Rousing Persisters through Ribosome Resuscitation and Rescue

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ABSTRACT

Persistence, the dormant, stress-tolerant state formed primarily through the cessation of protein synthesis after antibiotic and nutrient stress, is arguably one of most vital phenotypes for bacteria since nearly all cells experience nutrient stress. Persisters are also responsible for many recurring infections. However, how persister cells wake to reconstitute infections is not understood well, beyond that the level of ribosomes governs their waking speed in rich medium. Here, using single cell observations, we determined that alanine as the sole carbon source wakes *Escherichia coli* persister cells and that persister cells do not wake stochastically but wake primarily when presented with a carbon source. By screening all *E. coli* proteins, we found that the mechanism of persister cell waking is through sensing nutrients by the chemotaxis and flagellum systems and by reducing levels of the secondary messenger cAMP, which leads to ribosome resuscitation and rescue. Hence, persister cells wake using pathways evolved for nutrient sensing.

INTRODUCTION

The persister cell phenotype was first noted by Hobby *et. al.* in 1942[1] when penicillin did not completely sterilize a *Staphylococcus aureus* culture (1% of the population remained intact). The surviving subpopulation was deemed 'persisters' by Bigger *et. al* in 1944[2]. Both groups determined that persisters are dormant[1,2], which has been corroborated[3,4], and further research has demonstrated persister cells are not mutants[5,6] but instead acquire their antibiotic tolerance through this dormancy.

The persister cell phenotype is ubiquitous and has been well described in many bacteria such as *Escherichia coli*[7], *Pseudomonas aeruginosa*[7], *S. aureus*[7] and in Archaea[8]. Critically, the persister state arises not only after antibiotic stress but nutrient stress also creates persister cells[9-11]; in fact, the classic viable but not culturable state appears to be the same as the persister state[12] so persisters form everywhere as all bacterial cells eventually face nutrient stress[13]. Hence, it may be argued that the persister state is one of the most fundamental bacterial phenotypes.

Persister cells form from a reduction in metabolism usually from activation of a toxin of a toxin/antitoxin system. Evidence of this is that the deletion of several toxins of toxin/antitoxin systems such as MqsR[14,15], TisB[16], and YafQ[17] leads to a reduction in persistence. Similarly, production of toxins unrelated to toxin/antitoxin systems can also increase persistence[18].

Persister cells resuscitate as soon as instantaneously in rich medium[19] and wake based on their ribosome content[19]. For example, persister cells with four fold less ribosomes are delayed by several hours in their resuscitation while ribosome levels increase[19]. However, it is still unknown what pathway is involved in persister cell waking in regard to nutrient sensing.

To study persister cells without introducing traits of more prevalent cell phenotypes (e.g., slow-growing, tolerant stationary cells), their concentration needs to be increased so they are the dominant phenotype. Previously, we showed how to create a high percentage of *E. coli* persister cells (up to 80%) via rifampicin-pretreatment to stop transcription, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) to stop ATP production, or tetracycline to stop translation[3]; these cells were shown to be *bona fide* persister cells via eight different assays (multi-drug tolerance, immediate change from persistence to non-persistence in

the presence of nutrients, dormancy based on lack of cell division in the absence of nutrients, dormancy via metabolic staining and cell sorting, no change in MIC compared to exponential cells, no resistance phenotype, similar morphology to ampicillin-induced persisters, and similar resuscitation as ampicillin-induced persisters)[19]. Several other research groups have used our methods to make persister cells[20-24]; for example, Cui *et. al.* used rifampin and tetracycline to induce persistence for screening the *E. coli* Keio library and found several DNA repair mutants (e.g., *recA*, *recC*, *ruvA*, and *uvrD*) with reduced persistence, Grassi *et. al.*[21] used CCCP to generate *P. aeruginosa* and *S. aureus* persister cells to determine that antibiotic-tolerant phenotypes depend on different classes of antibiotics, and Narayanaswamy *et. al.*[23] used CCCP to generate *P. aeruginosa* persister cells and found poly (acetyl, arginyl) glucosamine is effective for killing them.

In the present study, we found that rather than waking spontaneously, persister cells wake primarily as a result of sensing nutrients. We then elucidated the waking pathway and found persister cells wake via chemotaxis and flagella pathways and by reducing cAMP which results in activating ribosomes.

RESULTS

Persister cells wake by recognizing Ala. We showed previously persister cells can wake immediately in rich medium based on their ribosome content[19]. Corroborating the importance of ribosomes, we found here that chloramphenicol (30 µg/mL), which inhibits bacterial protein synthesis by binding to the 50S ribosome subunit[25], prevents the waking of *E. coli* persister cells.

In order to elucidate insights about waking using defined medium, we hypothesized that a single amino acid could wake *E. coli* persister cells since *L*-alanine revives spores of *Bacillus subtilis*[26]. Initially, four groups of five amino acids were tested (#1: Ala, Arg, Cys, Phe, Ser; #2: Gly, His, Thr, Val, Tyr; #3: Asn, Ile, Lys, Pro, Trp; and #4: Asp, Glu, Gln, Leu, Met) by plating *E. coli* persister cells on M9 agar plates with the amino acid combinations as the sole carbon source and checking for growth after four days (**Supplemental Fig. 1**). We reasoned that if specific persister cells wake faster, larger colonies would form on M9 agar plates since we found previously that once they wake, persister cells grow at the same rate as

exponentially-growing cells[19].

65 Larger colonies were obtained from group #1 compared to groups #3 and #4, indicating faster waking, and persister cells did not wake for group #2 (**Supplemental Fig. 1**). As expected, there was no persister cell growth with M9 agar that lacks amino acids (negative control). For the two best groups (#1 and #3), each amino acid was tested separately for persister cell waking, and clearly substantially more persister cells were revived with Ala (**Supplemental Fig. 2**).

70 To confirm that the macroscopic colonies seen on M9 agar plates reflected faster waking, single *E. coli* persister cells were observed on M9 5X Ala agarose gel pads using light microscopy. We found that 18 ± 1% persister cells began to wake, as evidenced by dividing or elongating within 6 hours (**Fig. 1**, **Table S1**). As a negative control, single cell waking was monitored using Asn (5X) from group #3. Unlike with Ala, only 2 ± 2 % divided after 6 hours (**Fig. 1**, **Table S1**). Hence, persister cells with Ala wake 9-times more

75 frequently than those with Asn. As an additional negative control, waking on agarose gel pads that lack amino acids was investigated, and we found no persister cells wake after 6 h without nutrients (**Fig. 1**). This lack of persister cell waking without nutrients also confirms that the persister cells used are *bone fide* dormant cells since, in contrast, exponential cells can wake in 6 h on agarose pads that lack nutrients (12 ± 4%, **Table S1**).

80 **Persister cells wake primarily by sensing nutrients rather than spontaneously.** Along with waking as a response to a change in environmental conditions (e.g., the addition of nutrients[19]), it has been reported that persister cells wake spontaneously[27]; hence, we investigated whether persister cells wake in the absence of Ala. To test this, single persister cells were observed on NaCl, NaCl + Amp, and NaCl + Amp + Ala agarose gel pads. The rationale is that if the persisters wake, Amp lyses the cells[28] which is seen as a

85 disappearing single cell.

We found that persister cells wake primarily in the presence of Ala since there was 4.4-fold more waking followed by lysis with Ala + Amp in 6 hours compared to gel pads that lacked Ala but contained Amp (**Fig. 2**, **Table S2**). As expected, for the negative control (NaCl-only), there was no cell death seen in 6 hours (**Fig. 2A**). Therefore, most persister cells do not spontaneously awake but instead sense the carbon source

90 through some pathway.

Alanine is a waking signal. Since persister cells wake in the presence of Ala, we investigated whether Ala acts as a true signal rather than as a carbon and energy source by utilizing an isogenic *dadA* mutation that abolishes growth on alanine[29]. We confirmed the *dadA* mutation abolishes growth (**Supplemental Fig. 3**) and found 4% of the single *dadA* cells wake with Ala whereas no cells wake without Ala (**Supplemental**
95 **Fig. 3**, **Table S3**); hence, Ala wakes persister cells in the absence of growth.

Persister cells wake through the chemotaxis apparatus. Since most persister cells are waking by responding to the presence of Ala as a signal, we investigated the mechanism of how persister cells wake by looking for faster waking when individual proteins are produced. Hence, we pooled all the ASKA plasmids (each pCA24N-derived plasmid produces one *E. coli* protein) and electroporated them into *E. coli*
100 BW25113, formed persister cells, and selected for faster growth on M9 5X Ala agar (Cm) plates. After 3 days, some colonies could be seen and after 8 days, different phenotypes were clearly seen (**Supplemental Fig. 4**). The faster (bigger) colonies were purified and sequenced and six proteins were identified: PsiF, PanD, YmgF, YjcF, PptA, and CheY.

For these six putative proteins that stimulate persister cell waking, single cells harboring the ASKA
105 plasmid were observed on M9 5X Ala agarose gel pads and compared with persister cells with an empty plasmid (*E. coli* BW25113/pCA24N) (**Supplemental Fig. 5, Table S4**). We found single cells that produce PanD (23-fold) and YmgF (18-fold) wake at a higher frequency with Ala, compared to cells containing the empty plasmid (**Table S4**). Remarkably, all the single persister cells that produced the chemotaxis response regulator CheY resuscitated (33-fold enhancement, **Fig. 3, Table S4**), and 82% of the single persister cells
110 that produced response regulator CheA resuscitated (27-fold enhancement) (**Fig. 3, Table S5**); In addition, single cells of deletion mutants Δ*cheY* and Δ*cheA* are completely inhibited in their waking on M9 Ala (**Supplemental Fig. 6**). Critically, the CheY and CheA strains and all the strains used in the remaining experiments produce roughly the same number of persister cells (so there is no defect in the formation of persister cells) and grow similar to the wild-type (**Table S6)**. Together, these results demonstrate the
115 importance of the chemotaxis system in nutrient recognition and waking in persister cells.

To investigate further the link between chemotaxis and persister cell waking, we tested all five of the methyl-accepting chemotaxis proteins (Tsr, Tar, Trg, Tap, and Aer) for waking on agar plates by using isogenic mutants and found inactivating Tar and Trg decreased waking compared to the wild type (**Supplemental Fig. 7)**. Deleting *cheY* also reduced waking (**Supplemental Fig. 7)**. Corroborating these
120 results, persister cells that produce Tar and Trg woke faster than those with the empty plasmid and faster than cells producing Aer, Tsr, and Tap (**Supplemental Fig. 8)**.

Single cells producing Tar (9-fold) and Trg (8-fold) also woke with a higher frequency than cells containing the empty plasmid, and single cells producing Tsr woke the same as those with the empty plasmid (**Fig. 3, Table S5**). Hence, persister cells wake via the chemotaxis system utilizing methyl-
125 accepting chemotaxis proteins Tar and Trg and response regulators CheY and CheA.

Persister cells waking requires the flagellar regulators FhlD and FliN. Since the chemotaxis system involves the flagellum, we investigated its role in persister cell waking. Using agar plates, there was no effect of the flagellum of inactivating flagella motor MotA (**Supplemental Fig. 9**), and single persister cells with inactivated flagella motor MotB did not wake significantly differently than wild-type persisters
130 (**Supplemental Fig. 10, Table S7**). Corroborating these results, inactivating the hook protein FlgE[30] had no effect on persister waking (**Supplemental Fig. 11**), so the flagellum is not involved in persister cells waking.

In contrast, inactivating FhlD and FliN reduced the frequency of single cell waking by 3-fold (**Supplemental Fig. 10, Table S7)**. In addition, producing FliN in single cells increased the waking
135 frequency by 4.3-fold (**Fig. 3, Table S5)**. Hence, the master transcriptional regulator of flagellar proteins is involved in persister cell waking.

Persister cells wake by reducing cAMP. Having determined that persister cells wake by utilizing the chemotaxis apparatus, we explored how the external nutrient signal was propagated beyond the chemotaxis and flagellum system. Since cells can sense their environment (e.g., surfaces) by cAMP[31] and cAMP has
140 been linked to persistence[32], we reasoned that persister cell waking may involve this secondary message.

To test the importance of cAMP, we produced the only adenylate cyclase in *E. coli,* CyaA[33] via

BW25113/pCA24N-*cyaA* and found using agar plates that cells with elevated cAMP have reduced waking compared to cells with the empty plasmid (**Supplemental Fig. 12**). Consistently, cells producing the cAMP-specific phosphodiesterase CpdA[34] via BW25113/pCA24N-*cpdA* have increased waking on M9-
145 Ala agar plates; we showed previously CpdA can eliminate cAMP by reducing its concentration 323-fold [32]. Corroborating these results, cells lacking CpdA (BW25113 Δ*cpdA*) have reduced waking on M9-Ala plates (**Supplemental Fig. 12**).

Verifying these agar plate results, single cells producing adenylate phosphodiesterase CpdA before persister formation had a 15-fold greater (0.5 mM IPTG) and 8-fold greater (0 mM IPTG) frequency of
150 waking (**Fig. 3, Table S5)** by reducing cAMP, and producing adenylate cyclase CyaA reduced the frequency of waking by 30% compared to the empty plasmid (**Fig. 3, Table S5**). In a consistent manner, increasing cAMP by inactivating CpdA completely eliminated single cell waking (**Supplemental Fig. 13, Table S8)**, and decreasing cAMP by inactivating CyaA led to 4.3-fold greater frequency of waking (78%) (**Supplemental Fig. 13, Table S8)**. Hence, reducing cAMP levels increases waking dramatically. As
155 additional proof, we used the cAMP inhibitor atropine[35] at 3 mM and found reducing cAMP concentrations remarkably increased waking since colonies appeared in ½ the time as wild-type cells on agar plates (**Supplemental Fig. 14**); similar results; i.e., faster waking by reducing cAMP was also seen with 1 mM atropine. Confirming these results, we found single cells wake with atropine at twice the frequency (**Supplemental Fig. 15**). Moreover, combining atropine with production of CpdA to reduce cAMP
160 increased the frequency of waking 4-fold such that 92% of the cells woke in 6 h (**Fig. 3, Table S5)**.

Persister cells wake by resuscitating and rescuing ribosomes. Since reducing cAMP led to faster resuscitation, we explored further the mechanism for their waking. We hypothesized that since elevated cAMP increases ribosome hibernation during starvation[36], reducing cAMP should lead to ribosome resuscitation; hence, we tested the impact of changing the concentration of ribosome resuscitation factor
165 HflX[37]; HflX also rescues ribosomes from mRNA[38]. We utilized ribosome rescue factor ArfB[39] as a negative control since it is positively regulated by cAMP[40]. In addition, since trans-translation is used to rescue ribosomes from mRMA that lack a stop codon[39] , we also investigated the impact of SsrA on persister

cell waking.

For M9 Ala agar plates, we found cells producing HflX woke faster, compared to cells with the empty
170 plasmid whereas cells producing ArfB did not wake, indicating ArfB reduces waking dramatically (**Supplemental Fig. 16**). Similarly, for single cells, we found inactivating HflX completely inhibited waking (**Supplemental Fig. 17, Table S9**). In contrast, inactivating ArfB in single cells had no effect (**Supplemental Fig. 17, Table S9**). Corroborating these results, producing HflX in single cells increased the frequency of waking dramatically (7-fold) (**Fig. 3, Table S5**).

175 For ribosome rescue, using agar plates, we found inactivating SsrA led to significantly slower waking (**Supplemental Fig. 16**). Similarly, for single cells, inactivating SsrA almost completely inhibited waking (frequency of waking reduced to less than 1%) (**Supplemental Fig. 17, Table S9**). Therefore, resuscitating ribosomes via HflX and SsrA is vital for persister cell resuscitation.

