Rapid conversion of *Pseudomonas aeruginosa* to a spherical cell morphotype facilitates tolerance to carbapenems and penicillins but increases susceptibility to antimicrobial peptides

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Running title: β-lactam tolerance enhances AMP susceptibility

Abstract

The Gram negative human pathogen *Pseudomonas aeruginosa* is able to tolerate high concentrations of β-lactam antibiotics. Despite inhibiting the growth of the organism, these cell wall-targeting drugs exhibit remarkably little bactericidal activity. However, the mechanisms underlying β-lactam tolerance are currently unclear. Here we show that *P. aeruginosa* undergoes a rapid *en masse* transition from normal rod shaped cells to viable, cell wall defective spherical cells when treated with β-lactams from the widely used carbapenem and penicillin classes. When the antibiotic is removed, the entire population of spherical cells quickly converts back to the normal bacillary form. Our results demonstrate that these rapid population-wide cell morphotype transitions function as a strategy to survive antibiotic exposure. Taking advantage of these findings, we have developed a novel approach to efficiently kill *P. aeruginosa* by using carbapenem treatment to induce *en masse* transition to the spherical cell morphotype and then exploiting the relative fragility and sensitivity of these cells to killing by antimicrobial peptides (AMPs) that are relatively inactive against *P. aeruginosa* bacillary cells. This approach could broaden the repertoire of antimicrobial compounds used to treat *P. aeruginosa* and serve as a basis for developing new therapeutics to combat bacterial infections.

29 **Introduction**

30 *Pseudomonas aeruginosa* is a major human pathogen and a leading cause of hospital-acquired
31 infections. *P. aeruginosa* infections are difficult to eradicate and are often fatal, which is in part
32 due to the organism's high intrinsic resistance to a variety of different antimicrobials (1). The
33 mechanisms underlying intrinsic antibiotic resistance in *P. aeruginosa* are largely well
34 understood. However, an important and as yet unexplained observation is the ability of *P.*
35 *aeruginosa* to survive in the presence of high concentrations of the cell wall-targeting β-lactam
36 antibiotics.

37

38 β-lactams are a broad class of antibiotics that include penicillin derivatives, cephalosporins,
39 monobactams, and carbapenems. They are the most widely used group of antibiotics in the world
40 (2) and mediate bacterial killing primarily by inhibiting the enzymes, known as penicillin-binding
41 proteins or PBPs, that catalyse the formation of peptidoglycan cross-links in the bacterial cell
42 wall (3). β-lactams drugs typically exhibit bactericidal activity against susceptible organisms (4),
43 and this is often associated with changes in bacterial morphology and the formation of spherical
44 or filamentous cells that are prone to lysis (3, 5-8).

45

46 Interestingly, despite inhibiting cell growth, β-lactams have been found to have very little
47 bactericidal activity against *P. aeruginosa* (9-13). This intrinsic tolerance of β-lactams likely
48 accounts for the fact that when treating *P. aeruginosa* infections, optimal microbiological
49 outcomes occur when β-lactam concentrations are maintained at 4-6.6 x the minimal inhibitory
50 concentration (MIC) throughout the majority of the dosing period (13). Furthermore, β-lactam
51 tolerance by *P. aeruginosa* can lead to the emergence of spontaneous β-lactam resistant strains

52 (12, 14). Thus β-lactam tolerance provides significant challenges for clinicians when treating *P.*
53 *aeruginosa* infections. The mechanisms underlying this tolerance, however, are unclear.
54
55 Here we show that tolerance of *P. aeruginosa* to several β-lactams, including carbapenems and a
56 penicillin derivative, is mediated by a rapid population-wide transition to a viable, cell wall
57 defective spherical form. We demonstrate that these spherical cells can survive in the presence of
58 antibiotic and quickly revert to the normal bacillary mode of growth following its removal. Our
59 results indicate that transition to the spherical cell morphotype likely does not require the
60 acquisition of specific mutations but rather is an intrinsic response to antibiotic exposure that
61 serves as a survival strategy. Significantly, we further demonstrate that *P. aeruginosa* can be
62 efficiently killed by combining the β-lactam meropenem with antimicrobial peptides that show
63 little to no activity against bacillary cells of *P. aeruginosa*. We propose that such combination
64 treatments could provide a novel approach for antibacterial therapy.
65
66 **Materials and Methods**
67 *Bacterial strains, media and growth conditions*
68 *P. aeruginosa* strains used in this study were PA14, PAO1, PA103, PAK, ATCC27853, PAE27
69 (otitis externa isolate, Gribbles Pathology, Melbourne, Australia) and PA911 (cystic fibrosis lung
70 isolate, Monash Medical Centre, Melbourne, Australia). All experiments were performed with
71 strain PA14 unless otherwise indicated. Cells were grown either in cation-adjusted Mueller
72 Hinton broth (CAMHB; Fig. 1), synthetic cystic fibrosis sputum medium (15) (Fig. S1A), fetal
73 bovine serum (Fig. S1B) or CAMHB supplemented with 0.5 M sucrose (Fig. 2-7 and Fig. S2-S3).
74 To induce spherical cell formation, cultures were grown to early-exponential phase at 37°C with

75 vigorous shaking, antibiotic was added and cells were then incubated without shaking at 37°C.
76 For reversion, spherical cells were pelleted by centrifugation at 3,000 *g* for 10 min, resuspended
77 in antibiotic-free media and grown with static incubation at 37°C. Antibiotics were used at 5x
78 MIC, as determined against strain PA14 using standard methods (16). MIC values for PA14 were
79 as follows: meropenem, 1 μg/mL; imipenem, 2 μg/mL; carbenicillin, 128 μg/mL. MIC values for
80 meropenem were also determined against each strain used in the study and were found to be
81 between 1-2 μg/mL for all strains.
82
83 *Phase-contrast and fluorescence microscopy*
84 To prepare samples for phase-contrast imaging, a 1.0 X 1.0 cm square well was set up on the
85 surface of a glass microscope slide by attaching a 25 μL Gene Frame (ABgene). The well was
86 filled with 25 μL of culture and a coverslip was placed on top. For fluorescence microscopy
87 (live/dead staining and super resolution 3D-SIM), 200 μL of cells were applied to a 35 mm sterile
88 glass bottom FluoroDish (World Precision Instruments). Membrane stain FM1-43FX (5 μg/mL;
89 Life Technologies) was added to the sample before applying to the dish for 3D-SIM. Syto9 (5
90 □M; Life Technologies) and propidium iodide (30 □M; BD) were added for live/dead staining.
91
92 An Olympus IX71 wide-field inverted microscope was used for phase-contrast imaging, a Zeiss
93 Axioplan 2 fluorescence microscope was used for imaging of live/dead stained cells and a
94 DeltaVision OMX-Blaze™ (Applied Precision Inc.) was used for 3D-SIM (17). Images were
95 processed as described previously (17), analysed and presented using AnalySIS Research Pro
96 (Olympus), AxioVision (Zeiss) or IMARIS (Bitplane) software.
97

98 *Transmission electron microscopy (TEM)*

99 To prepare samples for TEM, cells were pelleted by centrifugation at 3,000 *g* for 10 min and
100 resuspended in a fixative solution of 2% glutaraldehyde in phosphate buffered saline (PBS; 0.1M,
101 pH 7.2) containing 0.1 M sucrose. Cells were fixed overnight at 4°C, then washed three times in
102 PBS. Cell pellets were embedded in 1% low melt agarose (J.T. Baker Inc.) and agarose blocks
103 were cut into 1 mm cubes, transferred to glass vials and post-fixed in 1% osmium tetroxide
104 (O_SO_4) in PBS for 1 h at room temperature. Following post-fixation, samples were washed in
105 distilled water three times for 5 min each wash and immersed in 2% aqueous uranyl acetate for
106 30 min. Cell pellets were dehydrated through a graded series of ethanols (50-100%), infiltrated
107 and subsequently embedded in L. R. White Resin (London White Resin Company). Ultra-thin
108 (70 nm) sections were cut using a Reichert ultramicrotome (Ultracut S, Leica Microsystems) and
109 mounted onto pioloform-coated, 300 mesh, thin-bar copper grids. Grids were stained with
110 saturated aqueous uranyl acetate (7.7%) for 30 min and Reynold's lead citrate for 4 min. Samples
111 were examined using a Philips CM0 transmission electron microscope equipped with a
112 Megaview III TEM CCD camera and iTEM image capture software (Olympus). Digital images
113 were processed for publication using Adobe Photoshop CS version 8.0 (Adobe Systems). All
114 TEM materials were supplied from ProScitech, Australia.

