicative of a high degree of stochasticity involved in persister  
175 awakening. The persister awakening rate in fresh medium without antibiotics ( $b = 0.31-0.35$ ; [Figure](#)  
176 [3b](#)) is higher than in the presence of cephalexin ( $k_p = 0.04-0.18$ ; [Figure 2a](#); [Suppl. Figure S1](#)), although  
177 both rates were measured in different setups and therefore not perfectly comparable. While these  
178 findings corroborate existing assumptions and hypotheses about persister awakening [17,18], this is,  
179 to our knowledge, the first study that provides conclusive experimental evidence of stochastic  
180 awakening at single-cell level with such a high throughput. In addition to the awakening times, we also  
181 derived individual growth rates of freshly-awakened persisters ([Suppl. Figure S3](#)). Strikingly, these data  
182 reveal that persisters instantaneously divide at a rate that does not differ from the growth rate of  
183 normal cells. Furthermore, individual growth rates do not correlate with awakening times, indicating  
184 that cells with a long lag time do not necessarily grow slower than cells with a short lag time ([Figure](#)  
185 [3c](#)). It should be noted that the majority of the cells did not start dividing within the course of the  
186 experiment (20 hours). As these cells are too numerous to be completely covered by the tail of the  
187 exponential distribution, they can presumably be classified as viable but non-culturable cells (VBNCs).  
188 The high abundance of VBNCs in *E. coli* cultures has already been reported before [19,20] and  
189 represents a prominent source of contamination in most persistence enrichment protocols, including  
190 ours. As VBNCs cannot be distinguished from persisters based on their antibiotic tolerance, our method  
191 is only able to discriminate between both by visualizing regrowth in fresh medium.

192 Cephalexin exhibits activity against a wide range of bacteria, including both Gram-positive and Gram-  
193 negative bacteria, where it elicits similar effects of filamentation and lysis. Our isolation protocol can  
194 therefore easily be extended to species other than *E. coli*. Nevertheless, the size of the inoculum used  
195 to initiate the exponential phase culture, the duration of exponential growth, and the duration and  
196 concentration of cephalexin treatment should be optimized for every strain or species, as these  
197 parameters are highly dependent on growth rate and lag phase. Ideally, an optimal balance should be  
198 found to ensure that all normal cells escaped the lag phase, while the cell density after exponential



199

200 **Figure 3. Single-cell analysis of isolated persisters in the mother machine reveals that persister awakening is a stochastic**  
201 **process.** (a) Microscopic images of isolated persisters dividing in the mother machine. The time until first division varies  
202 strongly among individual cells (left channel: short lag time; middle channel: medium lag time; right channel: long lag time).  
203 (b) Single-cell distributions of persister awakening times measured in the mother machine, for the wild type strain MG1655  
204 ( $n = 168$ ) and the high-persistence strain *hipA7* ( $n = 129$ ). An exponential distribution was fitted onto the binned data,  
205 revealing an awakening rate  $b$  that is similar for both strains. (c) Scatterplot of single-cell awakening times and growth rates  
206 of MG1655 and *hipA7* persisters, exponential phase cells, and stationary phase cells. While awakening times show a large  
207 variation, growth rates cluster more tightly around the average value. Both parameters are not correlated.

208 growth remains sufficiently low for an effective cephalixin treatment. We anticipate that the wide  
209 applicability of our size-separation-based persister isolation method will boost single-cell persistence  
210 studies, potentially with important consequences for the persistence field.

## 211 **Materials and Methods**

### 212 **Strains, culture conditions and antibiotics**

213 Experiments were performed with *E. coli* K-12 MG1655, except when stated otherwise. MG1655 *hipA7*  
214 was constructed by Pearl et al. [21]. Strains were grown at 37 °C in Mueller-Hinton broth (MHB) with  
215 orbital shaking (200 rpm) or on Luria-Bertani (LB) agar.

### 216 **Isolation of persisters**

217 A 20-hour overnight culture was diluted 1:10,000 in 100 ml Mueller-Hinton broth (MHB) and incubated  
218 for 20 hours. This culture was diluted 1:5,000 in 100 ml MHB and grown for 90 minutes, to a density  
219 of  $1-2 \times 10^6$  CFU/ml. Next, the culture was treated with cephalixin (50 µg/ml) for 60 minutes, after  
220 which it was poured twice over a polyvinylidene fluoride membrane filter (Merck Millipore) with a pore  
221 size of 5 µm. The filtrate was collected in falcons and spun down (4,000 rpm - 5 min). After pouring off  
222 the supernatant, the remaining volume was transferred to a microcentrifuge tube and centrifuged  
223 twice (6,000 rpm - 5 min) to wash away the remaining antibiotic. The pellet was resuspended in MgSO<sub>4</sub>  
224 (10 mM).