DISCUSSION

180 Spores are the ultimate in bacterial resting state, and *L*-alanine revives spores of *Bacillus subtilis*[26]. Hence, we hypothesized that *L*-alanine or another amino acid may wake *E. coli* persisters. By investigating all 20 amino acids, we found that *L*-alanine wakes *E. coli* persister cells much faster than other amino acids (**Supplemental Fig. S1, S2**). To determine insights into how *L*-alanine wakes *E. coli* persisters, we pooled the ASKA clones, isolated their plasmids, electroporated them into *E. coli* BW25113, made persister cells,
185 and selected for faster waking. From this selection, six proteins were identified that increased waking: PsiF, PanD, YmgF, YjcF, PptA, and CheY. From these six proteins, we found PanD and YmgF increase waking 20-fold. PanD (aspartate 1-decarboxylase)[41] which catalyzes the reaction of *L*-aspartate to β-alanine; hence, PanD produces an alanine-like compound. YmgF is related to an inner membrane division septum protein[42]; therefore, the persister cells wake and are primed for cell division. Critically, we determined the chemotaxis
190 response regulator CheY and the chemotaxis methyl-accepting chemotaxis proteins Tar and Trg are utilized for persister resuscitation. Surprisingly, the chemotaxis methyl-accepting protein Tsr, which is responsible for Ala chemotaxis[43], was not involved in persister waking. Therefore, persister cells wake through the

same nutrient sensors they utilize for chemotaxis. This also suggests that once resuscitated, the cells can rapidly move toward nutrients since the chemotaxis system is primed immediately, and the lack of energy
195 to make proteins (i.e., lack of nutrients to drive protein production) is what caused the dormancy in the first place[3].

cAMP activates ribosome hibernation as 100S dimers during starvation by activating ribosome-binding protein RMF[36] so it makes sense for cells to reverse this process (as we find here) and reduce cAMP to wake in the presence of nutrients since we identified that the activity of ribosomes dictates the speed of
200 persister resuscitation[19]. Supporting this insight, we found that inactivation of the ribosome rescue factor HflX (heat shock-induced ribosome-dependent GTPase) completely stopped waking of single cells and production of HflX increased the frequency of waking dramatically; *hflX* (in the same operon as *hfq*) is repressed by cAMP-Crp[44]. In contrast, ribosome rescue factor Arfb (peptidyl-tRNA hydrolase) had no effect because it is induced by cAMP-Crp[40]. Critically, all bacteria have mechanisms for rescuing ribosomes
205 since without rescue, all ribosomes would be depleted in less than one generation[45], and we found that without ribosome rescue via the trans-translation system (SsrA), persister cell waking is almost completely inhibited. Hence, ribosome rescue during persister cell resuscitation may be a general mechanism and should provide ample ribosomes for resuscitation.

Although it is counter intuitive, in regard to the *formation* of persister cells, high concentrations of cAMP
210 allow ribosomes to hibernate and prepare the cell for nutrient-depleted conditions; yet, high concentrations of cAMP actually *reduce* persistence[32] (reduce cell dormancy). This is because cells with high cAMP concentrations are more prepared for stress and have less need to become dormant; i.e., more fit cells have dramatically less persistence[46].

Overall, we have identified that (i) persister cells wake primarily in response to nutrient stimulus (rather
215 than spontaneously), (ii) Ala is the most suitable amino acid signal for waking *E. coli* persister cells, (iii) this nutrient signal is perceived through the chemotaxis system via CheY, Trg, and Tar, (iv) second messenger cAMP concentrations are reduced for waking, and (v) ribosomes are restored for waking (**Fig. 4**). It remains to be determined if waking via other nutrients follows this pathway and how the chemotaxis

system results in a reduction in cAMP concentrations, but it clear that persister cell waking is an elegant
220 (i.e., highly-regulated) response to perceived environmental signals.

METHODS

Bacterial strain and growth conditions. The bacterial strains and plasmids used in this study are listed in **Table S10**. *E. coli* BW25113 its isogenic mutants[47], and the pCA24N-based plasmids from the *E. coli* ASKA collection[48] were used. Lysogeny broth (LB)[49] was used for routine cell growth, and M9 medium
225 supplemented with amino acids[50] was used for waking studies; strains were grown at 37 °C. Each of the 20 amino acids stock solutions were prepared as indicated in **Table S11**[50].

Microscopic observation of persister cells on agarose gel pads. We utilized our previous method for generating persister cells[3,12,19]: exponentially-growing cells (turbidity of 0.8 at 600 nm) were treated with rifampicin (100 µg/mL for 30 min) to form persister cells by stopping transcription and non-persister cells
230 were removed by lysis via a 3 ampicillin treatment (100 µg/mL): Cells were harvested at 17,000 g for 1 min and washed with 1x PBS buffer twice to remove all possible carbon sources, then re-suspended with 1 mL of 1x phosphate buffered saline buffer[51]. Gel pads of 1.5 % agarose were prepared[19] and 5 µL of persister cells were added, kept at 37°C, and observed using a light microscope (Zeiss Axio Scope.A1, bl_ph channel at 1000 ms exposure) every hour.

235 **Persister waking screen.** All 4,267 ASKA clones (GFP-)[48] were combined, grown to a turbidity of 2 at 600 nm in LB medium, and their plasmids isolated using a plasmid DNA Mini Kit I (OMEGA bio-tek, inc., Norcross, GA, USA). The pooled ASKA plasmids (1 µL containing 30 ng DNA) was electroporated into 50 µL of *E. coli* BW25113 competent cells, 1 mL LB medium was added, and the cells were grown to a turbidity of 0.5 at 600 nm. Chloramphenicol was added (30 µg/mL) to the culture, and the cells were
240 incubated at 250 rpm to a turbidity of 0.8. Rifampicin followed by ampicillin was added to make persister cells, the persister cells were washed twice with 1x PBS buffer, diluted 100-fold 1x PBS buffer, and 100 µL was plated on M9 5X Ala (Cm) agar plates and incubated at 37°C; faster colony appearance indicated faster persister resuscitation.

AUTHOR'S CONTRIBUTIONS

245 RY and SYS performed the experiments. RY, SYS, MJB, and TKW designed the experiments.

AVAILABILITY OF DATA AND MATERIALS

All data are present in the manuscript.

COMPETING INTERENSTS

Authors declare no competing interests.

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FIGURE LEGENDS

Fig. 1. Single persister cells wake with Ala. *E. coli* BW25113 persister cells were incubated at 37 °C on M9 5X Ala agarose gel pads and images were captured at 0 h and 6 h. Black arrows indicate cells that resuscitate. Scale bar indicates 10 µm. Representative results from two independent cultures are shown. Tabulated cell numbers are shown in **Table S1**.

Fig. 2. Single persister cells wake primarily by sensing nutrients rather than spontaneously. *E. coli* BW25113 persister cells were incubated at 37 °C on NaCl, NaCl + Amp, and NaCl + Amp + Ala agarose gel pads for 6 hours. **Panel I**: Cells first resuscitate (black arrows) and then lyse in the presence of ampicillin (red arrows). Scale bar indicates 10 µm. Representative results from two independent cultures are shown. **Panel II:** Persister cell waking (%) after 6 hours incubation. Tabulated cell numbers are shown in **Table S2**.

Fig. 3. Single persister cell waking after producing chemotaxis-related, cAMP-related, and ribosome-resuscitation-related proteins. Panel I: *E. coli* BW25113 persister cells containing (**A**) pCA24N, (**B**) pCA24N_*cheA*, (**C**) pCA24N_*cheY*, (**D**) pCA24N_*tar*, (**E**) pCA24N_*trg*, (**F**) pCA24N_*tsr*, (**G**) pCA24N_*cpdA*, (**H**) pCA24N_ *cpdA* induced by 0.5 mM IPTG, (**I**) pCA24N_*cpdA* with 3 mM atropine, (**J**) pCA24N_*cyaA*, (**K**) pCA24N_*fliN*, and (**L**) pCA24N_*hflX* were incubated at 37 °C on M9 5X Ala agarose gel pads for 18 h. Black arrows indicate cells that resuscitate. Scale bar indicates 10 µm. Representative results from two independent cultures are shown. Prior to forming persister cells, cultures were grown without IPTG with 30 µg/mL Cm. **Panel II**: Persister cell waking (%) after incubating for 18 h. Tabulated cell numbers are shown in **Table S4**.

Fig. 4. Schematic of persister cell waking. ① Tar and Trg sense Ala and ② activate CheA and CheW which activate ③ CheY. CheY (P-CheY) binds to FliM and FliN. ④ The P-CheY-FliM-FliN complex activates the FlhD-FlhC via an unknown mechanism. ⑤ The FlhD-FlhC complex activates Crp-cAMP via an unknown mechanism. ⑥ The cell wakes by resuscitating dimerized ribosomes/stalled ribosomes via HflX due to reduced CRP-cAMP and by rescuing ribosomes on corrupt mRNA via SsrA. Circles indicates proteins, diamonds indicate amino acids, boxes indicate RNA, Ⓟ indicates phosphate, → indicates induction, and ⊣ indicates repression.

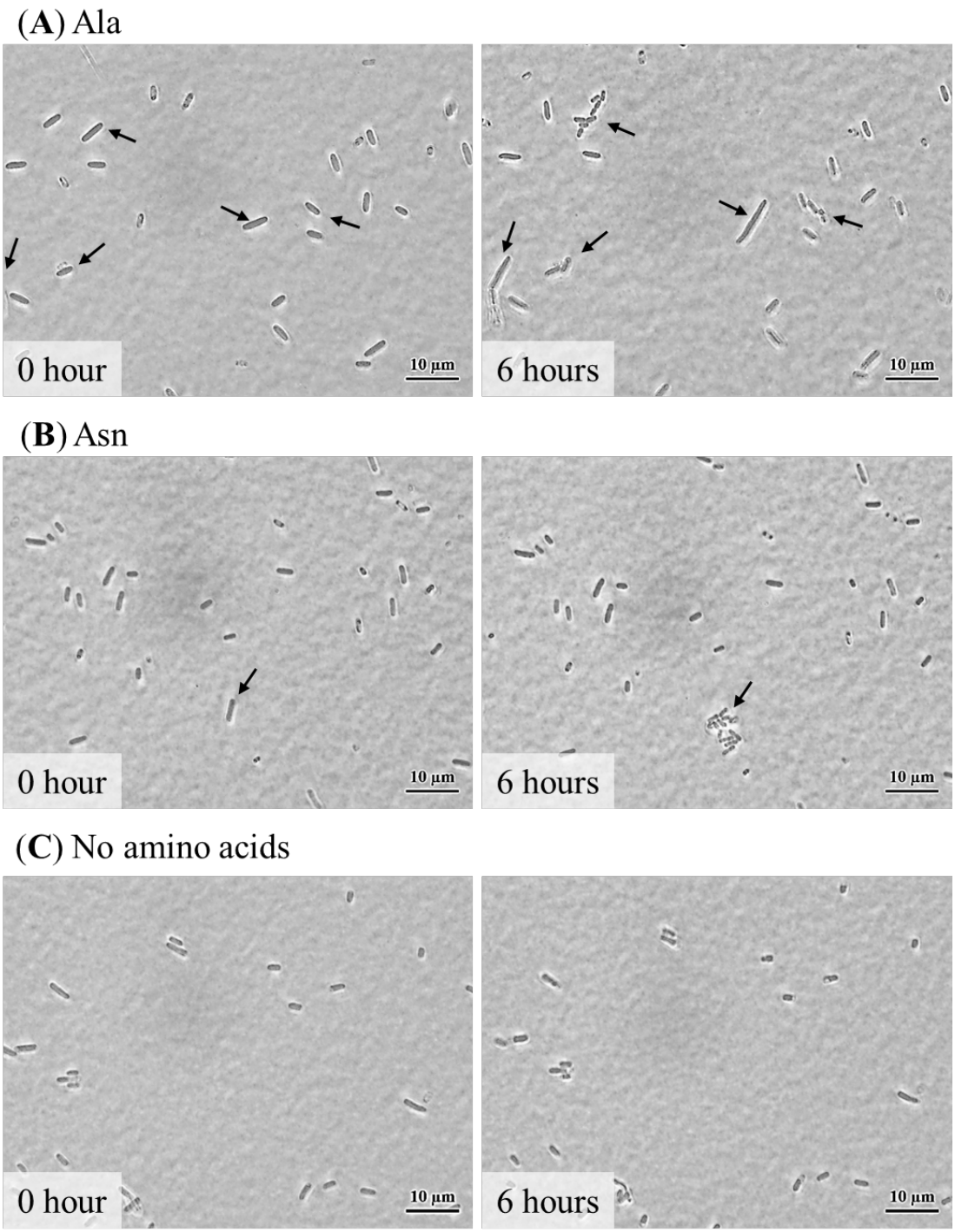

(A) Ala
0 hour
10 µm
6 hours
10 µm
(B) Asn
0 hour
10 µm
6 hours
10 µm
(C) No amino acids
0 hour
10 µm
6 hours
10 µm

Figure 1

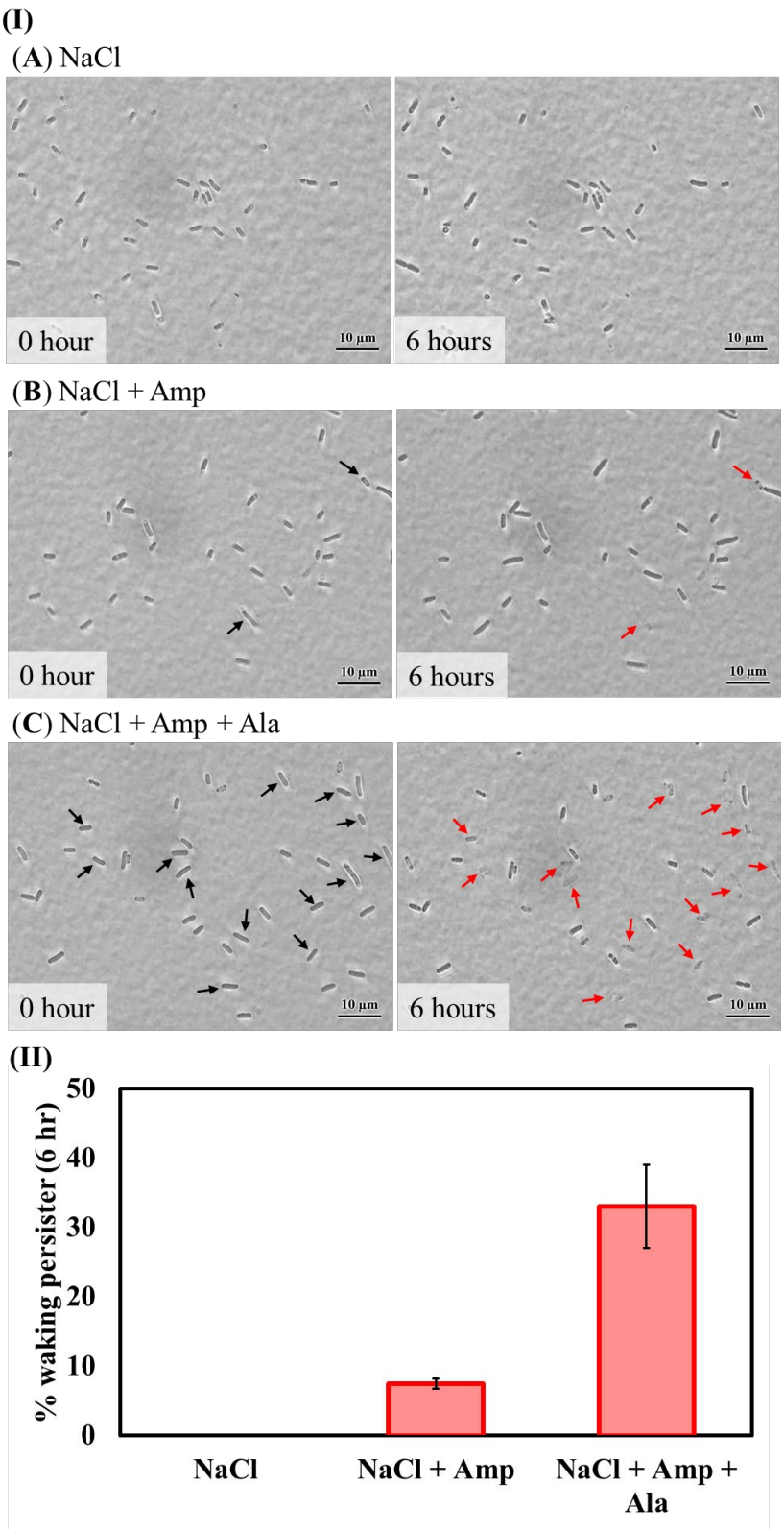

(I)
(A) NaCl
0 hour
10 µm
6 hours
10 µm
(B) NaCl + Amp
0 hour
10 µm
6 hours
10 µm
(C) NaCl + Amp + Ala
0 hour
10 µm
6 hours
10 µm
(II)
% waking persister (6 hr)
50
40
30
20
10
0
NaCl
NaCl + Amp
NaCl + Amp + Ala