115

116 *Viability assays*

117 To estimate the number of viable cells in culture, 10-fold dilutions were plated in triplicate onto
118 LB medium containing 1.6% agar (without antibiotics) and incubated overnight at 37°C.
119 Colonies were counted and viability was calculated as the number of colony forming units
120 (CFUs) per mL of culture.

121

122 **Results**

123 *P. aeruginosa cells rapidly convert to a viable spherical morphotype when treated with*
124 *meropenem*

125 As an initial approach to understand β-lactam tolerance by *P. aeruginosa*, we monitored *P.*
126 *aeruginosa* cells under the microscope following treatment with meropenem, a broad-spectrum
127 carbapenem antibiotic that is commonly used to treat *P. aeruginosa* infections in humans. It has
128 been noted in previous studies that *P. aeruginosa* cells switch to a spherical morphology when
129 exposed to meropenem (7, 8). We hypothesized that this spherical morphotype may be a viable
130 alternative form of the bacterium that mediates survival in the presence of the antibiotic.

131

132 To test this, *P. aeruginosa* strain PA14 was first cultured in a standard laboratory growth
133 medium (cation-adjusted Mueller Hinton broth) and treated with a clinically relevant dose of
134 meropenem (5x MIC). Cells were then visualized at regular intervals using conventional phase-
135 contrast microscopy. In the presence of meropenem, we observed a rapid and efficient conversion
136 of the entire population from normal rod shaped cells to apparently spherical cells (Fig. 1A-E).
137 Just 1 h after the addition of the antibiotic a large proportion of cells (65%) had clearly begun
138 transitioning to the spherical morphology. Most cells (67%) had completed the transition by 4 h,
139 and after 24 h the entire population consisted only of spherical cells (Fig. 1E). To determine if
140 these spherical cells were viable we co-stained PA14 with the fluorescent dyes Syto9 and
141 propidium iodide after 24 h exposure to meropenem. This approach differentiates viable cells,
142 which only stain with Syto9 (green), from dead cells that also stain with propidium iodide (red)
143 (Fig. 1F, G). These assays showed that the vast majority of spherical cells in the population
144 (84%) were viable and thus able to tolerate exposure to 5x MIC of the antibiotic.

145

146 The remaining 16% of cells were lysed, and could be distinguished from viable cells not only by
147 live/dead staining but also by a characteristic "ghost"-like appearance in phase-contrast images
148 (Fig. 1F, arrow). Interestingly, we found that the addition of 0.5M sucrose to the growth medium
149 as an osmoprotectant reduced the number of lysed cells from 16% to 3% after 24 h exposure to
150 meropenem (Fig. 1H, I). Osmoprotection was clearly not essential for spherical cell formation or
151 viability, however, unless otherwise indicated, we chose to add 0.5M sucrose to the growth
152 medium in subsequent experiments (Fig. 2-7) simply to minimize the small amount of cell lysis
153 observed in standard laboratory broth.

154

155 Importantly, we also tested whether spherical cells can form under conditions relevant to
156 infection and the *in vivo* environment. To this end PA14 cells were treated with meropenem (5x
157 MIC) in a synthetic sputum medium designed to mimic the cystic fibrosis lung (15), as well as in
158 fetal bovine serum. Note, sucrose was not added to these media. In both synthetic sputum and
159 fetal bofine serum we observed a rapid transition of the cellular population from rods to spherical
160 cells following treatment with meropenem, similar to that seen in standard growth media (Fig.
161 S1). Therefore, *P. aeruginosa* spherical cells form in response to meropenem exposure under
162 physiologically relevant conditions.

163

164 To confirm the spherical shape of meropenem-treated cells we used 3D-structured illumination
165 microscopy (3D-SIM), a super resolution fluorescence technique that enables genuine 3D images
166 of bacterial cells and reveals morphological details not visible with conventional microscopy
167 through increases in both lateral and axial resolution (18) (Fig. 2). We also used 3D-SIM to
168 examine the transition from rod to sphere, and found that it begins with the emergence of a small

169 membrane protrusion on the cell surface (Fig. 2B). This gradually bulges out to form a larger
170 sphere, which becomes completely enclosed in its own membrane. The original rod then lyses,
171 leaving a mature, spherical cell (Fig. 2B, C).
172
173 *P. aeruginosa spherical cells have a defective cell wall and disrupted outer membrane*
174 The bacterial cell wall is a key structural feature of the cell that determines its shape and serves as
175 an important osmotic barrier to the external environment. The loss of cell shape observed after
176 meropenem treatment of *P. aeruginosa*, together with the slight osmosensitivity of the resulting
177 spherical cells (see above), strongly suggests that these cells lack a functional cell wall. In
178 addition, we found that spherical cells rapidly burst when placed directly between a microscope
179 slide and a coverslip, indicating a mechanical fragility consistent with loss of integrity in the cell
180 envelope.
181
182 To further examine the cell envelope structure of the spherical cell morphotype, we performed
183 transmission electron microscopy (TEM) on thin sections of PA14 cells after 24 h exposure to
184 meropenem. We also examined untreated cells as a control, and as expected these cells exhibited
185 a regular double membrane cell envelope structure characteristic of Gram negative bacteria (Fig.
186 3A, B). In spherical cells, however, the outer membrane was clearly disrupted in all cells
187 examined (Fig. 3C, D; 82 cells were analysed in total). While fragments of outer membrane
188 remained associated with the cell (Fig. 3C), there were extensive regions in which the inner
189 membrane appeared to be exposed on the cell surface. Interestingly, membrane vesicles were
190 often observed to form apparently from outer membrane material that had detached from the cell
191 envelope (Fig. 3C, D). Taken together, these results indicate that *P. aeruginosa* cells can convert

192 to an alternative form deficient in an intact cell wall and outer membrane when challenged with a
193 cell wall-targeting antibiotic.
194
195 *The transition to spherical cells is readily reversible*
196 To test whether *P. aeruginosa* PA14 spherical cells induced by meropenem treatment can revert
197 to normal rod shaped cells in the absence of antibiotic selection, we resuspended spherical cells
198 formed after 24 h exposure to meropenem in fresh media lacking the drug. Cells were then
199 visualized at regular intervals by phase-contrast microscopy and 3D-SIM. Under these
200 conditions, we observed a rapid population-wide transition of the cellular population from
201 spherical cells back to normal bacillary cells (Fig. 4). The first visible sign of this reversion was a
202 change from a spherical to an ellipsoid morphology, which could be seen in some cells after just
203 30 min in the absence of drug. This was followed by a gradual elongation in cell length and
204 reduction in width to produce a normal cylindrical cell. High resolution 3D-SIM images showed
205 that the cell surface was highly irregular, sometimes even branched, during this reversion
206 process, but ultimately became smooth and uniform as the normal cell wall was restored (Fig.
207 4E). After 6 h in the absence of antibiotic, the vast majority of the cellular population (>90%) had
208 completed the transition to rods (Fig. 4F). These cells were able to grow and divide normally, as
209 evidenced by a rapid increase in the density of cells in culture following reversion to the rod
210 shape (Fig. 4C-D). This indicates that the transition to spherical cells following exposure to
211 meropenem is readily reversible.
212
213 *Spherical cell formation is a conserved mechanism of meropenem tolerance*
214 Importantly, we observed that the ability to transition into and out of the spherical cell state
215 occurred independently of strain background. An identical response to meropenem treatment was