### 225 **Time-kill curves and measurement of multidrug tolerance**

226 A 20-hour overnight culture was diluted 1:10,000 in 100 ml MHB and incubated for 20 hours. This  
227 culture was then diluted 1:5,000 in 100 ml MHB and grown for 90 minutes, to a density of  $1-2 \times 10^6$   
228 CFU/ml. To measure time-kill curves of exponential phase cells, this culture was treated with  
229 cephalixin (50 µg/ml) for 16 hours. Alternatively, 1 ml of culture was transferred to a test tube and  
230 treated with cephalixin (50 µg/ml, 6x MIC), cefsulodin (320 µg/ml, 10x MIC), mecillinam (5 µg/ml, 40x

231 MIC), aztreonam (0.64 µg/ml, 10x MIC), ampicillin (40 µg/ml, 10x MIC), or ciprofloxacin (0.32 µg/ml,  
232 20x MIC) for 5 hours, with plating before and after treatment.

233 To measure time-kill curves of isolated cells, the filtration protocol was performed as stated above,  
234 after which the isolated cells were treated with cephalexin (50 µg/ml) for 8 hours. Alternatively,  
235 isolated cells were resuspended in fresh MHB and 1 ml was treated in a test tube with cephalexin (50  
236 µg/ml, 6x MIC), cefsulodin (320 µg/ml, 10x MIC), mecillinam (5 µg/ml, 40x MIC), aztreonam (0.64  
237 µg/ml, 10x MIC), ampicillin (40 µg/ml, 10x MIC), or ciprofloxacin (0.32 µg/ml, 20x MIC) for 5 hours,  
238 with plating before and after treatment.

### 239 **Flow cytometry**

240 A 20-hour overnight culture was diluted 1:10,000 in 100 ml MHB and incubated for 20 hours. This  
241 culture was diluted 1:5,000 in 100 ml MHB and grown for 90 minutes, to a density of  $1-2 \times 10^6$  CFU/ml.  
242 Next, the culture was treated with cephalexin (50 µg/ml) for 60 minutes. A sample was taken from this  
243 culture and washed in PBS, after which the scattering values were measured by flow cytometry using  
244 a BD Influx cell sorter. The remainder of the culture was poured twice over a polyvinylidene fluoride  
245 membrane filter (Merck Millipore) with a pore size of 5 µm. The filtrate was collected in falcons and  
246 spun down (4,000 rpm - 5 min). After pouring off the supernatant, the remaining volume was  
247 transferred to a microcentrifuge tube and washed in PBS. The scattering values of this sample were  
248 measured by flow cytometry.

### 249 **Microscopy of agarose pads**

250 To visualize killing by cephalexin, a 20-hour overnight culture was diluted 1:5,000 and incubated for 90  
251 minutes. The resulting exponential phase culture was washed with MgSO<sub>4</sub> and 2 µl of cells was seeded  
252 onto an MHB+agarose pad (2% w/v) containing cephalexin (50 µg/ml). Cells were incubated at 37 °C  
253 and killing was monitored for 6 hours. Images were obtained using a Nikon Ti-E inverted microscope  
254 with a 60x objective.

255 To visualize persisters, cells were isolated as described above. The resulting sample was resuspended  
256 in 10  $\mu$ l  $MgSO_4$  and 2  $\mu$ l of cells was seeded onto an MHB+agarose pad (2% w/v) with or without  
257 cephalixin (50  $\mu$ g/ml). Cells were incubated at 37 °C and growth was monitored for 12 hours. Images  
258 were obtained using a Nikon Ti-E inverted microscope with a 60x objective.

#### 259 **Fabrication of mother machine devices**

260 Master molds of the microfluidic devices were designed and fabricated using standard  
261 microfabrication techniques [16]. Microfluidic chips were made by casting polydimethylsiloxane  
262 (PDMS) onto the wafer. PDMS (Sylgard 184 kit; Dow Corning) was prepared by mixing polymer base  
263 and curing agent in a 10:1 ratio. After degassing the mixture in a vacuum chamber, it was poured over  
264 the wafer and cured overnight at 65 °C. Devices were peeled from the wafer and holes for the inlet  
265 and outlet were punched using a syringe and needle (0.9 mm), and bonded to a glass coverslip after  
266 plasma activation. The bonding was established for at least 15 minutes at 65 °C. The dimensions of the  
267 growth channels were approximately 25  $\mu$ m (L) x 1  $\mu$ m (W) x 1  $\mu$ m (D).