Figure 2

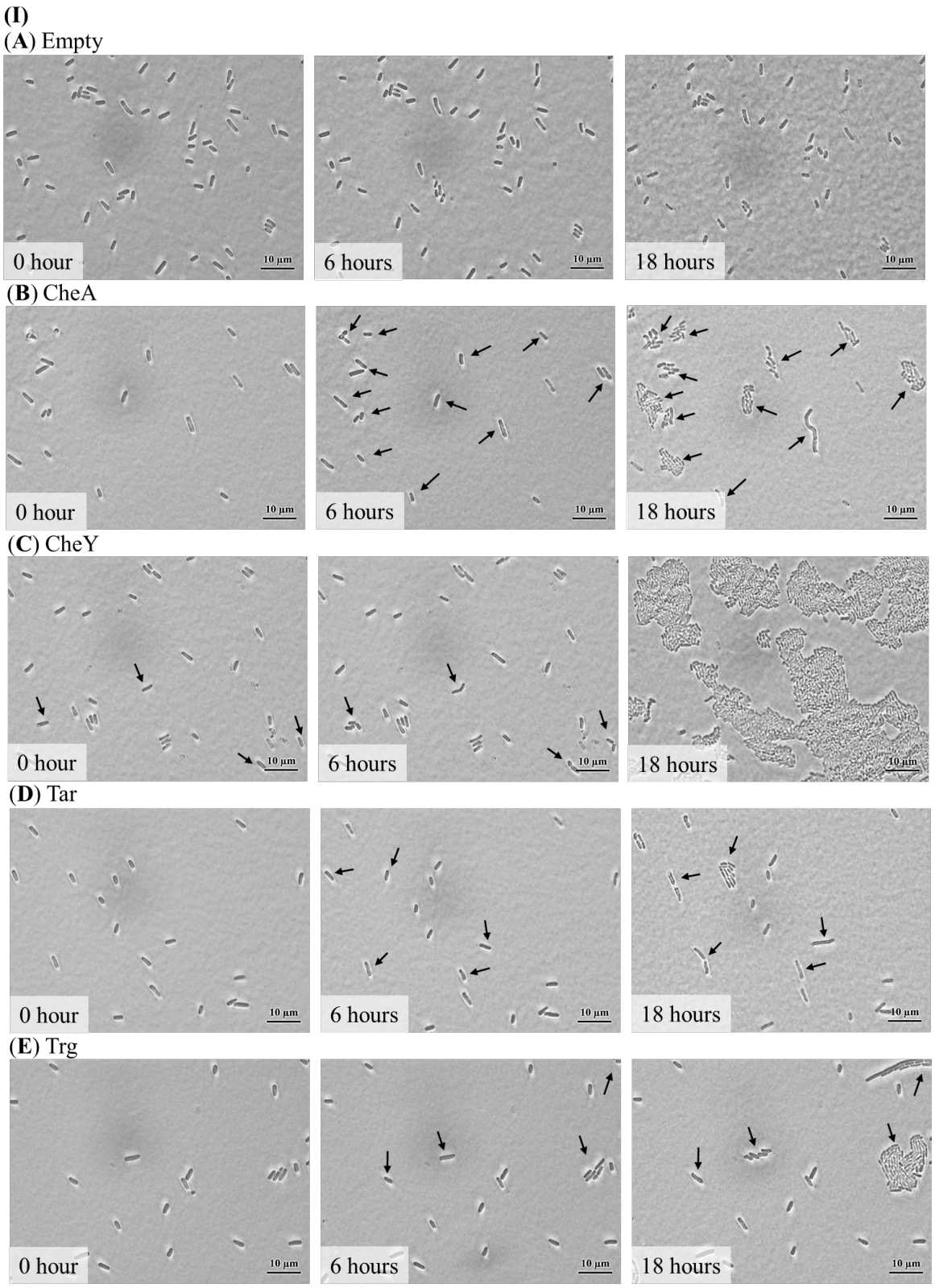

(I)
(A) Empty
0 hour
6 hours
18 hours
(B) CheA
0 hour
6 hours
18 hours
(C) CheY
0 hour
6 hours
18 hours
(D) Tar
0 hour
6 hours
18 hours
(E) Trg
0 hour
6 hours
18 hours
10 µm

Figure 3

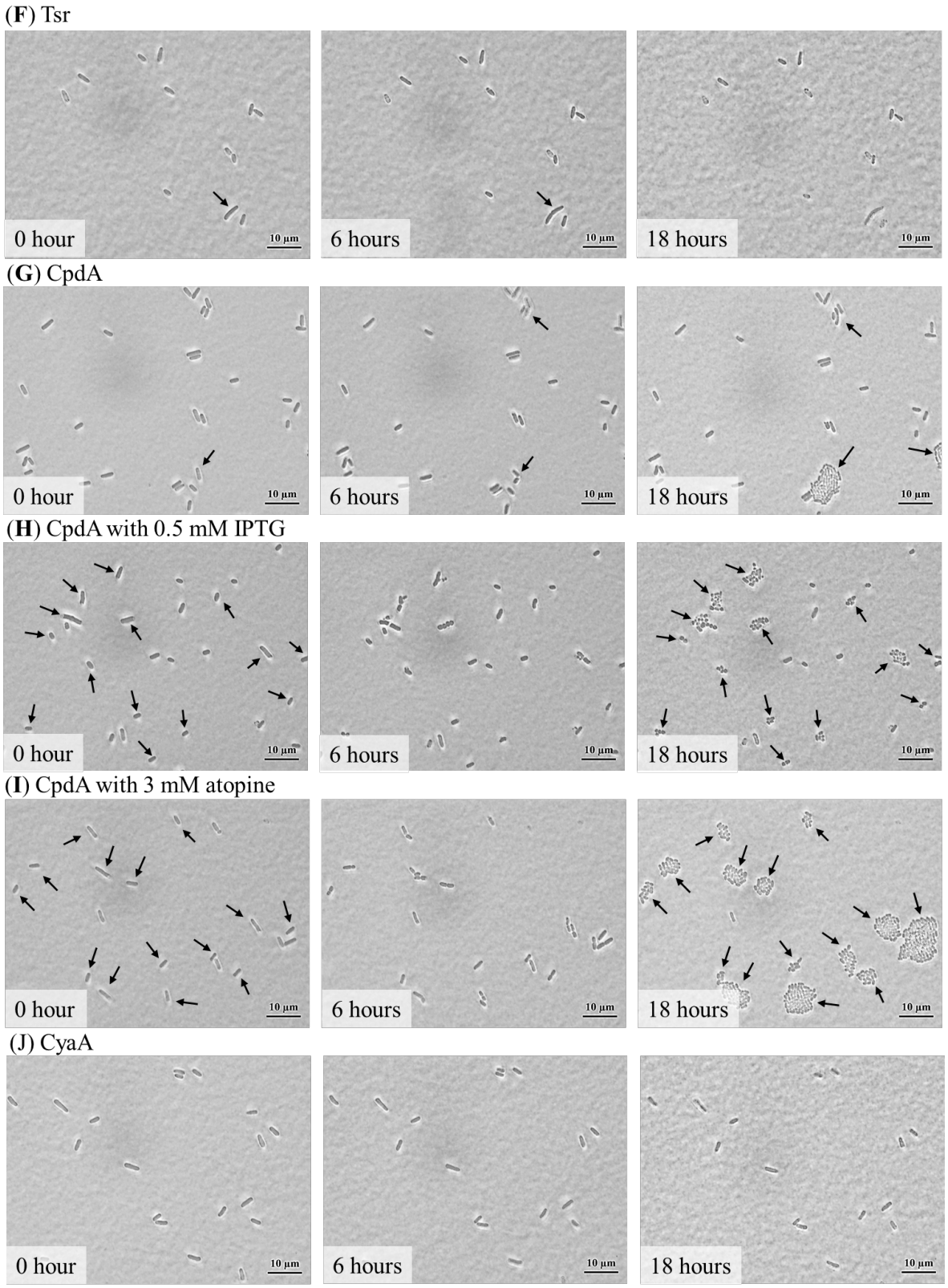
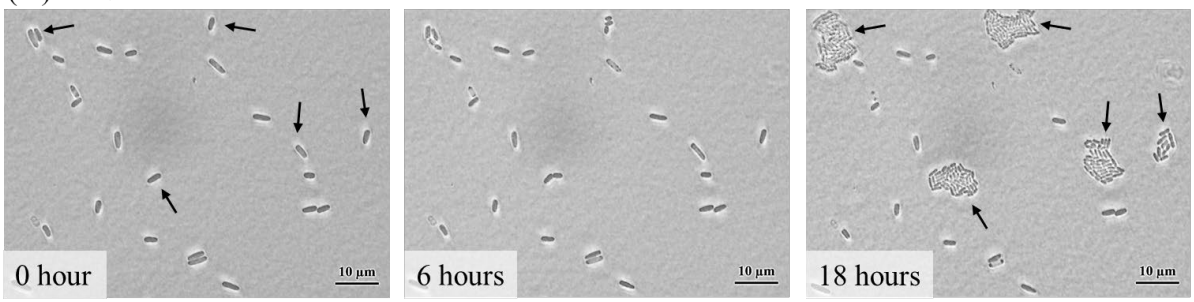
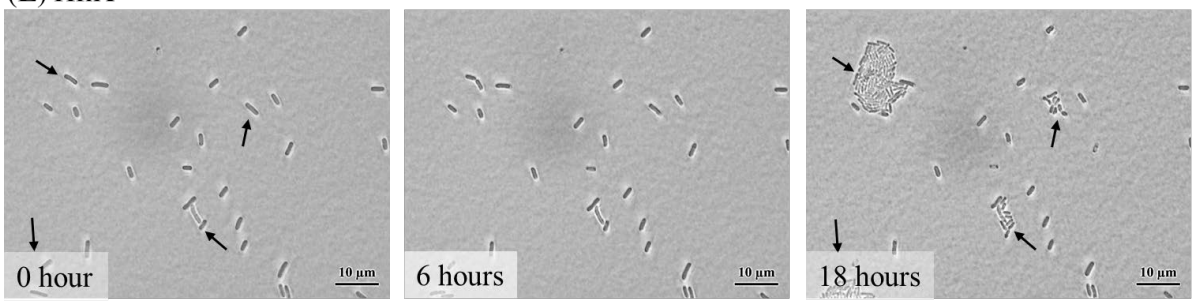


Figure 3 (cont.)

(**K**) FliN



(**L**) HflX



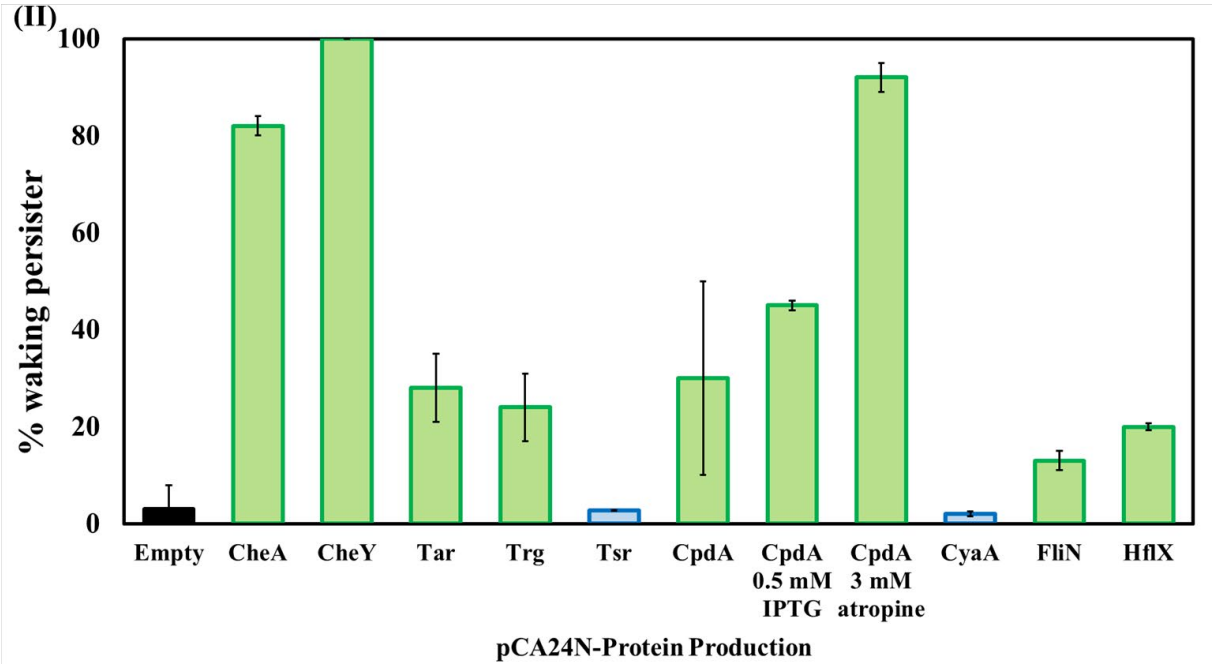


Figure 3 (cont.)