216 seen for a wide variety of *P. aeruginosa* strains, including well known lab strains (PA14, PAO1,
217 PA103, PAK, ATCC27853) and recent clinical isolates (data not shown). This observation, along
218 with the sheer speed, efficiency and reversibility of the rod to spherical cell transition, indicates
219 that formation of spherical cells in *P. aeruginosa* does not depend on the acquisition of
220 chromosomal mutations. Rather, our results suggest that spherical cell formation is an intrinsic
221 protective response. By undergoing rapid *en masse* phenotypic transitions between bacillary and
222 spherical cell morphotypes, the vast majority of the *P. aeruginosa* population are able to survive
223 exposure to meropenem and are poised to transition to the bacillary form when antibiotic levels
224 are reduced.
225
226 *Penicillins and carbapenems induce spherical cell-mediated antibiotic tolerance*
227 Having determined that *P. aeruginosa* PA14 cells quickly convert to a spherical cell morphotype
228 when treated with meropenem, we were interested to determine whether other cell wall-targeting
229 antibiotics can elicit a similar response. Interestingly, *P. aeruginosa* has previously been shown
230 to induce "spheroplasts" in response to carbenicillin (19), a penicillin derivative, and we reasoned
231 that these spherical cells could facilitate tolerance to the drug. We found that following treatment
232 of PA14 with carbenicillin (5x MIC), a large proportion of cells indeed converted to spherical
233 cells and did so via the same morphological pathway that we observed in response to meropenem
234 exposure (Fig. 5). In contrast to meropenem, however, some cells became filamentous rather than
235 converting to the spherical cell state (Fig. 5). While filamentation is a common phenotype
236 associated with β-lactam treatment in rod-shaped bacteria (3, 20), we found that the *P.*
237 *aeruginosa* filamentous cells arising under these conditions tended to lyse. Live/dead staining
238 with Syto9 and propidium iodide showed that the filamentous cells stained with propidium iodide

239 indicating that they were non-viable (Fig. 5C) whereas the spherical cell morphotypes stained
240 only with Syto9 and were therefore viable. As was observed after removal of meropenem, the
241 population of carbenicillin-induced spherical cells rapidly reverted to normal bacillary cells when
242 resuspended in fresh media lacking the antibiotic (data not shown).
243
244 We also examined the effect of imipenem (5x MIC) on *P. aeruginosa* PA14 cells. Like
245 meropenem, imipenem is a carbapenem antibiotic and we found that this also induced a rapid and
246 reversible transition of the entire cellular population to spherical cells (Fig. S2). Taken together,
247 these results demonstrate that spherical cell-mediated antibiotic tolerance in *P. aeruginosa* can
248 occur in response to β-lactams from at least two classes.
249
250 *Meropenem-induced spherical cells are efficiently killed by antimicrobial peptides*
251 Having established that *P. aeruginosa* cells convert very rapidly and efficiently to cell wall
252 deficient spherical cells when treated with β-lactam antibiotics, we hypothesized that the
253 combination of a β-lactam that induces spherical cell formation with a drug that kills spherical
254 cells could be feasible as a new and effective treatment approach for *P. aeruginosa* infections.
255 One class of compounds that we reasoned might exhibit strong spherical cell killing activity were
256 antimicrobial peptides (AMPs). These compounds function by direct interaction with membrane
257 lipids and are thought to induce cell death through the formation of pores in the cytoplasmic
258 membrane (21). Our TEM images show that there are vast stretches of cytoplasmic membrane
259 exposed on the surface of spherical cells (Fig. 3) which should therefore be much more accessible
260 to AMPs. Importantly, AMPs tend to have only weak antimicrobial activities against normal
261 bacillary cells of *P. aeruginosa* under physiological conditions (22).

262

263 To test our hypothesis, cultures of *P. aeruginosa* PA14 were treated with a combination of
264 meropenem to induce spherical cell formation (at a fixed concentration of 5 μg/mL) and one of
265 the AMPs LL-37 or nisin (at various concentrations between 0 and 128 μg/mL). After 24 h in the
266 presence of the drug combinations, viable cell counts were determined by serial dilution and
267 plating. While meropenem alone produced viable spherical cells, the addition of LL-37 or nisin
268 markedly reduced viability (Fig. 6A). In fact, at the highest AMP concentration tested (128
269 μg/mL), viable cell counts were more than 3-4 log lower than those of the meropenem only
270 control. IC50 values were 16 μg/mL for nisin and 32 μg/mL for LL-37 in combination with
271 meropenem. The strong bactericidal activity of the meropenem/AMP combinations was further
272 highlighted by a distinct absence of intact cells under the microscope, replaced with what
273 appeared to be cellular debris (Fig. 6B-D). Importantly, under our assay conditions the AMPs
274 alone (LL-37 or nisin) showed no detectable antibacterial ability (Fig. 6E-H) even at the highest
275 concentration tested (128 μg/mL). This is consistent with the high MIC values recently reported
276 for LL-37 against PA14 cells under similar conditions (23) (112 – 225 μg/mL). These
277 observations suggest, as predicted, that AMPs are more effective against spherical cells than
278 normal bacillary cells, which we further confirmed by demonstrating that the AMPs can
279 efficiently kill pre-formed *P. aeruginosa* spherical cells (Fig. S3).

280

281 Finally, time-kill assays were performed with PA14 to examine the rate of meropenem/AMP-
282 induced cell death (Fig. 7A). Both LL-37 and nisin triggered a rapid decline in viability when
283 administered in combination with meropenem. A 100-fold reduction of viable cell counts relative
284 to the meropenem only control was observed within just 1 h for LL-37 and 8 h for nisin and by

285 24 h viability was reduced by 4-5 log for both AMP/meropenem combinations. Interestingly, the
286 rapid killing by LL-37 suggests that only a partial transition of the cell to the spherical cell state
287 is required for the AMP to mediate killing. We confirmed this for LL-37 using time-lapse
288 microscopy, which showed that the peptide causes the cell to burst at the site of spherical cell
289 formation (Fig. 7B).
290

291 **Discussion**

292 Currently there is a desperate need for innovative approaches to antimicrobial therapy. This stems
293 from a relentless rise in the emergence of antibiotic resistant bacteria, a lack of new antibiotic
294 classes in the pharmaceutical pipeline, and greatly reduced investment within the pharmaceutical
295 sector (24-26). Our data show that it is possible to effectively kill bacteria by turning their own
296 biological responses against them, namely using drug combinations that induce the cell to
297 transition to an alternative form then exploit the inherent weaknesses of that form. Drug
298 combinations are commonly used in medicine to enhance the effectiveness of existing antibiotics
299 (24), and represent a promising avenue for the development of new treatments (27-29) with
300 reduced rates of resistance (30). In this study we have shown that combinations of meropenem
301 with AMPs LL-37 or nisin are potently bactericidal against *P. aeruginosa* at concentrations
302 where each drug alone has negligible bactericidal activity. Our findings provide a platform to
303 identify novel antibacterial drug combinations that induce and target spherical cells in a directed
304 and cost effective manner.
305

306 Significantly, our observations suggest that the ability to switch between bacillary and spherical
307 cell morphotypes is a response to stress invoked by exposure to β-lactam antibiotics. Transition