#### 268 **Mother machine experiments**

269 Persisters were isolated as described before and resuspended in a final volume of 20  $\mu$ l  $MgSO_4$ . After  
270 flushing the channels of the microfluidic device with  $MgSO_4$ , cells were loaded by syringe injection  
271 followed by chip centrifugation. A peristaltic pump was used to flow medium through the device at a  
272 flow rate of 90  $\mu$ l/min. The microscope chamber, which also contained the medium reservoir, was  
273 constantly held at 37 °C. Images were obtained using an Olympus XI71 inverted microscope with a  
274 100x objective.

#### 275 **Data analysis and statistics**

276 Images were analysed using NIS Elements D 4.60.00 (Nikon Instruments, Japan) and ImageJ  
277 (<https://imagej.nih.gov/>). Flow cytometry data were analysed with FlowJo V10. Statistical analyses  
278 were performed in R (<https://www.r-project.org/>).

279 *Time-kill curves*

280 Biphasic killing parameters were determined by fitting a bi-exponential mixed model to the  $\text{Log}_{10}$ -  
281 transformed, normally distributed number of surviving cells (CFU/ml) using the R package *nlme*  
282 (<https://cran.r-project.org/web/packages/nlme/index.html>). The model was based on the equation  
283  $\text{Log}_{10}(\text{CFU}) = \text{Log}_{10}(N_0 \cdot e^{-k_n \cdot \tau} + P_0 \cdot e^{-k_p \cdot \tau})$ , with  $\tau$  the treatment time (in hours),  $N_0$  and  $P_0$  the number of  
284 normal and persister cells at  $\tau=0$ , and  $k_n$  and  $k_p$  the rate at which normal and persister cells are killed  
285 (per hour) [22]. For uniphasic killing, the R package *lme4* ([https://cran.r-](https://cran.r-project.org/web/packages/lme4/index.html)  
286 [project.org/web/packages/lme4/index.html](https://cran.r-project.org/web/packages/lme4/index.html)) was used to fit the equation  $\text{Log}_{10}(\text{CFU}) = \text{Log}_{10}((N_0 + P_0) \cdot e^{-k \cdot \tau})$ .  
287 AIC (Akaike Information Criterion) was used to assess both models.

288 *Multidrug tolerance*

289 Surviving fractions were compared between conditions using one-way ANOVA and post-hoc  
290 comparisons with Sidak's correction for multiple testing.

291 *Distribution of awakening times*

292 The variable 'persister awakening time' was split into bins of 60 minutes. The number of observations  
293 in each bin was normalized, to obtain relative frequencies (*freq*) of awakening events. The *nls* function  
294 in R was used to fit an exponential distribution with equation  $\text{Log}_{10}(\text{freq}) = \text{Log}_{10}(b \cdot e^{-b \cdot \tau})$  onto the data,  
295 with  $\tau$  the time in fresh medium and  $b$  the rate of awakening. After checking normality with a Shapiro-  
296 Wilk test,  $\text{Log}_{10}$ -transformed awakening times were compared statistically among different strains or  
297 cell types using an unpaired, two-sided *t*-test, with Welch correction in the case of unequal variances  
298 (checked with an F-test).

299 *Growth rates*

300 A piecewise linear function was fitted to the cumulative number of divisions over time, for each  
301 individual cell. The number of knots was chosen by cross-validation and most often corresponds to  
302 one, dividing the growth curve into a lag phase and exponential growth phase. The slope of the second

303 curve was then used to derive the average growth rate of individual cells. After checking normality  
304 with a Shapiro-Wilk test, growth rates were compared statistically among different strains using an  
305 unpaired, two-sided *t*-test, with Welch correction in the case of unequal variances (checked with an F-  
306 test).

### 307 **Data availability**

308 The data that support the findings of this study are available from the corresponding author upon  
309 request.

### 310 **Acknowledgements**

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313 (PF/10/010; C16/17/006), FWO (G047112N; G0B2515N; G055517N), and the Flemish Institute for  
314 Biotechnology (VIB).

### 315 **Author contributions**

316 E.M.W. designed and performed the experiments, analyzed the data and wrote the manuscript. Z.B.M  
317 helped performing the experiments. T.Z., B.V.d.B., P.H. and J.M. helped designing the experiments and  
318 edited the manuscript. K.J.V. edited the manuscript.