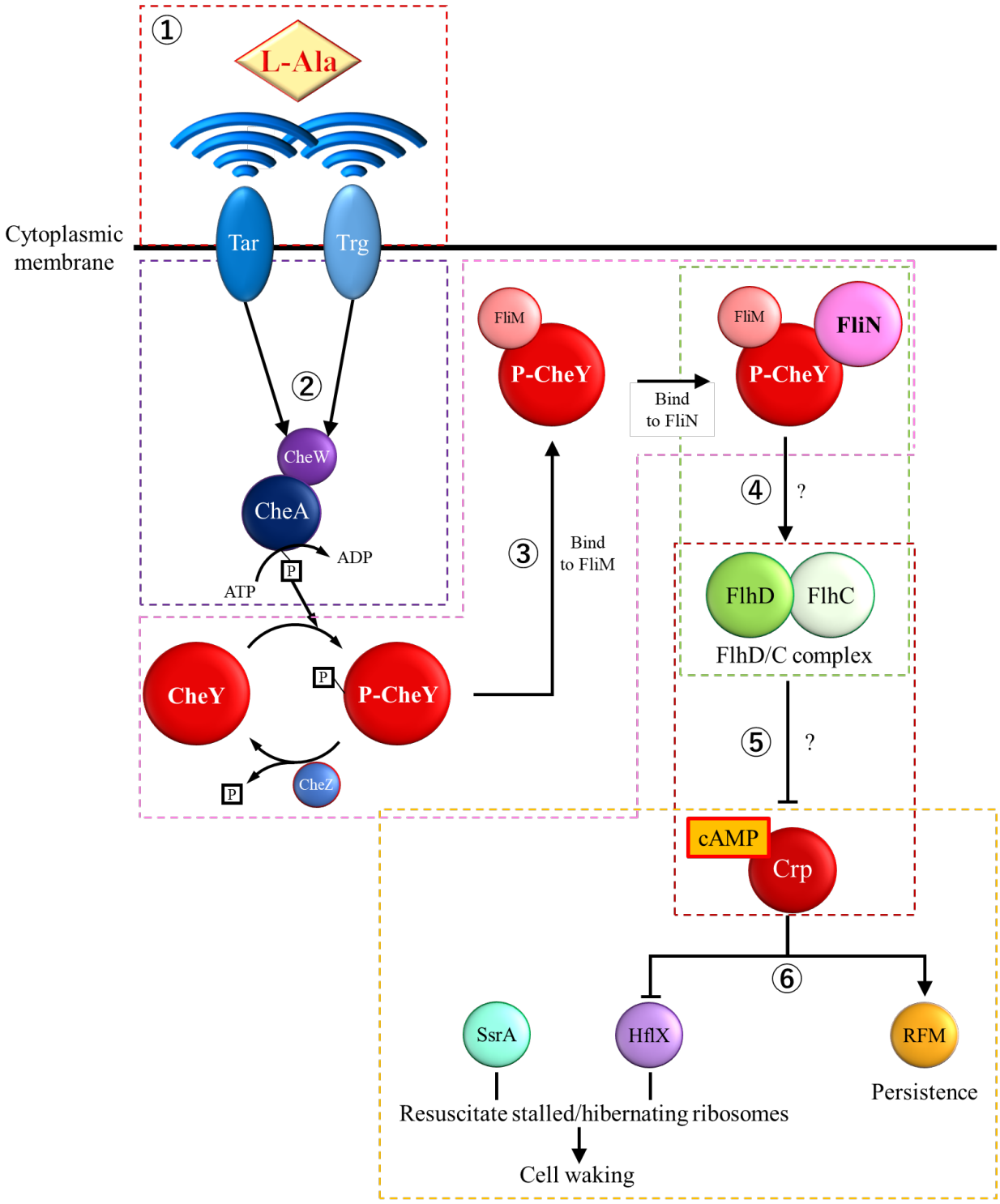

1
L-Ala
Cytoplasmic membrane
Tar
Trg
2
CheW
CheA
ADP
ATP
P
CheY
P-CheY
CheZ
3
Bind to FliM
FliM
P-CheY
Bind to FliN
FliM
FliN
P-CheY
4
?
FlhD
FlhC
FlhD/C complex
5
?
cAMP
Crp
6
SsrA
HflX
RFM
Persistence
Resuscitate stalled/hibernating ribosomes
Cell waking

Figure 4

Supporting Information

Rousing Persisters through Ribosome Resuscitation and Rescue

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Table S1. Single *E. coli* persister cell waking by Ala. Single persister cells were observed using light microscopy (Zeiss Axio Scope.A1). The total number of *E. coli* BW25113 persister cells that wake on M9 5X Ala, M9 5X Asn, and no amino acid (aa) gel pads is shown after 6 h at 37 °C. Also, growth of exponential cells on gel pads that lack amino acids is shown after 6 h at 37 °C. 5X Ala and 5X Asn is 375 µg/mL. Total waking cells indicates the number of dividing or elongating cells. These results are the combined observations from two independent experiments. The microscope images are shown in **Fig. 1**.

	Total cells	**Dividing cells**	**Elongating cells**	**Total waking cells**	**% waking**	**Fold-change**
Ala	51	7	2	9	18 ± 1	9
Asn	46	1	0	1	2 ± 2	1
no aa	56	0	0	0	0 ± 0	-∞
no aa (expon. cells)	104	12	0	12	12 ± 4	-

Table S2. Single persister cells wake primarily by sensing nutrients rather than spontaneously. Single persister cells were observed using light microscopy (Zeiss Axio Scope.A1). The total number of *E. coli* BW25113 wild type persister cells on the NaCl, NaCl + Amp, and NaCl + Amp + Ala agarose gel pads is shown after 6 h at 37 °C. Dead cells were identified by their disappearance. The concentration of NaCl was 0.85 %, Amp was 100 µg/mL, and Ala was 375 µg/mL. Fold-changes was based on NaCl + Amp. These results are the combined observations from two independent experiments. The microscope images are shown in **Fig. 2**.

	Total cells	**Dead cells**	**% of dead cells**	**Fold-change**
NaCl	144	0	0 ± 0	-
NaCl + Amp	164	13	7.5 ± 0.7	1
NaCl + Amp + Ala	135	47	33 ± 6	4.4

Table S3. Single persister cells wake with alanine as a signal. Single persister cells were observed using light microscopy (Zeiss Axio Scope.A1). The total number and waking number of persister cells of BW25113 Δ*dadA* on M9 10X Ala or no nutrient agarose gel pads are shown after 6 h at 37°C. These results are the combined observations from two independent experiments. The microscope images are shown in **Supplementary Figure 3**.

	Total cells	**Total waking cells**	**% waking**	**Fold-change**
Δ*dadA* + Ala	345	16	4 ± 1	1
Δ*dadA* (no nutrients)	223	0	0 ± 0	-∞

Table S4. Enhanced single persister cell waking via proteins identified by the ASKA library search. Single persister cells were observed using light microscopy (Zeiss Axio Scope.A1). The total number and waking number of persister cells of BW25113 producing the indicated proteins (PsiF, PanD, YmgF, YjcF, PptA, and CheY) are shown. Fold-change in waking is relative to BW25113 with the empty plasmid pCA24N. These results are the combined observations from two independent experiments after 18 hours. The microscope images are shown in **Supplementary Figure 5**.

	Total cells	Waking cells	% waking	Fold-change
Empty	103	2	3 ± 5	1
PsiF	91	10	11 ± 3	6.1
PanD	105	43	40 ± 30	22.8
YmgF	87	28	30 ± 10	17.9
YjcF	120	14	11.7 ± 0.3	6.5
PptA	118	15	8 ± 6	7.1
CheY	93	93	100 ± 0	33

Table S5. Single persister cell waking after producing chemotaxis-related, cAMP-related, and ribosome-resuscitation-related proteins. Single persister cells were observed using light microscopy (Zeiss Axio Scope.A1). The total number and waking number of persister cells are shown. Fold-change in waking is relative to BW25113 with the empty plasmid pCA24N. IPTG was used only for producing CpdA as indicted, otherwise, protein production was from the leaky promoter. M9 5X Ala with 3 mM atropine in the agarose gel pad was used with cells producing CpdA. These results are the combined observations from two independent experiments after 18 hours. The microscope images are shown in **Fig. 3**.

	Total cells	Waking cells	% waking	Fold-change
Empty	103	2	3 ± 5	1
CheA	93	77	82 ± 2	27
CheY	93	93	100 ± 0	33
Tar	123	33	28 ± 7	9
Trg	97	23	24 ± 7	8
Tsr	144	4	2.78 ± 0.05	0.9
CpdA	128	21	25 ± 24	8
CpdA (0.5 mM IPTG)	115	52	45 ± 1	15
CpdA + 3 mM atropine	77	71	92 ± 3	31
CyaA	137	3	2.1 ± 0.5	0.7
FliN	142	18	13 ± 2	4
HflX	126	25	20.0 ± 0.7	7

Table S6. Persister cell formation on LB. Persister cells were observed on LB plates after 1 day, and the percentage of persister cell formation indicates the number of persisters formed relative to the BW25113 wild-type strain. "+" indicates roughly the same growth of the strain compared with the wild type. These results are the combined observations from two independent experiments.

	% persister formation	Growth
Wild type	100.0 ± 0.1	+
Δ***arfB***	98.9 ± 0.9	+
Δ***cheA***	99 ± 1	+
Δ***cheY***	101.6 ± 0.1	+
Δ***cpdA***	100.8 ± 0.3	+
Δ***crp***	102 ± 1	+
Δ***cyaA***	93.9 ± 2.4	+
Δ***dadA***	97 ± 4	+
Δ***flgE***	101.2 ± 0.6	+
Δ***flhD***	101.1 ± 0.2	+
Δ***fliN***	98.1 ± 0.8	+
Δ***hflX***	100.4 ± 0.2	+
Δ***motB***	100.7 ± 0.4	+
Δ***motA***	99.1 ± 0.2	+
Δ***ssrA'***	101 ± 2	+
Δ***tar***	98.3 ± 0.1	+
Δ***trg***	98.7 ± 0.4	+
pCA24N	100.0 ± 0.5	+
ArfB	97.4 ± 0.7	+
CheA	95.6 ± 0.6	+
CheY	99.6 ± 0.5	+
CpdA	99.7 ± 0.5	+
Crp	98.2 ± 0.7	+
CyaA	99.7 ± 0.7	+
FlhD	98.0 ± 0.7	+
FliN	97.6 ± 0.6	+
HflX	97.7 ± 0.9	+
Tar	98.8 ± 0.8	+
Trg	97.9 ± 0.7	+

Table S7. Single persister cell waking after inactivating flagellar proteins MotB, FhlD, and FliN. Single persister cells were observed using light microscopy (Zeiss Axio Scope.A1). The total number and waking number of persister cells are shown. Fold-change in waking is relative to wild-type BW25113. These results are the combined observations from two independent experiments after 6 hours. The microscope images are shown in **Supplementary Figure 9**.

	Total cells	**Waking cells**	**% waking**	**Fold-change**
Wild type	51	9	18 ± 1	1
Δ*motB*	57	17	30 ± 10	1.7
Δ*flhD*	81	5	6.1 ± 0.4	0.3
Δ*fliN*	96	5	5 ± 2	0.3

Table S8. Single persister cell waking after inactivating CpdA and CyaA. Single persister cells were observed using light microscopy (Zeiss Axio Scope.A1). The total number and waking number of persister cells are shown. Fold-change in waking is relative to wild-type BW25113. These results are the combined observations from two independent experiments after 6 hours. The microscope images are shown in **Supplementary Figure 13**.

	Total cells	**Waking cells**	**% waking**	**Fold-change**
Wild type	51	9	18 ± 1	1
Δ*cpdA*	79	0	0 ± 0	-∞
Δ*cyaA*	93	68	74 ± 8	4.1

Table S9. Single persister cell waking after inactivating ribosome rescue proteins HflX and ArfB and inactivating trans-translation (SsrA). Single persister cells were observed using light microscopy (Zeiss Axio Scope.A1). The total number and waking number of persister cells are shown. Fold-change in waking is relative to wild-type BW25113. These results are the combined observations from two independent experiments after 6 hours. The microscope images are shown in **Supplementary Figure 16**.

	Total cells	**Waking cells**	**% waking**	**Fold-change**
Wild type	51	9	18 ± 1	1
Δ*hflX*	96	0	0 ± 0	-∞
Δ*arfB*	101	15	15 ± 2	0.8
Δ*ssrA*	123	1	0.8 ± 1.1	0.09

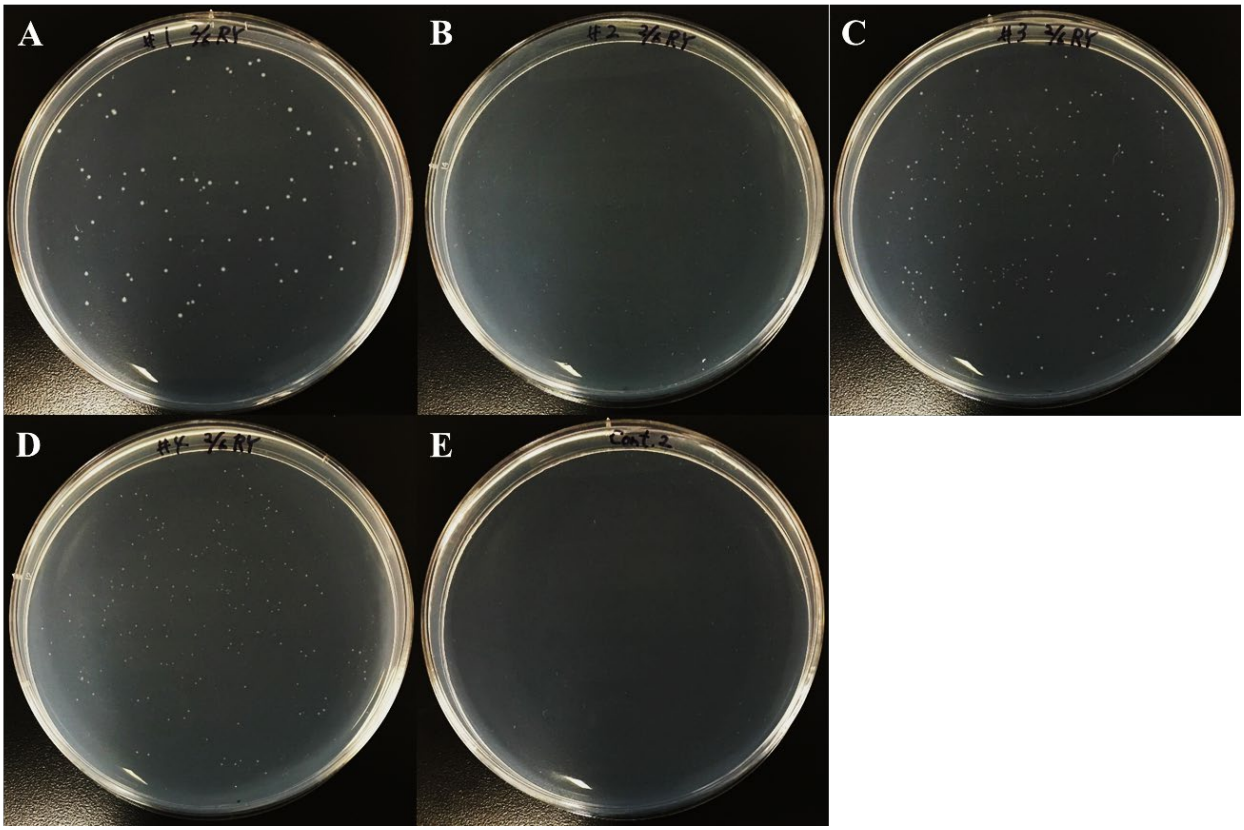
Table S10. Bacterial strains and plasmids used in this study.