308 of *P. aeruginosa* to the spherical state occurred rapidly in response to β-lactams from the
309 carbapenem and penicillin classes, and was found to be independent of genetic background.
310 These results suggest that spherical cell formation may be an intrinsic developmental response of
311 *P. aeruginosa* that is induced by antibiotic treatment. Whilst the production of spherical cells by
312 carbapenems and penicillins has been reported previously (7, 8, 19), our observations show for
313 the first time that spherical cell formation is reversible and occurs readily under physiological
314 conditions, indicating that this response may serve as a genuine mechanism of drug tolerance.
315
316 We propose that spherical cell-mediated β-lactam tolerance may underlie many of the
317 pharmacodynamic challenges associated with β-lactam treatment of *P. aeruginosa* infections (12-
318 14), at least in the case of carbapenems and penicillins. *P. aeruginosa* infections are notoriously
319 difficult to treat and are often chronic or recurrent in nature, particularly in immunocompromised
320 patients (31). It is tempting to speculate that the transition of *P. aeruginosa* to a viable alternative
321 spherical cell morphotype during exposure to β-lactams could lead to prolonged or recurrent
322 infections by allowing the bacterium to survive *in vivo* over the course of antibiotic treatment and
323 then to revert to the bacillary form when antibiotic therapy is ceased .
324
325 Significantly, spherical cell formation may also play an important role in the emergence of β-
326 lactam resistance. Spontaneous β-lactam resistant mutants of *P. aeruginosa* are known to arise
327 quite readily *in vitro* during antibiotic treatment (12, 14). Here we have shown that *P. aeruginosa*
328 can undergo *en masse* transition to cell wall defective spherical cells under these conditions.
329 While in the spherical form the population would be well poised to acquire β-lactam resistance,
330 either by mutation or lateral gene transfer (1), which would enable reversion to the bacillary form

331 and rapid cell proliferation. Consistent with this idea, early studies on carbenicillin-induced
332 spheroplasts of *P. aeruginosa* showed that cells spontaneously reverted to rods after extended
333 exposure to the drug (5-8 days), and that the MIC of carbenicilin increased as much as 150-fold
334 following reversion (19, 32). Critically, we now show that a carbapenem in combination with
335 antimicrobial peptides induces rapid killing of *P. aeruginosa* cells. This type of approach would
336 severely limit the potential for acquiring resistance during β-lactam therapy.
337
338 A classic problem in population and evolutionary biology is to understand how a population
339 copes with environmental changes that challenge survival. Common strategies that enable
340 survival of antibiotic exposure by bacterial populations include selection of a sub-population that
341 has a fitness advantage that can arise either through mutation, stochastic phenotypic
342 heterogeneity or regulated responses (33). Our observations suggest that tolerance to β-lactam
343 exposure by *P. aeruginosa* can occur through an adaptive response that involves *en masse*
344 phenotypic transitions between bacillary cells and spherical cells with defective cell walls.
345 Therefore, population-wide morphotype transitions add to the repertoire of strategies that can be
346 employed by bacteria to survive exposure to β–lactam antibiotics.
347
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438
439

440 **Figure Legends**

441 **Fig. 1. *P. aeruginosa* rapidly converts to spherical cells in response to a β-lactam antibiotic.**
442 PA14 cells cultured in cation-adjusted Mueller Hinton broth (CAMHB) were treated with
443 meropenem (5x MIC) and visualized by phase-contrast or fluorescence (live/dead) microscopy.
444 (A-D) Phase-contrast images taken immediately prior to meropenem addition (A) and after 1 h
445 (B), 4 h (C) and 24 h (D) in the presence of the drug. (E) Proportion of rods, spherical cells and
446 transitioning cells in the population over time. 200 cells were scored from phase-contrast
447 micrographs at each time point from 2 experiments. (F-I) Live/dead viability staining of
448 meropenem-treated cells after 24 h. Cells were grown in CAMHB (F-G) or CAMHB
449 supplemented with 0.5M sucrose (H-I). Samples were co-stained with SYTO9 (live, green) and
450 propidium iodide (dead, red) and visualized by phase-contrast (F, H) and wide-field fluorescence
451 microscopy (G, I). Arrow points to a lysed cell. Scale bars 5 μm.

452

453 **Fig. 2. Super resolution 3D-SIM microscopy showing the transition of *P. aeruginosa* to**
454 **meropenem-induced spherical cells.** Following treatment with meropenem, PA14 cells were
455 stained with membrane dye FM1-43 and visualized by 3D-SIM. Images were acquired at 0 h (A),
456 2 h (B) and 24 h (C) after meropenem addition. Asterisk highlights a developing spherical cell
457 that is fully surrounded by membrane. Arrow shows a cell at an earlier stage of the transition,
458 containing a surface bulge that is not yet membrane-enclosed. Scale bars 1 μm.

459

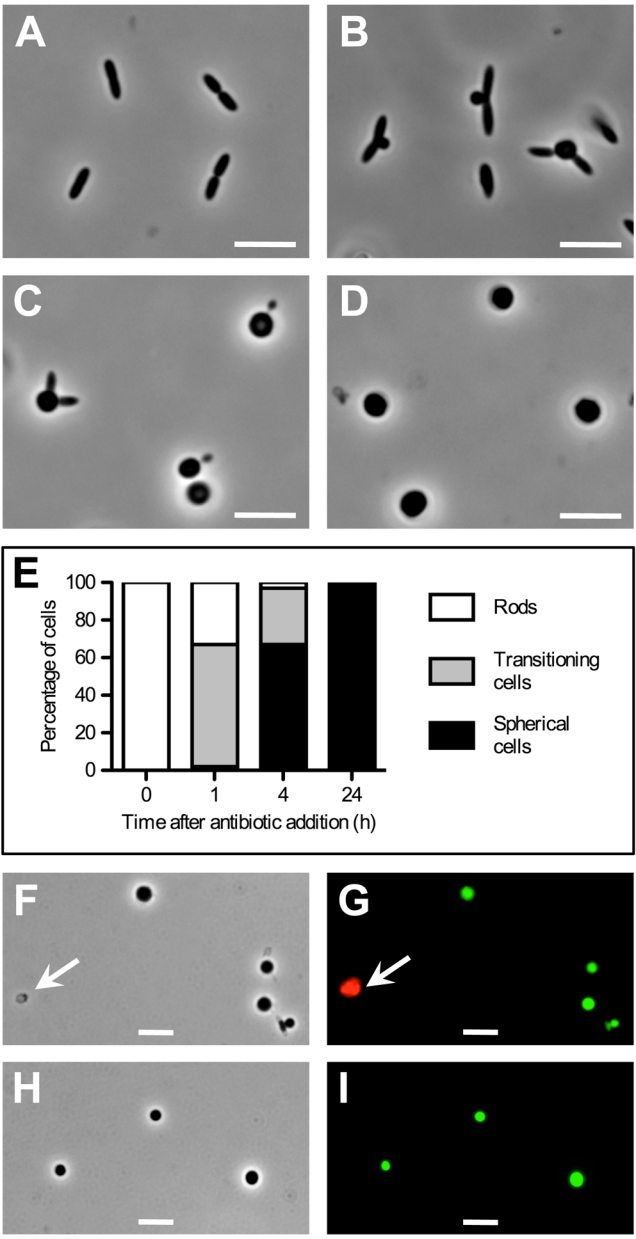
460 **Fig. 3. Meropenem-induced spherical cells of *P. aeruginosa* have a compromised cell**
461 **envelope.** Transmission electron microscopy (TEM) was performed on thin sections of PA14
462 cells immediately prior to the addition of meropenem (A, B) and after 24 h in the presence of the