### 319 **Competing interests**

320 The authors declare no conflict of interest.

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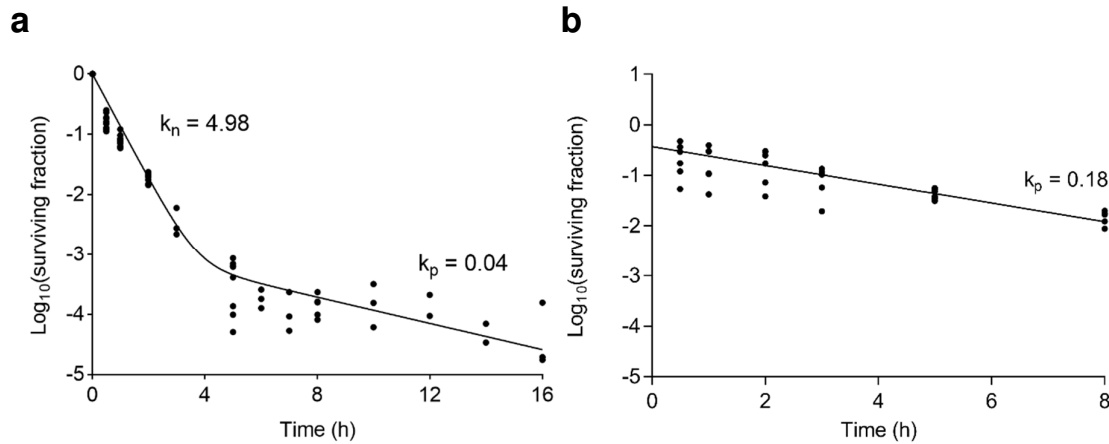
367 persistence promotes the evolution of antibiotic resistance by increasing survival and mutation

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## 370 Supplementary Figures