Strains and Plasmids	Features	Source
E. coli BW25113	Wild type	47
E. coli BW25113 Δ*arfB*	Δ*arfB*, Km^R	47
E. coli BW25113 Δ*cheA*	Δ*cheA*, Km^R	47
E. coli BW25113 Δ*cheY*	Δ*cheY*, Km^R	47
E. coli BW25113 Δ*cpdA*	Δ*cpdA*, Km^R	47
E. coli BW25113 Δ*crp*	Δ*crp*, Km^R	47
E. coli BW25113 Δ*cyaA*	Δ*cyaA*, Km^R	47
E. coli BW25113 Δ*dadA*	Δ*dadA*, Km^R	47
E. coli BW25113 Δ*flgE*	Δ*flgE*, Km^R	47
E. coli BW25113 Δ*flhD*	Δ*flhD*, Km^R	47
E. coli BW25113 Δ*fliN*	Δ*fliN*, Km^R	47
E. coli BW25113 Δ*hflX*	Δ*hflX*, Km^R	47
E. coli BW25113 Δ*motA*	Δ*motA*, Km^R	47
E. coli BW25113 Δ*motB*	Δ*motB*, Km^R	47
E. coli BW25113 Δ*ssrA'*	Δ*ssrA*, Km^R (161 of 363 nt deleted with attL intact)	52
E. coli BW25113 Δ*tar*	Δ*tar*, Km^R	47
E. coli BW25113 Δ*trg*	Δ*trg*, Km^R	47
E. coli BW25113 Δ*arfB*	Δ*arfB*, Km^R	47
Plasmids		
pCA24N	Cm^R; *lacI*^{q}	48
pCA24N_*aer*	Cm^R; *lacI*^{q}, $P_{T5\text{-}lac}$::*aer*$^+$	48
pCA24N_*arfB*	Cm^R; *lacI*^{q}, $P_{T5\text{-}lac}$::*arfB*$^+$	48
pCA24N_*cheA*	Cm^R; *lacI*^{q}, $P_{T5\text{-}lac}$::*cheA*$^+$	48
pCA24N_*cheY*	Cm^R; *lacI*^{q}, $P_{T5\text{-}lac}$::*cheY*$^+$	48
pCA24N_*cpdA*	Cm^R; *lacI*^{q}, $P_{T5\text{-}lac}$::*cpdA*$^+$	48
pCA24N_*crp*	Cm^R; *lacI*^{q}, $P_{T5\text{-}lac}$::*crp*$^+$	48
pCA24N_*cyaA*	Cm^R; *lacI*^{q}, $P_{T5\text{-}lac}$::*cyaA*$^+$	48
pCA24N_*flhD*	Cm^R; *lacI*^{q}, $P_{T5\text{-}lac}$::*flhD*$^+$	48
pCA24N_*fliN*	Cm^R; *lacI*^{q}, $P_{T5\text{-}lac}$::*fliN*$^+$	48
pCA24N_*hflX*	Cm^R; *lacI*^{q}, $P_{T5\text{-}lac}$::*hflX*$^+$	48
pCA24N_*panD*	Cm^R; *lacI*^{q}, $P_{T5\text{-}lac}$::*panD*$^+$	48
pCA24N_*pptA*	Cm^R; *lacI*^{q}, $P_{T5\text{-}lac}$::*pptA*$^+$	48
pCA24N_*psiF*	Cm^R; *lacI*^{q}, $P_{T5\text{-}lac}$::*psiF*$^+$	48
pCA24N_*tap*	Cm^R; *lacI*^{q}, $P_{T5\text{-}lac}$::*tap*$^+$	48
pCA24N_*tar*	Cm^R; *lacI*^{q}, $P_{T5\text{-}lac}$::*tar*$^+$	48
pCA24N_*trg*	Cm^R; *lacI*^{q}, $P_{T5\text{-}lac}$::*trg*$^+$	48
pCA24N_*tsr*	Cm^R; *lacI*^{q}, $P_{T5\text{-}lac}$::*tsr*$^+$	48
pCA24N_*yjcF*	Cm^R; *lacI*^{q}, $P_{T5\text{-}lac}$::*yjcF*$^+$	48
pCA24N_*ymgF*	Cm^R; *lacI*^{q}, $P_{T5\text{-}lac}$::*ymgF*$^+$	48

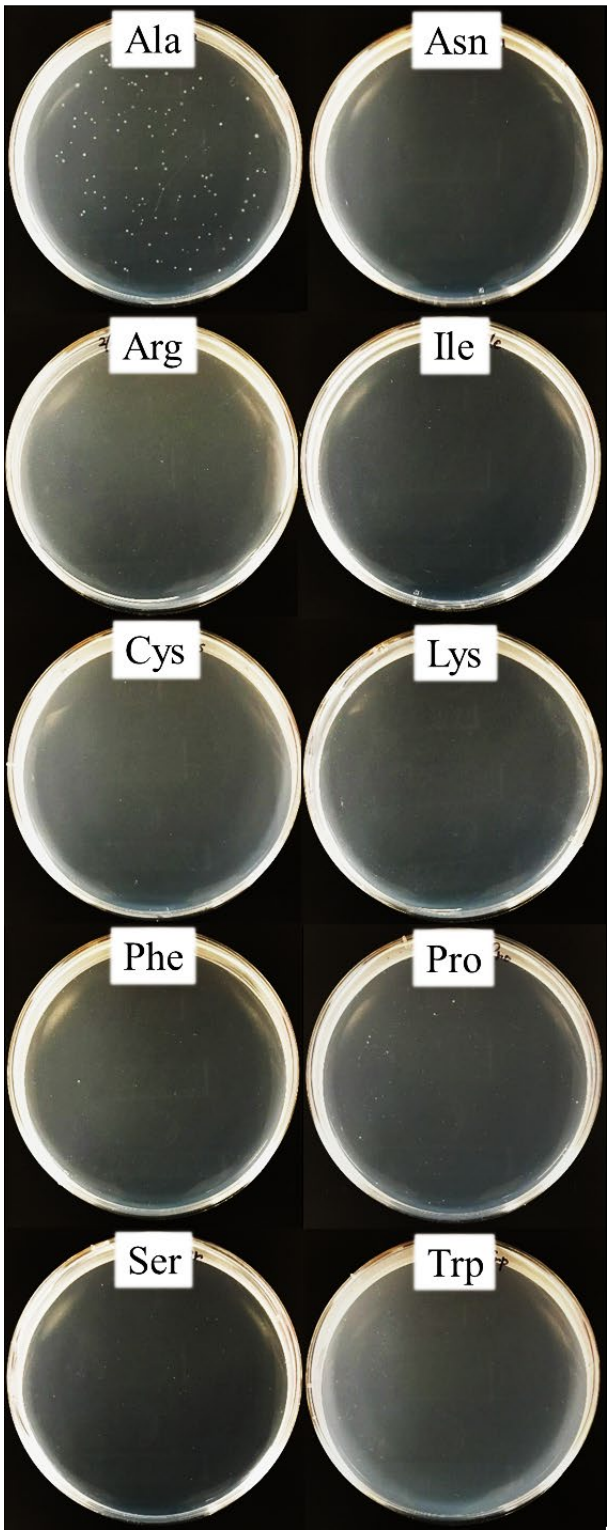
Km^R and Cm^R indicate kanamycin and chloramphenicol resistance, respectively.

Table S11. Supplementation levels (1X) for amino acids in M9 minimal medium.

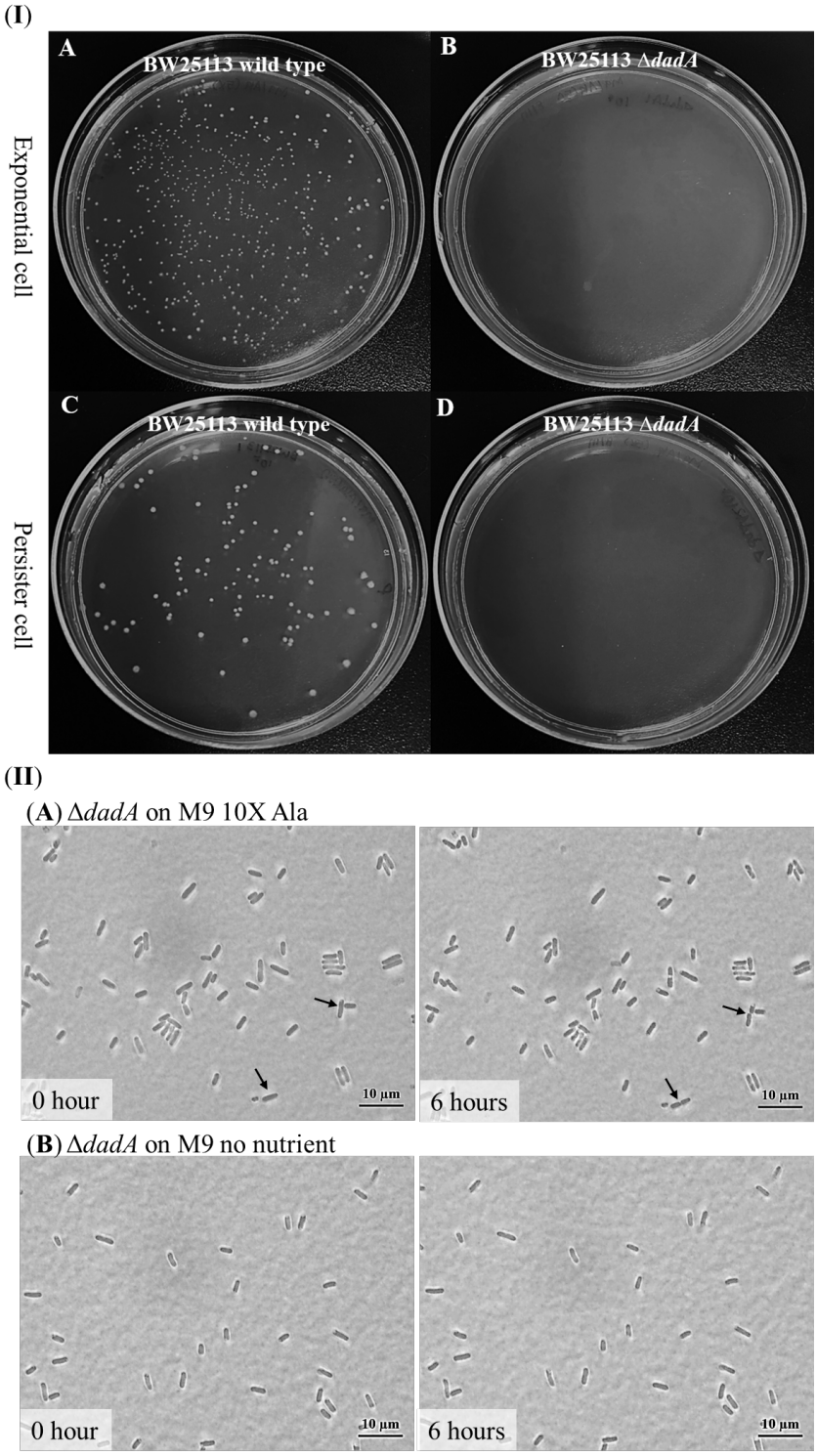
No.	Amino acid	Final concentration (µg/mL)
1	1 % L-alanine	75
2	2 % L-arginine	145
3	1 % L-asparagine	75
4	1 % L aspartic acid	75
5	2 % L-cysteine	50
6	2 % L-histidine	42
7	1 % L-glutamic acid	75
8	1 % L-glutamine	75
9	2 % glycine	110
10	1 % L-isoleucine	42
11	1 % L-leucine	41
12	1 % L-lysine	75
13	2 % L-methionine	25
14	1 % L-phenylalanine	75
15	4 % L-proline	164
16	2 % L-serine	42
17	2 % L-threonine	82
18	0.25 % L-tryptophan	18
19	1 % L-tyrosine	75
20	1 % L-valine	42



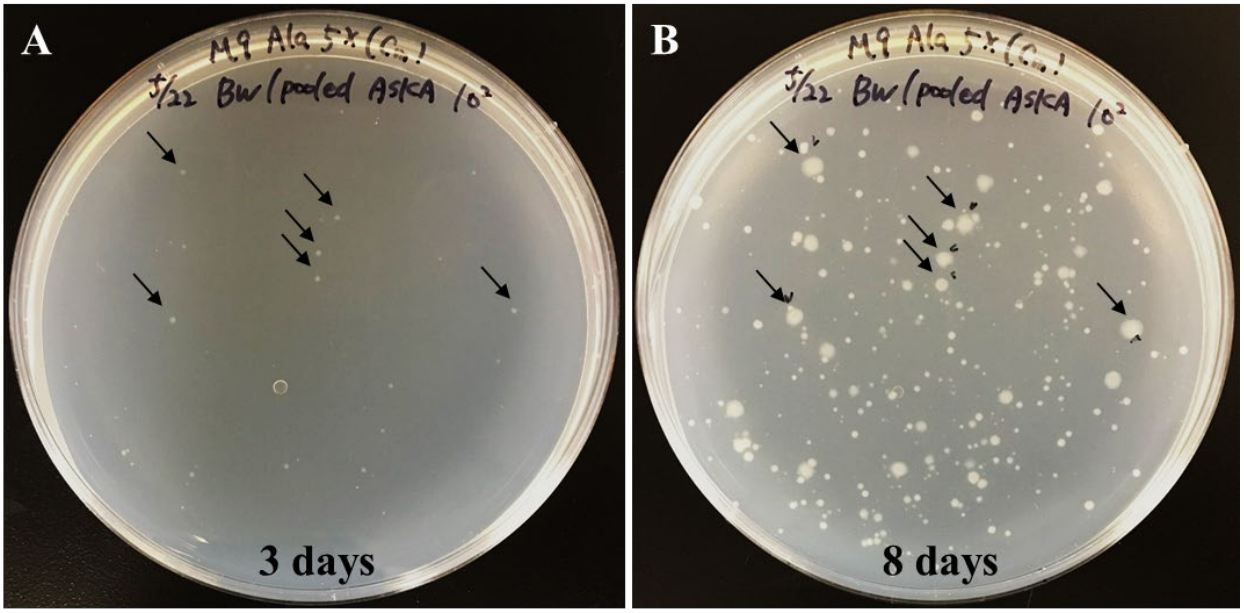
Supplementary Figure 1. Persister cells waking on agar plates with groups of amino acids. *E. coli* BW25113 persister cells were incubated at 37 °C for 4 days on M9 agar plates with groups of five different amino acids: **(A) #1**: Arg, Ser, Cys, Ala, and Phe, **(B) #2**: His, Thr, Gly, Val, and Tyr, **(C) #3**: Lys, Asn, Pro, Ile, and Trp, **(D) #4**: Asp, Glu, Gln, Leu, and Met, and **(e)** no amino acids. These amino acids were used at 1X concentration (**Table S11**). One representative plate of two independent cultures is shown.



Supplementary Figure 2. Persister cells waking on agar plates with alanine. *E. coli* BW25113 persister cells were incubated at 37 °C for 4 days on M9 agar plates with 10 different amino acids (Arg, Ser, Cys, Ala, Phe, Lys, Asn, Pro, Ile, and Trp). These amino acids were used at 1X concentration (**Table S11**). One representative plate of two independent cultures is shown.



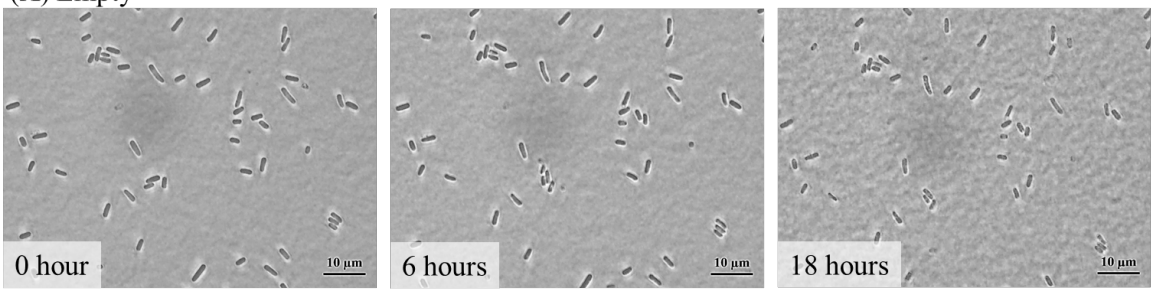
Supplementary Figure 3. Alanine is a waking signal. (**I**) Exponential wild-type *E. coli* BW25113 (**A**), exponential BW25113 Δ*dadA* (**B**), persister wild-type *E. coli* BW25113 (**C**), and persister BW25113 Δ*dadA* (**D**) incubated at 37 °C on M9 5X Ala agar plates for 3 days. (**II**) Persister cells of BW25113 Δ*dadA* on M9 10X Ala gel pads (**A**) and on M9 no nutrient gel pads (**B**) after 6 h at 37°C. Black arrows indicate cells that resuscitate. Scale bar indicates 10 µm. One representative plate of two independent cultures is shown. Tabulated cell numbers are shown in **Supplementary Table 3**.