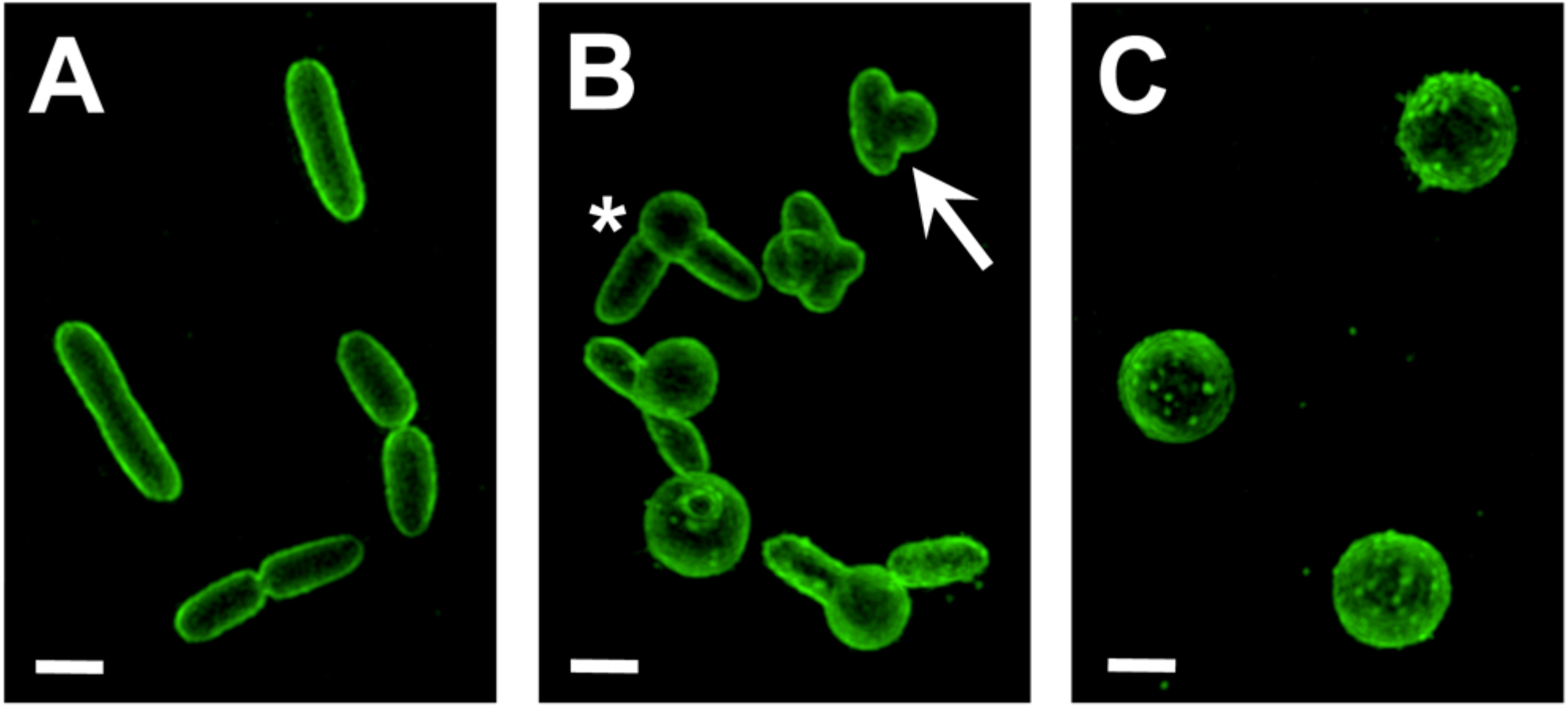
463 drug (C, D). Panels B and D are shown at high magnification to illustrate the cell envelope
464 structure, revealing an intact outer membrane (black arrow) and inner membrane (white arrow) in
465 the bacillary form (B) and a clearly disrupted outer membrane in the spherical cell (D). Arrow in
466 C shows a vesicle that appears to have formed from detached outer membrane material. Scale
467 bars 100 nm.
468
469 **Fig. 4. Spherical *P. aeruginosa* cells rapidly revert to normal rods in the absence of**
470 **antibiotic.** Meropenem-induced spherical cells of PA14 were resuspened in antibiotic-free media
471 and visualized by phase-contrast microscopy or 3D-SIM. (A-D) Phase-contrast images taken at 0
472 h (A), 2 h (B), 6 h (C) and 24 h (D) after removal of meropenem. (E) 3D-SIM images of FM1-43
473 stained cells captured at various stages in the transition from spherical cell (i) to normal rod (vi).
474 (F) Proportion of spherical cells, rods and transitioning cells in the population over time. 200
475 cells were scored from phase-contrast micrographs at each time point from 2 experiments. Scale
476 bars 5 μm (A-D) or 1 μm (E).
477
478 **Fig. 5. Spherical cells induced by carbenicillin.** (A-B) 3D-SIM images of PA14 cells stained
479 with membrane dye FM1-43 after 2 h (A) and 24 h (B) in the presence of carbenicillin. (C)
480 Live/dead viability staining of carbenicillin-treated cells after 24 h, showing viable spherical cells
481 and a dead filamentous cell. Cells were co-stained with SYTO9 (live, green) and propidium
482 iodide (dead, red) and visualized by wide-field flourescence microscopy. (D) Proportion of rods,
483 spherical cells, transitioning cells and filaments in the population over time. 200 cells were
484 scored from phase-contrast micrographs at each time point. Scale bars 1 μm (A-B) or 5 μm (C).
485

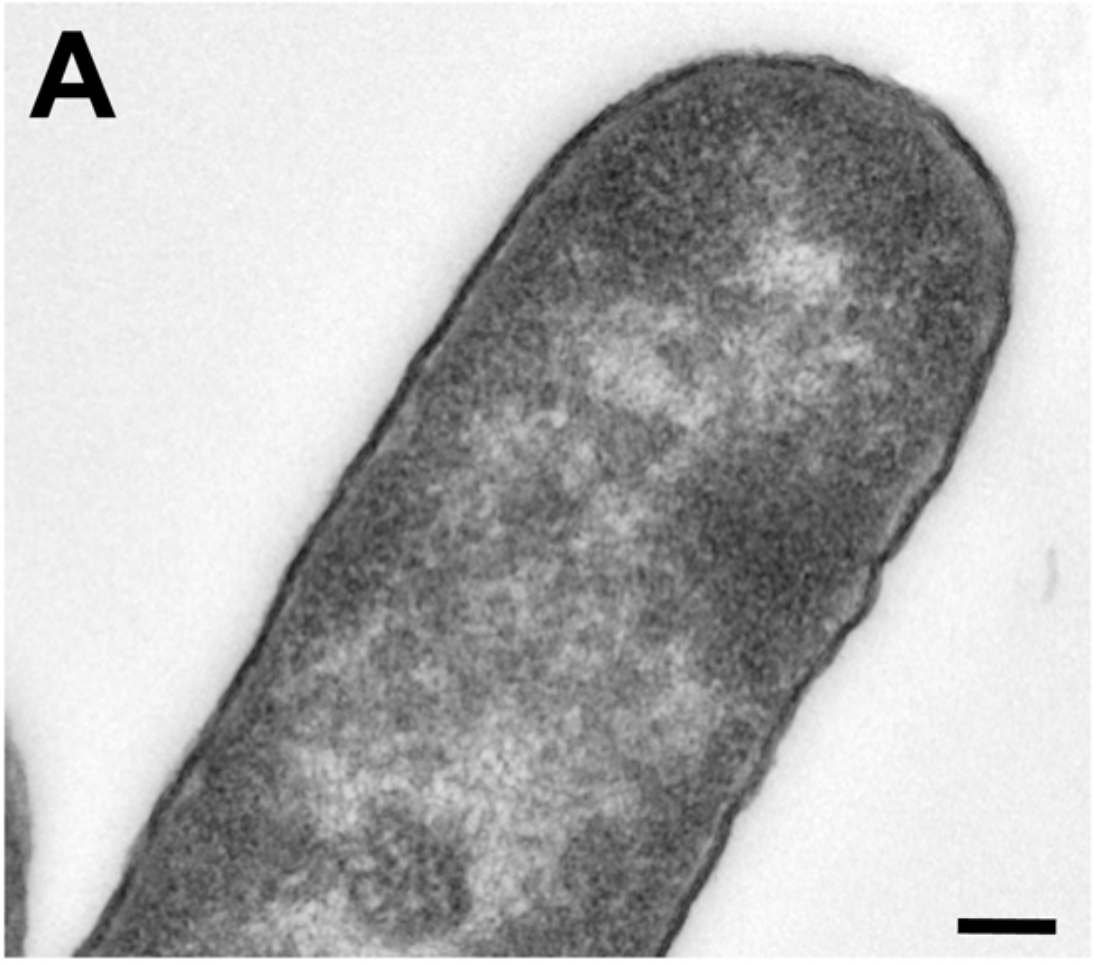
486 **Fig. 6. AMPs selectively kill meropenem-induced spherical cells of *P. aeruginosa*.** (A) Viable
487 cell counts after treatment of PA14 for 24 h with a combination of meropenem (5 μg/mL) and
488 either LL-37 or nisin (0-128 μg/mL). (B-D) Representative phase-contrast images of cells treated
489 under the same conditions as in A. (B) Meropenem only control (no peptide), showing intact
490 spherical cells. (C) LL-37 (128 μg/mL) with meropenem. (D) Nisin (128 μg/mL) with
491 meropenem. Cellular debris can be seen in place of intact cells for both AMP/meropenem
492 combinations. (E) Viability counts for untreated cells and cells treated with LL-37 or nisin alone
493 (128 μg/mL). (F-H) Corresponding phase-contrast images. (F) Untreated control, showing
494 normal rod shaped cells. (G) LL-37. (H) Nisin. Neither AMP alone appears to have any effect on
495 cellular morphology. Scale bar 5 μm. For (A) and (E) one representative data set is shown from
496 duplicate (A) or triplicate (E) experiments. Error bars correspond to the standard error of the
497 mean for triplicate measurements.
498
499 **Fig. 7. Meropenem and AMP combinations induce rapid cell death.** (A) Time-kill assay.
500 Viable cell counts were measured at regular intervals after the addition of meropenem (5 μg/mL),
501 both alone and in combination with LL-37 or nisin (128 μg/mL), to a culture of PA14 cells. One
502 representative data set from duplicate experiments is shown. Error bars correspond to the
503 standard error of the mean for triplicate measurements. (C) Time-lapse microscopy capturing the
504 peptide-mediated death of a developing spherical cell. PA14 cells were treated with meropenem
505 (5 μg/mL) for 1 h to induce a partial transition to the spherical state. LL-37 (128 μg/mL) was
506 then added, and phase-contrast images were collected at 2 s intervals over a period of 30 min. In
507 the example shown, panel i was acquired 13 min after the addition of the peptide, and each