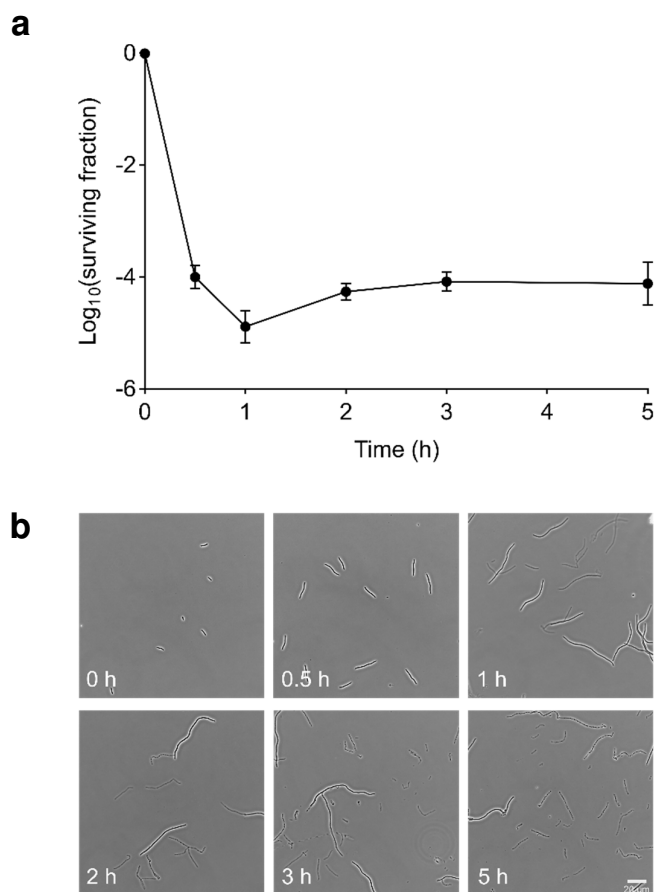
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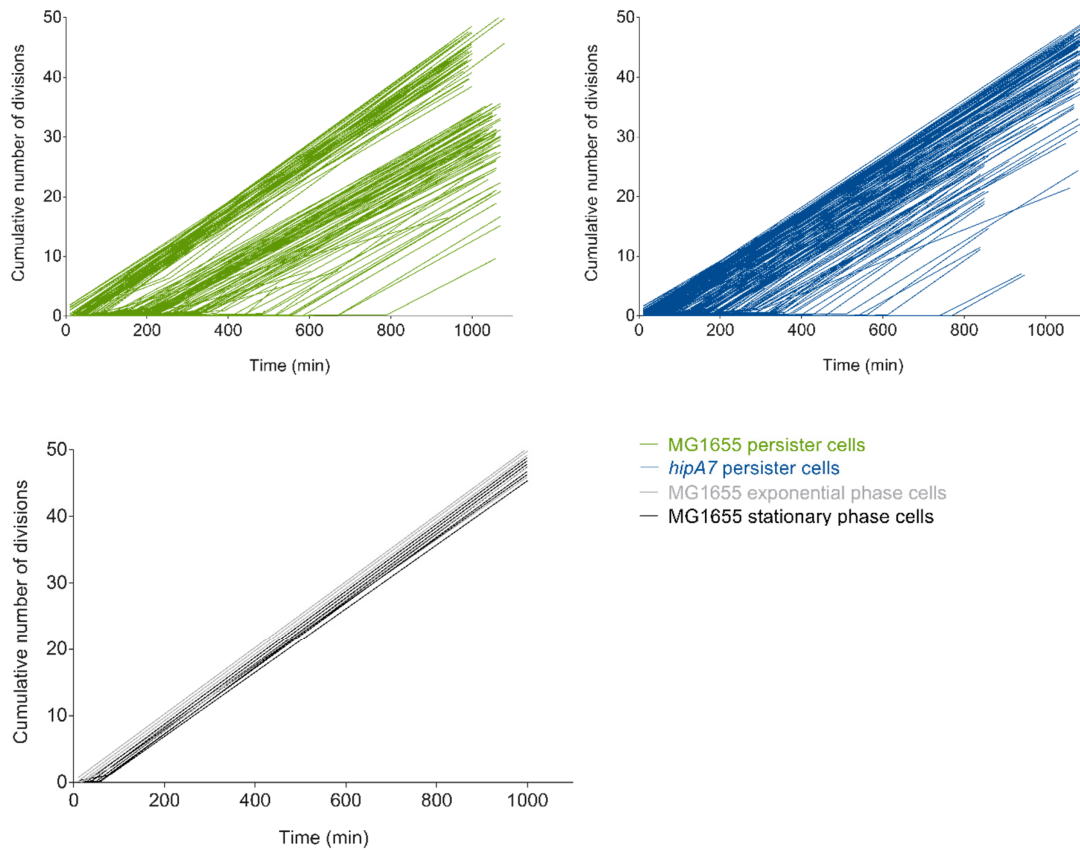
373 **Figure S1. Persisters are killed slowly in the presence of cephalexin.** (a) Time-kill kinetics of an exponential phase culture  
374 treated with cephalexin (50 µg/ml) for 16 hours. A biphasic exponential curve was fitted onto the data, with the first phase  
375 representing the fast killing rate of susceptible cells ( $k_n$ ), and the second phase showing the slow killing rate of tolerant  
376 persisters ( $k_p$ ). (b) A culture was first treated with cephalexin (50 µg/ml) for 5 hours to kill all susceptible cells (not shown).  
377 The remaining persisters were then exposed to an 8-hour cephalexin treatment (50 µg/ml). A uniphase exponential curve  
378 was fitted onto the data, with the killing rate ( $k_p$ ) presumably representing the awakening rate of persisters. This rate does  
379 not differ significantly from the killing rate of cells isolated by cephalexin treatment and filtration ( $p = 0.399$ ; Figure 2a of the  
380 main text). Best-fit estimated values of the killing parameters are indicated on the graphs.

381



382

383 **Figure S2. A 1-hour cephalixin treatment is optimal for persister isolation by filtration.** (a) Filtration was performed at  
384 regular time points during a 5-hour cephalixin treatment (50  $\mu\text{g}/\text{ml}$ ). After 30 minutes of treatment, the sample after  
385 filtration is still contaminated with susceptible cells due to insufficient filamentation. A treatment of 1 hour is sufficient to  
386 obtain a sample only containing persisters, as the number of cells does not decrease further when the treatment is extended  
387 ( $n=3$ ). The latter was confirmed by fitting a linear model to the data (time  $\geq 1$  h). The slope of this model is not significantly  
388 different from zero ( $p = 0.17$ ). (b) Microscopy images of samples taken at different time points during treatment of an  
389 exponential phase culture with cephalixin (50  $\mu\text{g}/\text{ml}$ ), without performing filtration. A treatment longer than 1 hour results  
390 in an increasing amount of debris from lysed cells and might therefore hamper subsequent single-cell studies.



393 **Figure S3. Fittings to the cumulative number of divisions of awakened persisters in the mother machine.** Linear splines  
394 were fitted onto the cumulative number of divisions for each awakened persister, as well as for exponential and stationary  
395 phase cells observed in the mother machine. Individual growth rates were derived from the slopes of the fitted curves  
396 (MG1655 persisters: n=168; *hipA7* persisters: n=129; MG1655 exponential phase cells: n=10; MG1655 stationary phase cells:  
397 n=11).