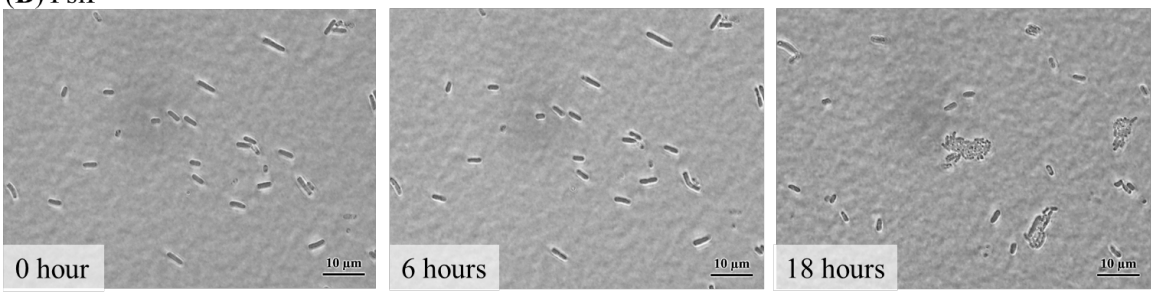
Supplementary Figure 4. Screening persister cell waking with the pooled ASKA plasmids. *E. coli* BW25113 persisters containing each of the pooled ASKA plasmids were incubated at 37 °C on M9 5X Ala agar plates with 30 µg/mL Cm for (**A**) 3 days and (**B**) 8 days. Faster growing colonies are indicated with black arrows. Prior to forming persister cells, cultures were grown without IPTG with 30 µg/mL Cm. One representative plate of two independent cultures is shown.

(I)

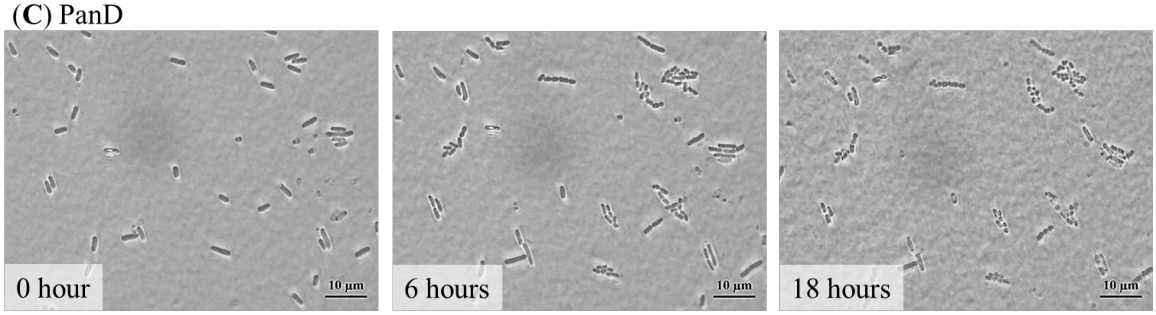
(**A**) Empty



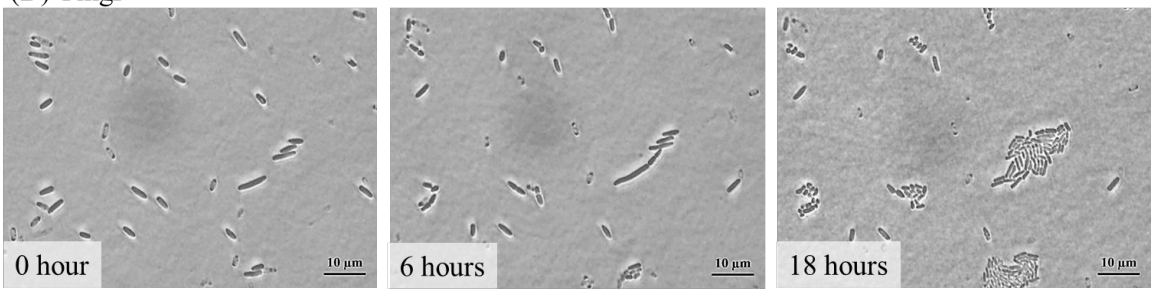
(**B**) PsiF



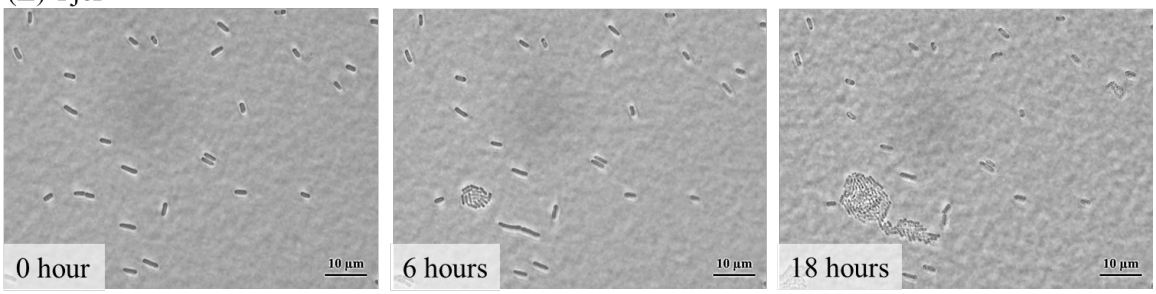
(**C**) PanD



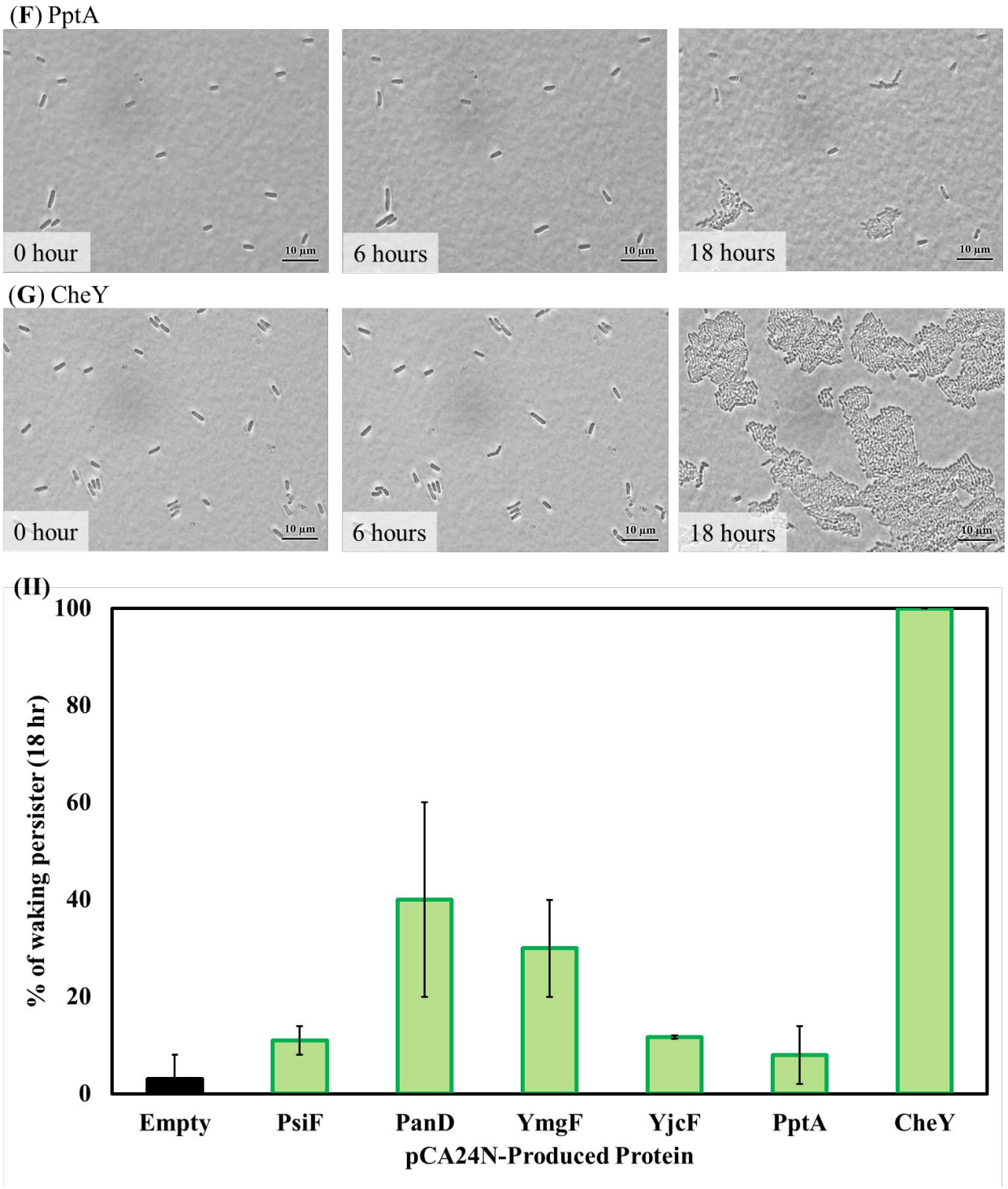
(**D**) YmgF



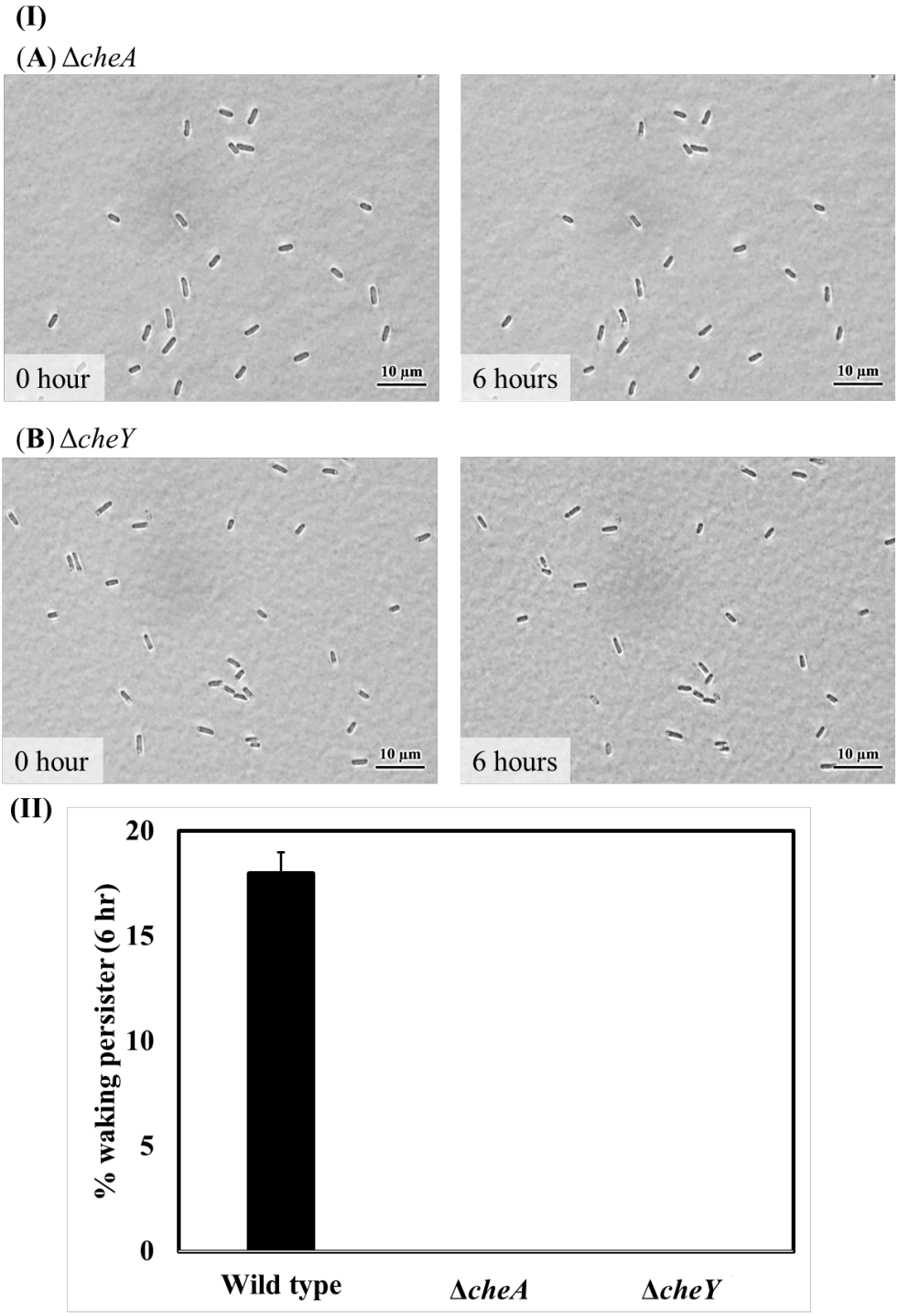
(**E**) YjcF



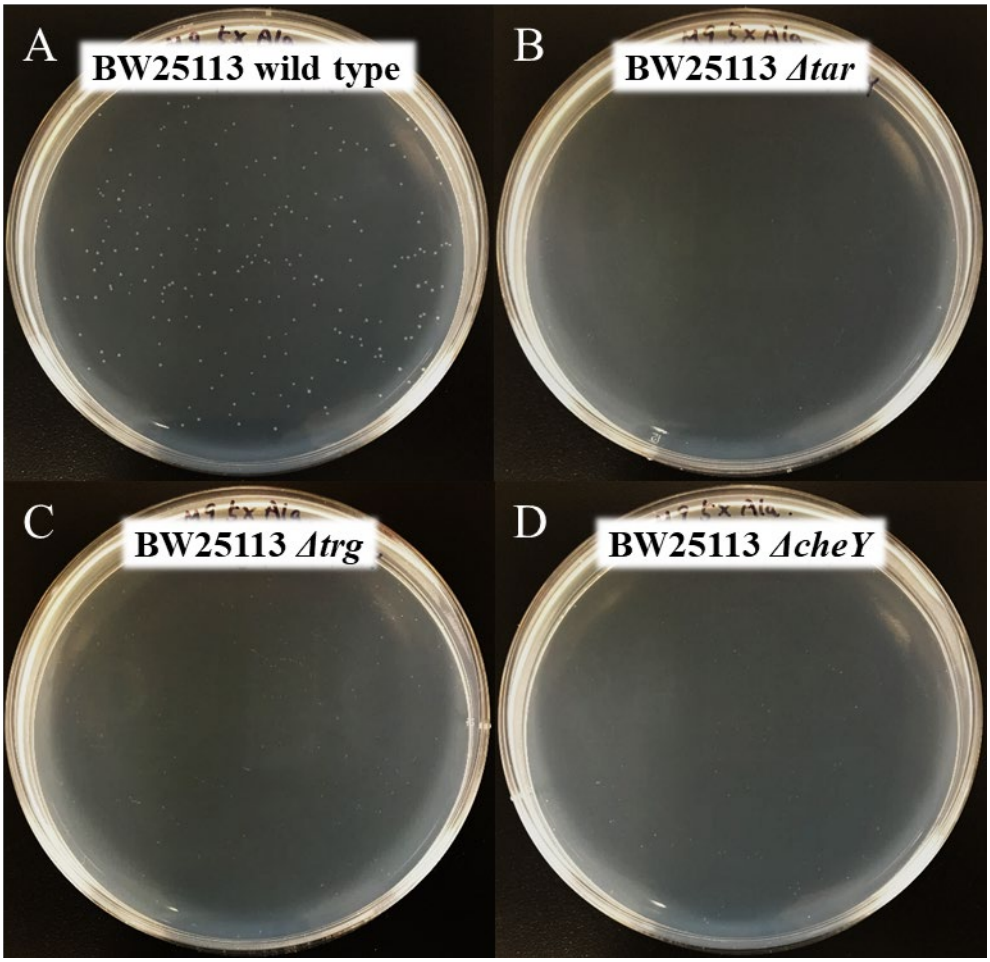
Supplementary Figure 5. (continued next page)