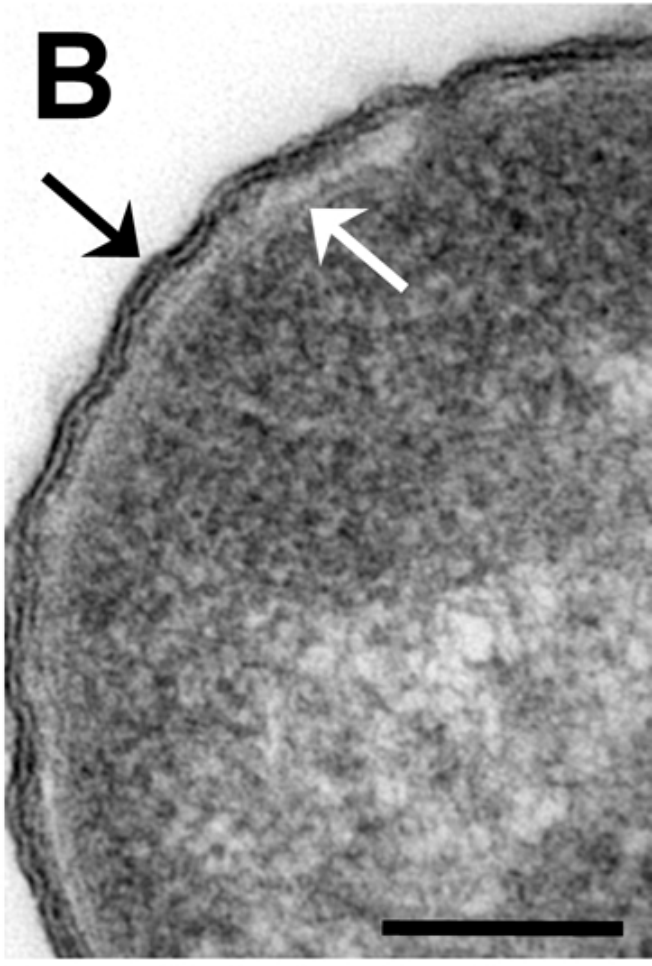
508 subsequent image after an additional 2 s. A total of 13 individual cell death events were observed

509 over four separate experiments. Scale bar 1 μm.


A
B
C
D
E
Percentage of cells
100
80
60
40
20
0
0
1
4
24
Time after antibiotic addition (h)
Rods
Transitioning cells
Spherical cells
F
G
H
I