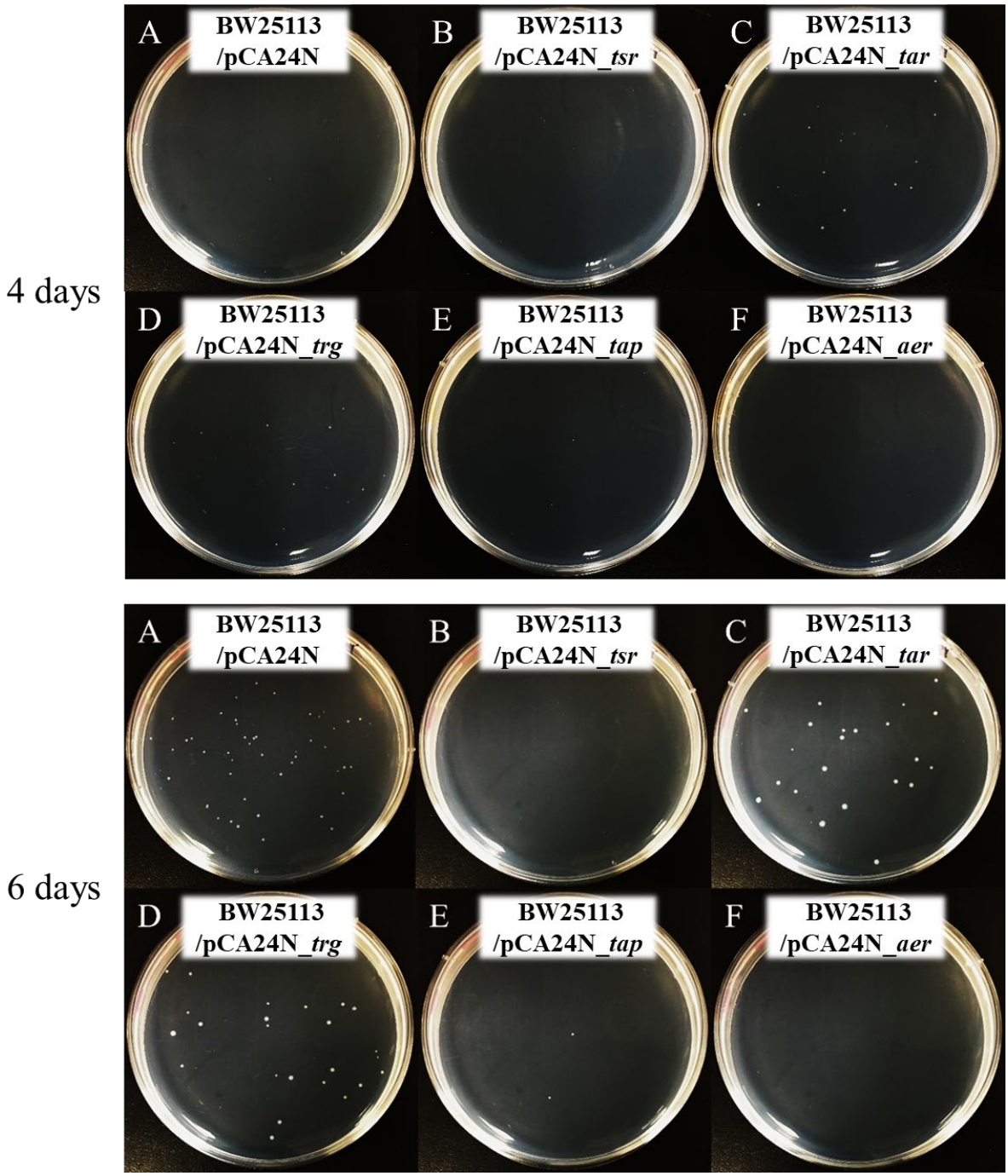
Supplementary Figure 5. Single persister cell waking related to the ASKA screen. (**I**) *E. coli* BW25113 persister cells containing (**A**) pCA24N (empty plasmid control), (**B**), pCA24N_*psiF,* (**C**) pCA24N_*panD*, (**D**) pCA24N_*ymgF*, (**E**) pCA24N_*yjfF*, (**F**) pCA24N_*pptA,* and (**G**) pCA24N_*cheY* were incubated at 37°C on M9 5X Ala agarose gel pads for 18 h. Scale bar indicates 10 µm. Representative results from two independent cultures are shown. Prior to forming persister cells, cultures were grown without IPTG with 30 µg/mL Cm. (**II**) Persister cell waking (%) after 18 hours.



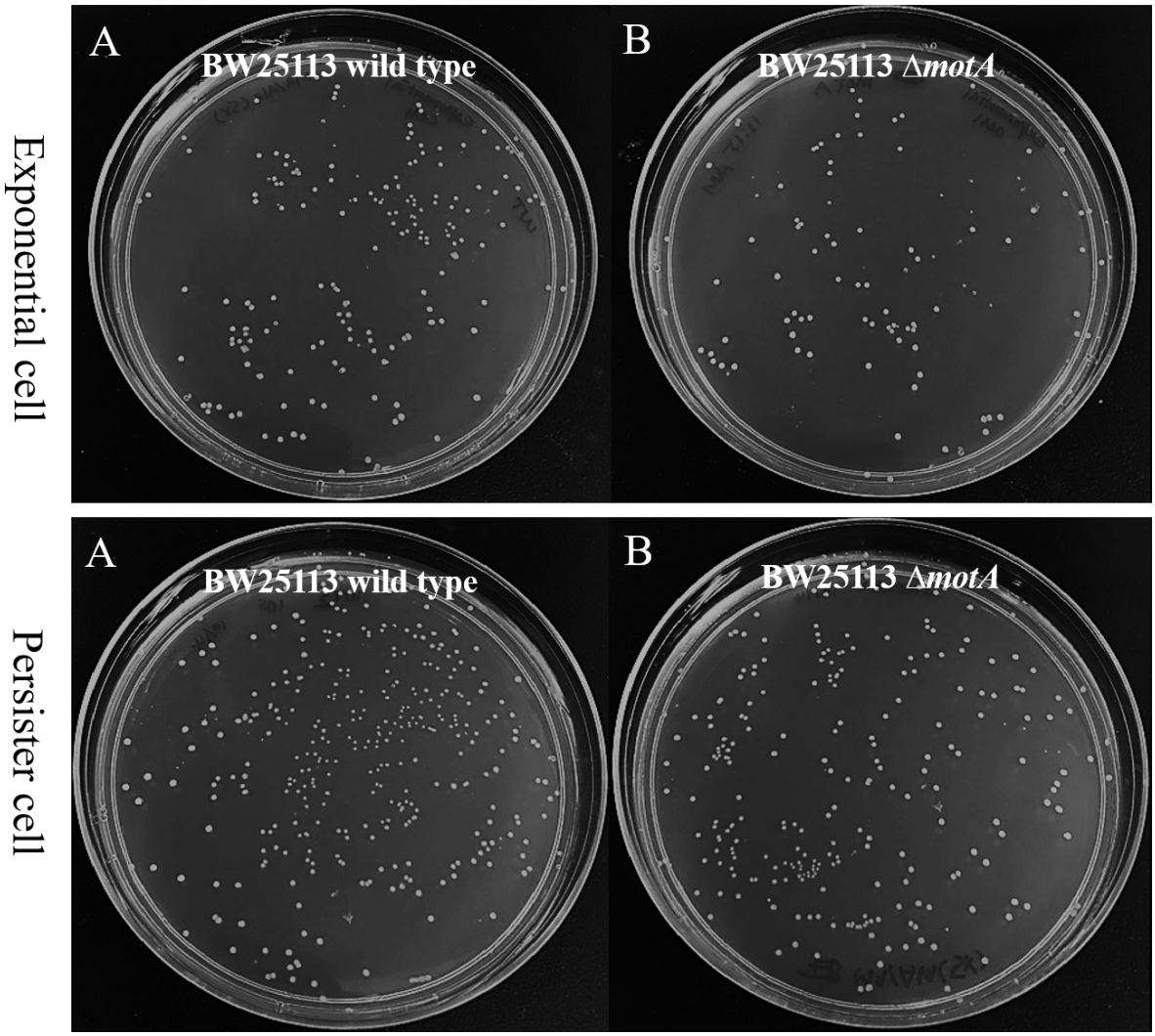
Supplementary Figure 6. Single persister cell waking after inactivating chemotaxis proteins CheA and CheY. (**I**) Persister cells of (**A**) BW25113 Δ*cheA* and (**B**) BW25113 Δ*cheY* waking on M9 5X Ala agarose gel pads after 6 h at 37°C. Scale bar indicates 10 µm. Representative results from two independent cultures are shown. (**II**) Persister cell waking (%) after 6 hours.



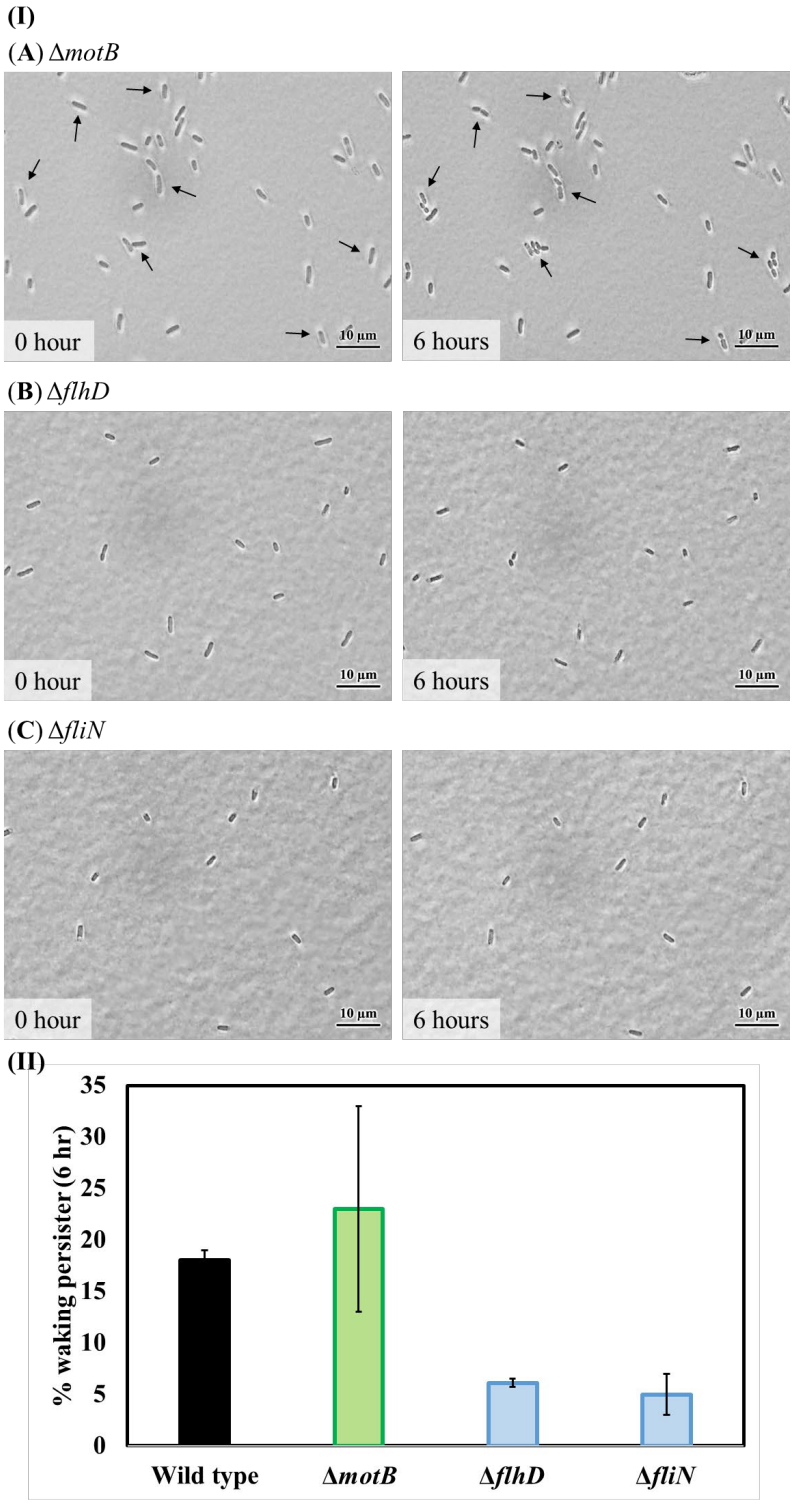
Supplementary Figure 7. Persister cell waking on agar plates after inactivating the chemotaxis-related proteins Tar, Trg, and CheY. Persister cells were incubated at 37 °C on M9 5X Ala agar plates for 2 days. (**A**) wild type BW25113, (**B**) BW25113 Δ*tar*, (**C**) BW25113 Δ*trg*, and (**D**) BW25113 Δ*cheY*. One representative plate of two independent cultures is shown.



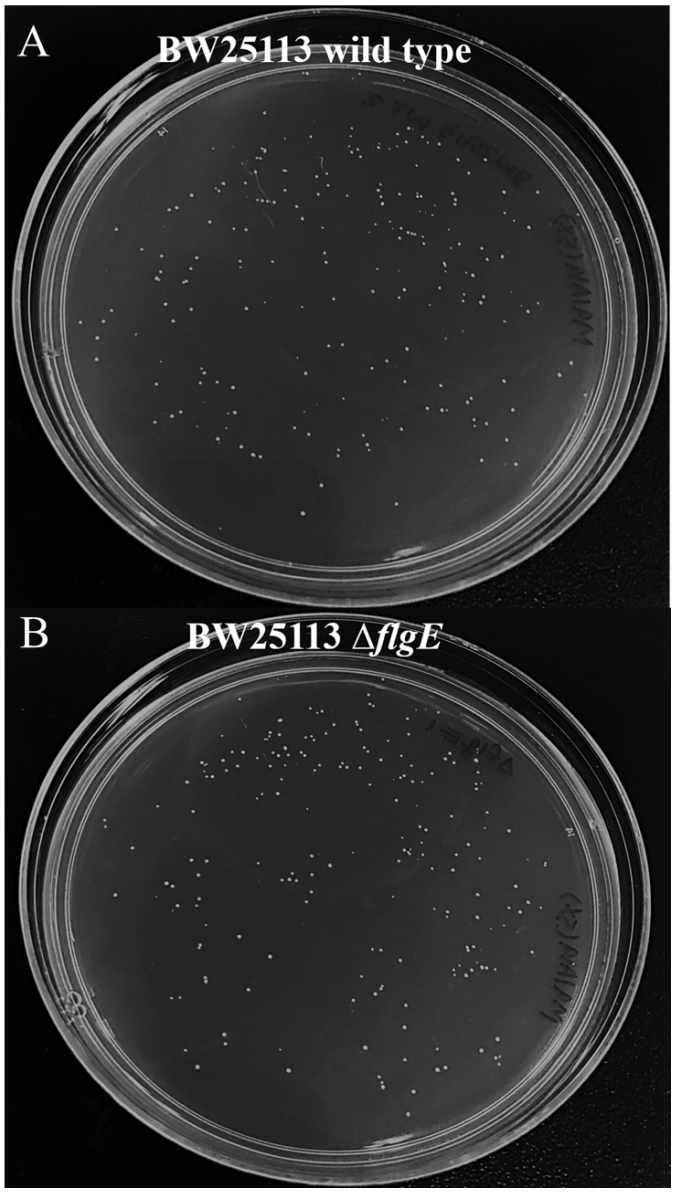
Supplementary Figure 8. Persister cell waking on agar plates after producing chemotaxis proteins. Persister waking after 4 days (upper panel) and after 6 days (lower panel) incubation at 37 °C on M9 5X Ala agar plates. (**A**) BW25113/pCA24N, (**B**) BW25113/pCA24N_*tsr*, (**C**) BW25113/pCA24N_*tar*, (**D**) BW25113/pCA24N_*trg*, (**E**) BW25113/pCA24N_*tap*, and (**F**) BW25113/pCA24N_*aer*. Prior to forming persister cells, cultures were grown without IPTG with 30 µg/mL Cm. One representative plate of two independent cultures is shown.