A
B
*
C


A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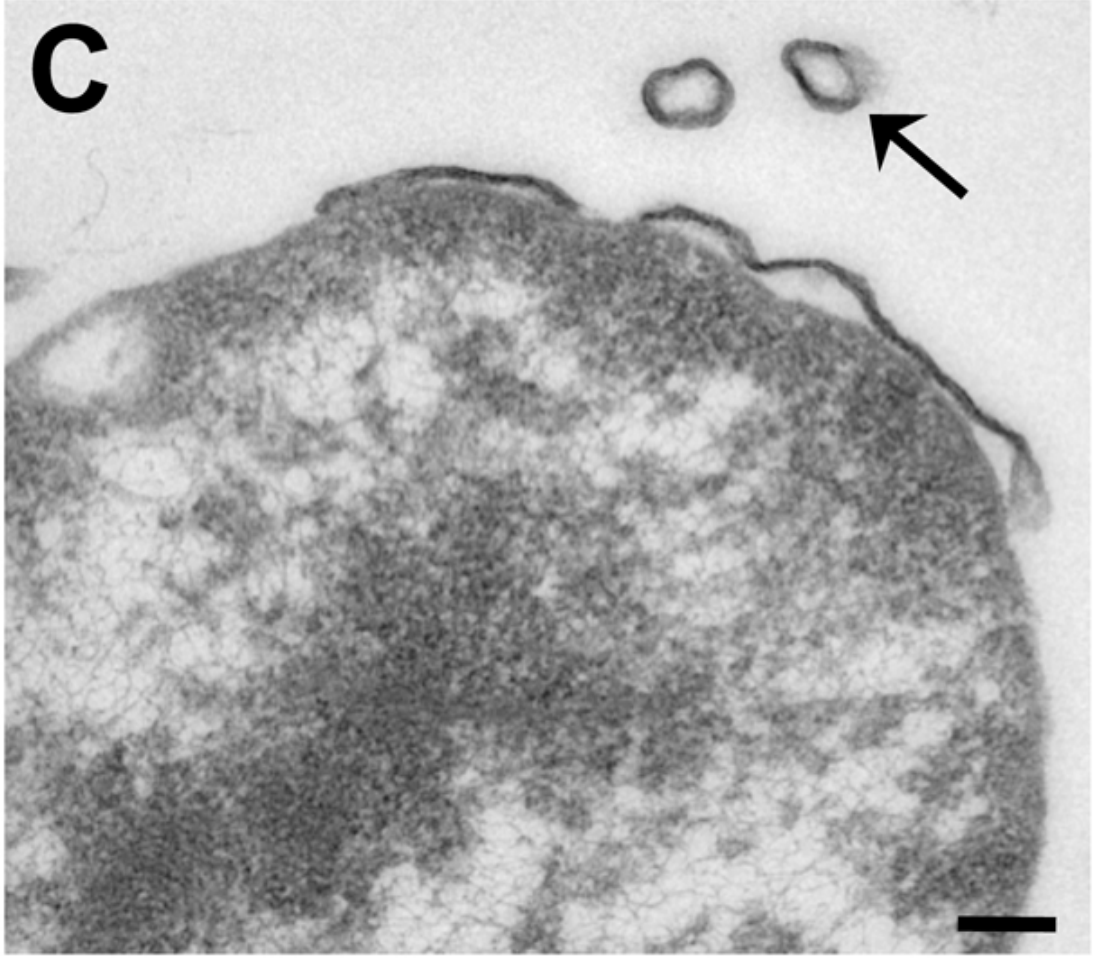
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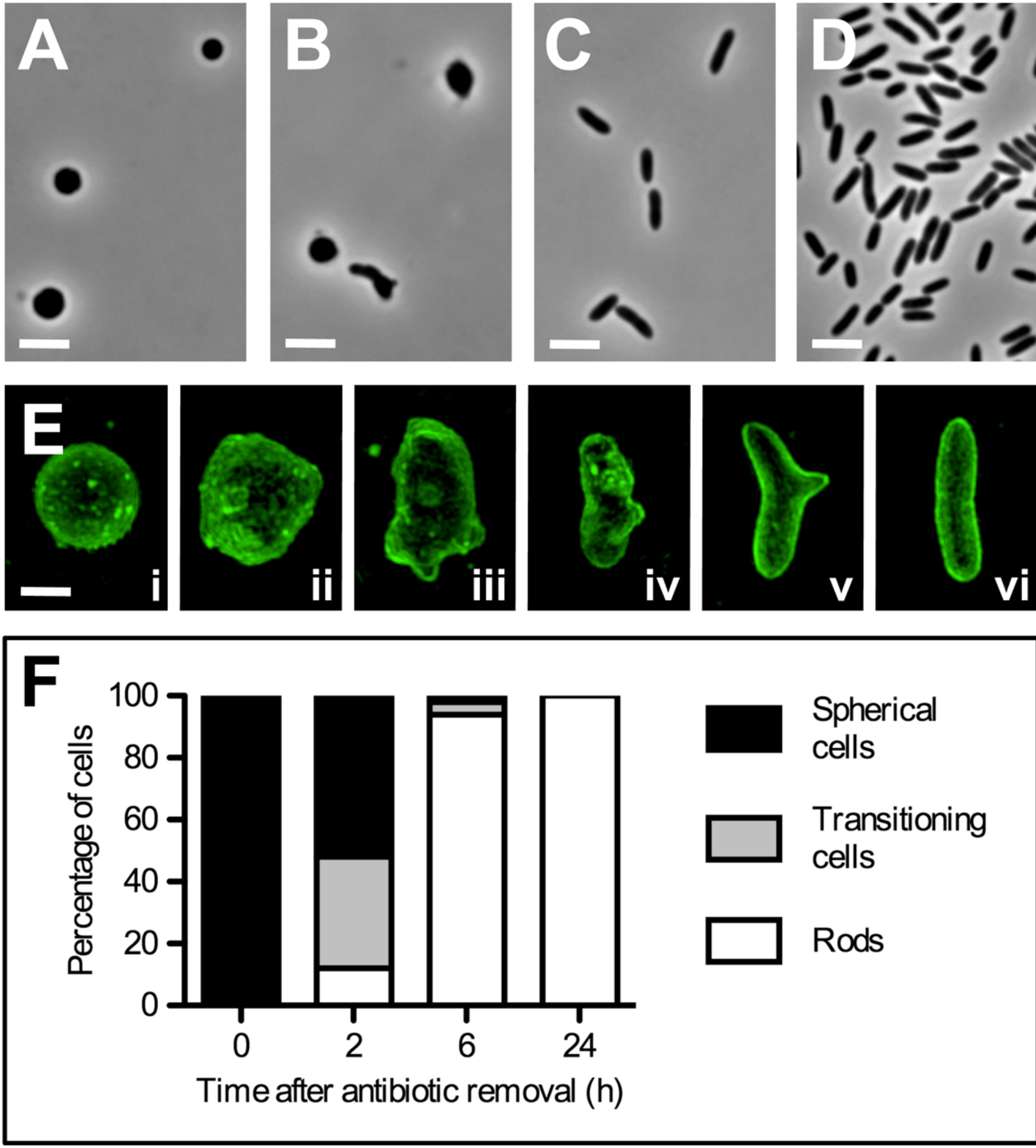
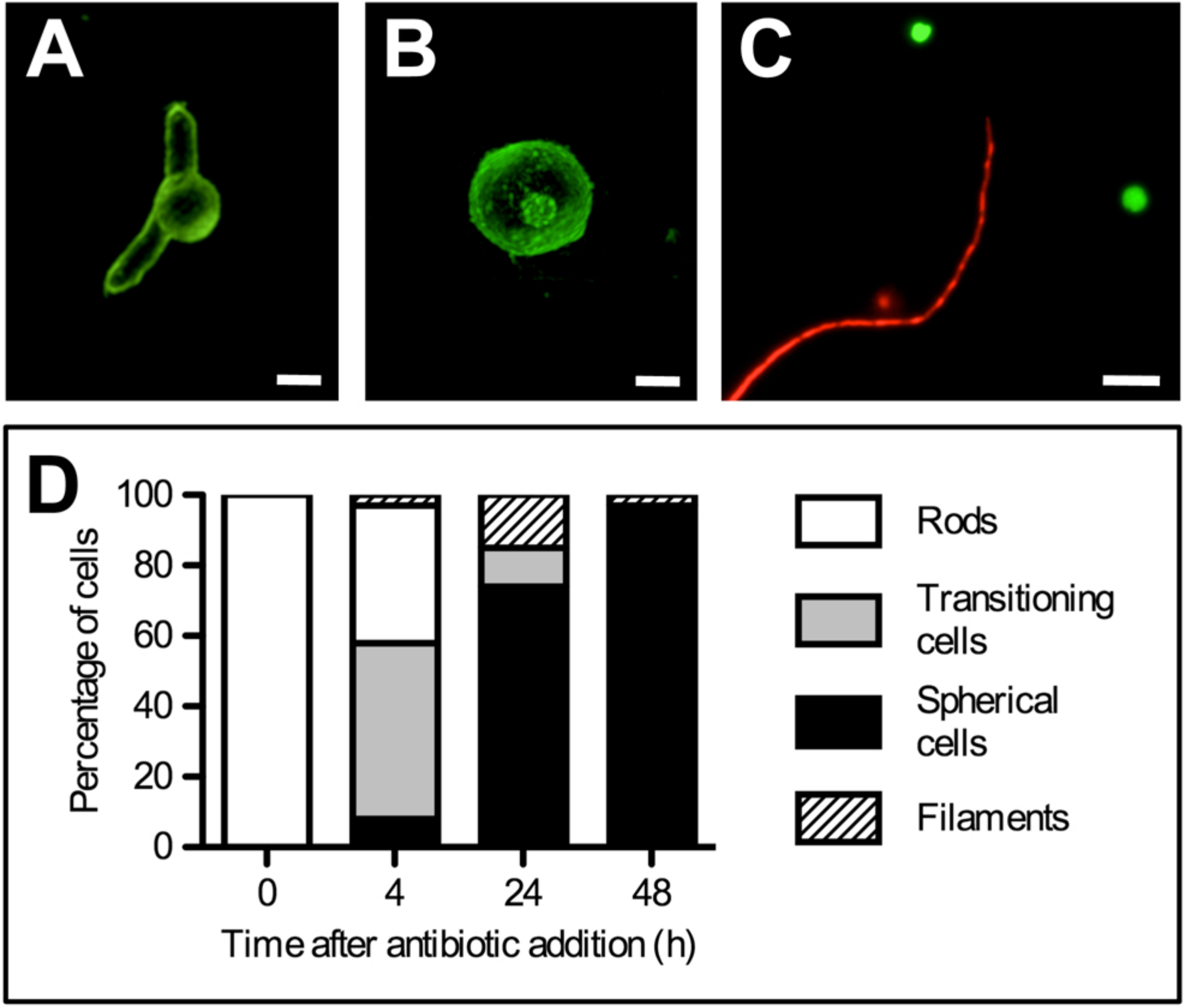
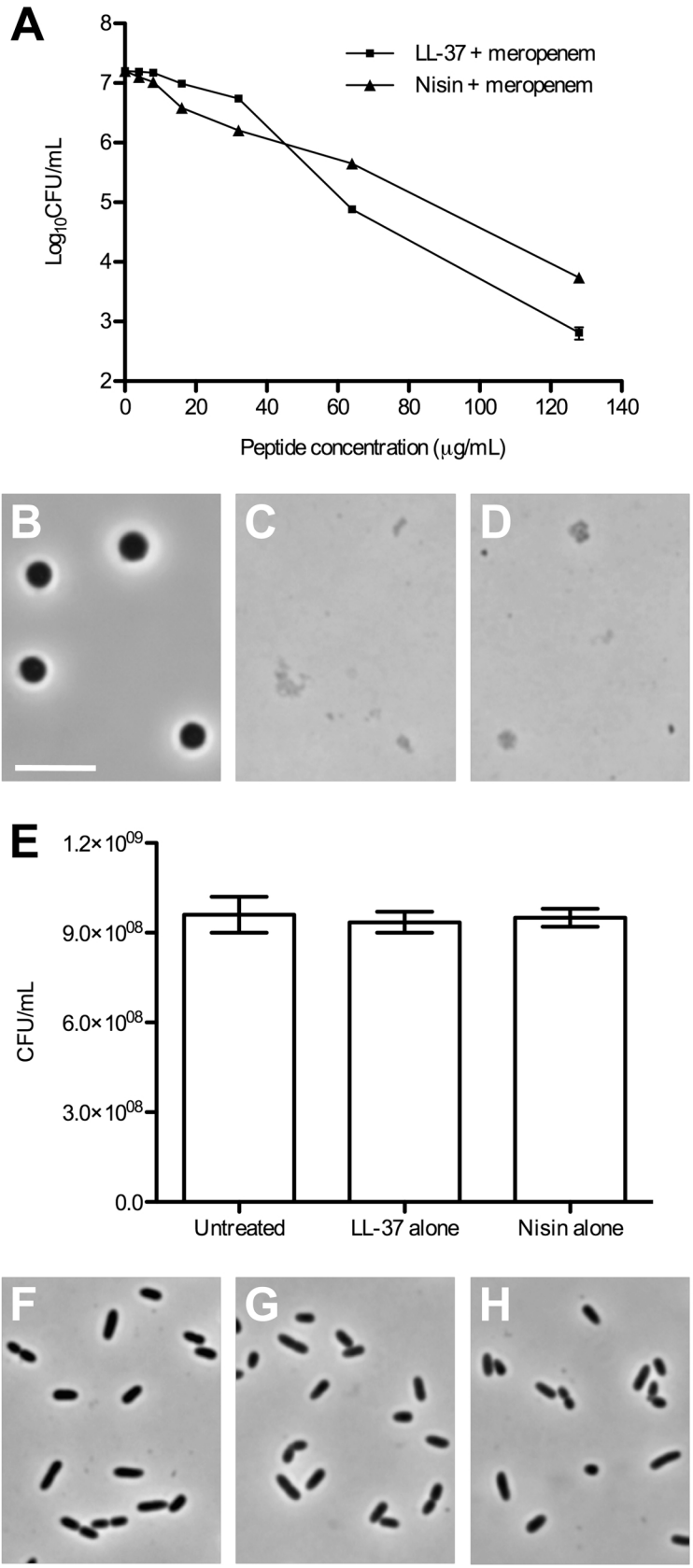
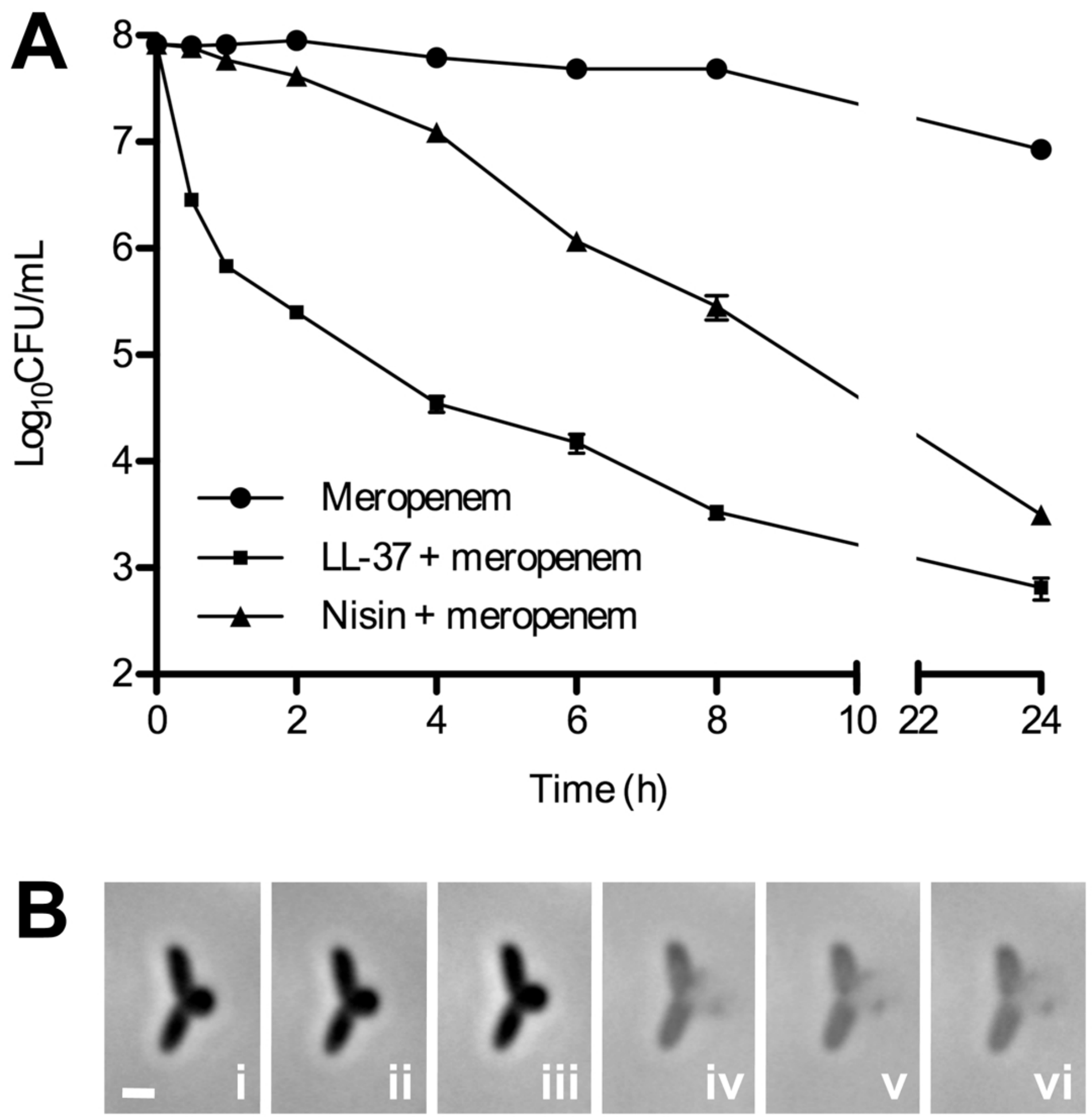
D


A
B
C
D
E
i
ii
iii
iv
v
vi
F
Percentage of cells
100
80
60
40
20
0
0
2
6
24
Time after antibiotic removal (h)
Spherical cells
Transitioning cells
Rods


A
B
C
D
Percentage of cells
100
80
60
40
20
0
0
4
24
48
Time after antibiotic addition (h)
Rods
Transitioning cells
Spherical cells
Filaments


A
LL-37 + meropenem
Nisin + meropenem
Log10CFU/mL
Peptide concentration (μg/mL)
B
C
D
E
CFU/mL
1.2×10^09
9.0×10^08
6.0×10^08
3.0×10^08
0.0
Untreated
LL-37 alone
Nisin alone
F
G
H


A
8
7
6
5
4
3
2
Log10CFU/mL
Meropenem
LL-37 + meropenem
Nisin + meropenem
0
2
4
6
8
10
22
24
Time (h)
B
i
ii
iii
iv
v
vi