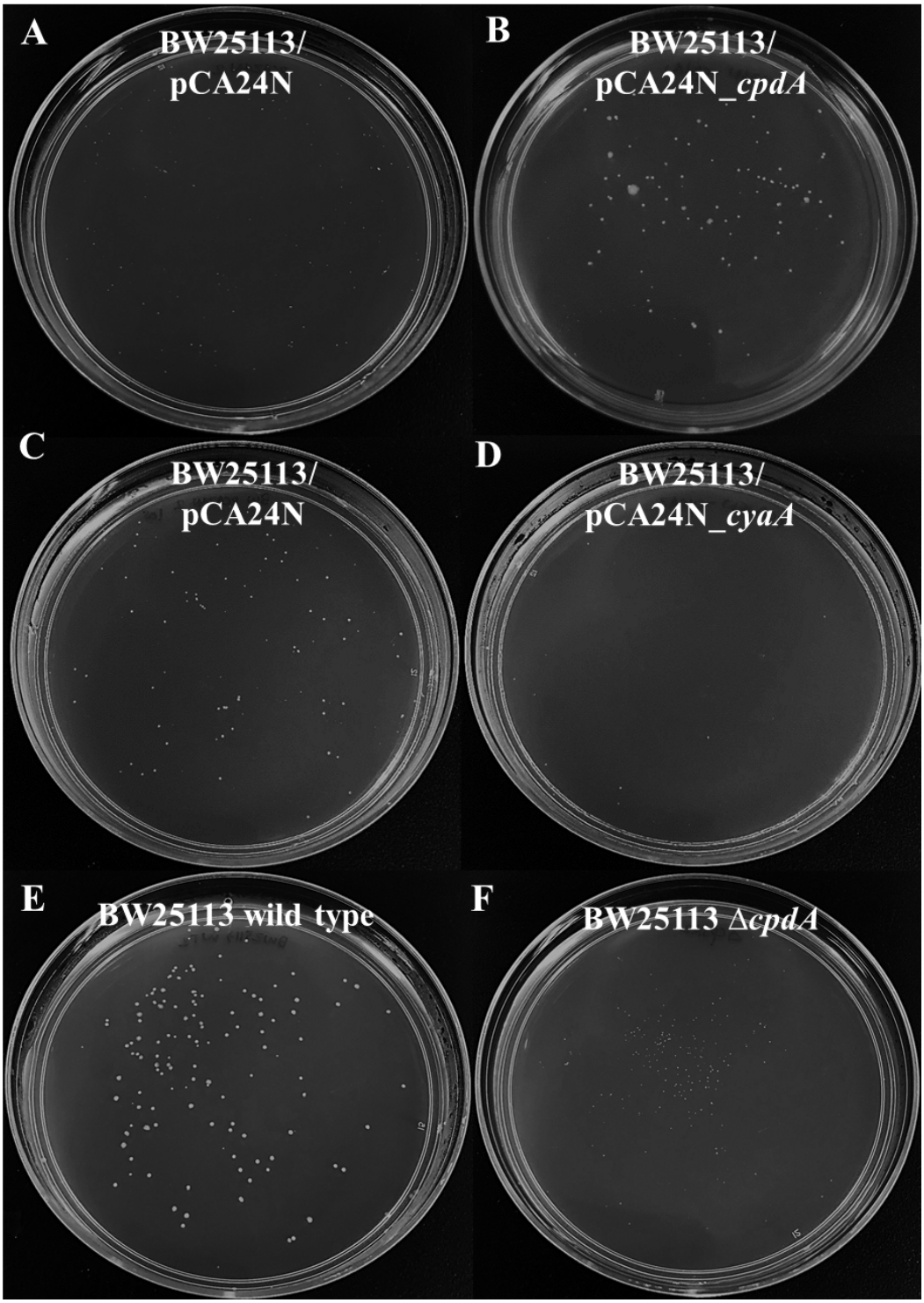
Supplementary Figure 9. Persister cell waking on agar plates after inactivating flagellar motor complex proteins MotA. Cells were incubated at 37 °C on M9 5X Ala agar plates for 3 days. Upper panel: Exponential cells (**A**) wild type BW25113 and (**B**) BW25113 Δ*motA*. Lower panel: Persister cells of (**A**) wild type BW25113 and (**B**) BW25113 Δ*motA*. One representative plate of two independent cultures is shown.



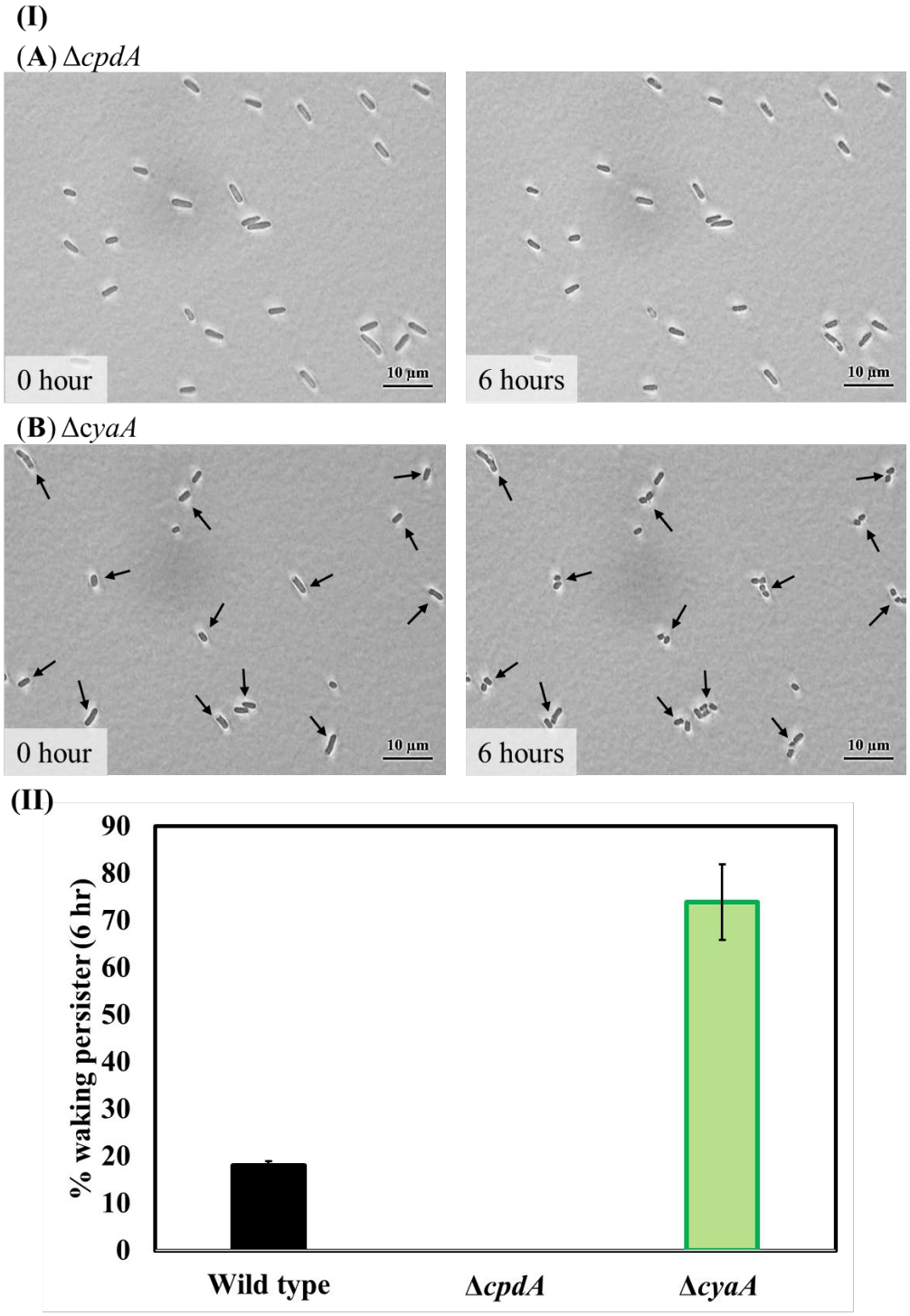
Supplementary Figure 10. Single persister cell waking after inactivating flagellar proteins MotB, FhlD, and FliN. (**I**) Persister cells of (**A**) BW25113 Δ*motB*, (**B**) BW25113 Δ*flhD*, and (**C**) BW25113 Δ*fliN* waking on M9 5X Ala agarose gel pads after 6 h at 37°C. Black arrows indicate cells that resuscitate. Scale bar indicates 10 µm. Representative results from two independent cultures are shown. (**II**) Persister cell waking (%) after 6 hours.



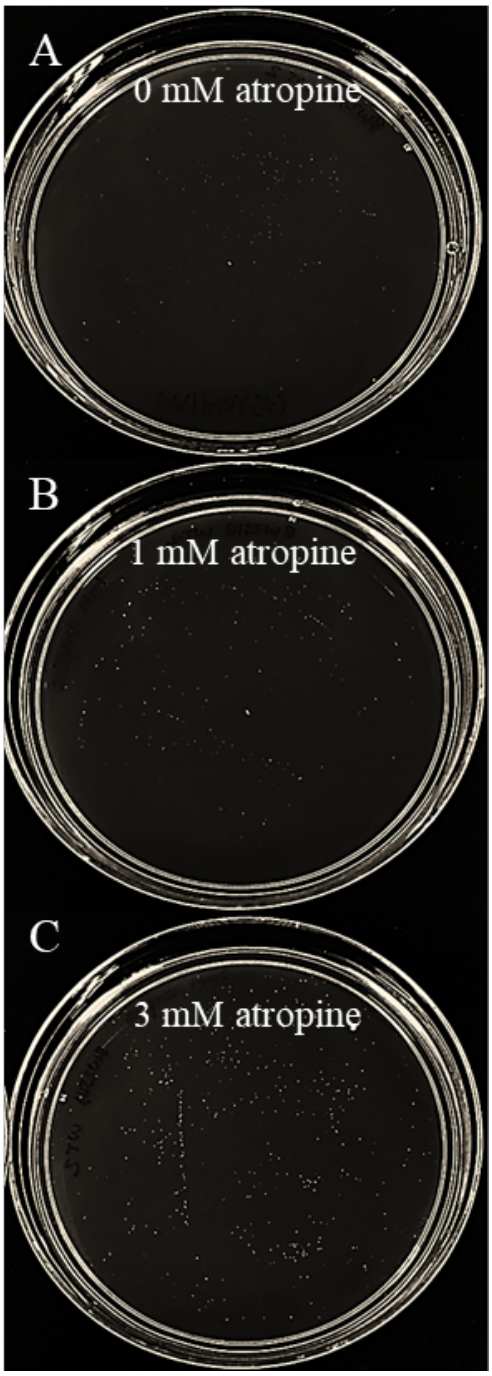
Supplementary Figure 11. Persister cell waking on agar plates after inactivating the FlgE flagellum hook protein. *E. coli* BW25113 persister cells of (**A**) wild type BW25113 and (**B**) BW25113 Δ*flgE* were incubated at 37 °C on M9 5X Ala agar plates for 2 days. One representative plate of two independent cultures is shown.



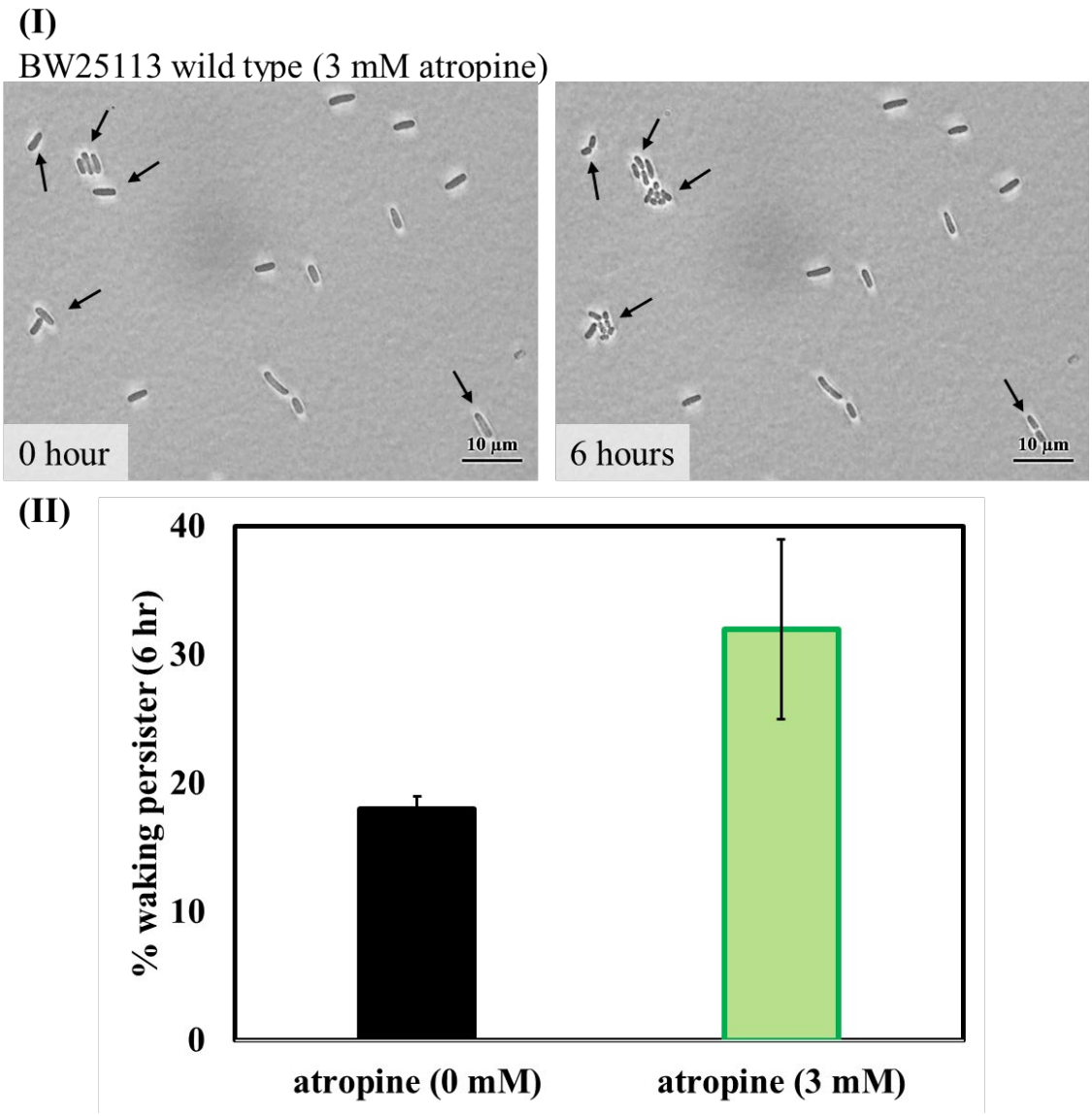
Supplementary Figure 12. Persister cell waking on agar plates after inactivating or producing proteins related cAMP. *E. coli* BW25113 persister cells with (**A**) pCA24N after 4 days, (**B**) pCA24N_*cpdA* after 4 days, **(C)** pCA24N after 5 days, **(D)** pCA24N_*cyaA* after 5 days, (**E**) wild type BW25113 after 3 days, and **(F)** BW25113 Δ*cpdA* after 3days. Plates were incubated at 37 °C and contain M9 5X Ala. One representative plate of two independent cultures is shown.



Supplementary Figure 13. Single persister cell waking after inactivating cAMP-related proteins CpdA and CyaA. (**I**) Persister cells of (**A**) BW25113 Δ*cpdA* and (**B**) BW25113 Δ*cyaA* waking on M9 5X Ala agarose gel pads after 6 h at 37°C. Scale bar indicates 10 µm. Representative results from two independent cultures are shown. (**II**) Persister cell waking (%) after 6 hours.

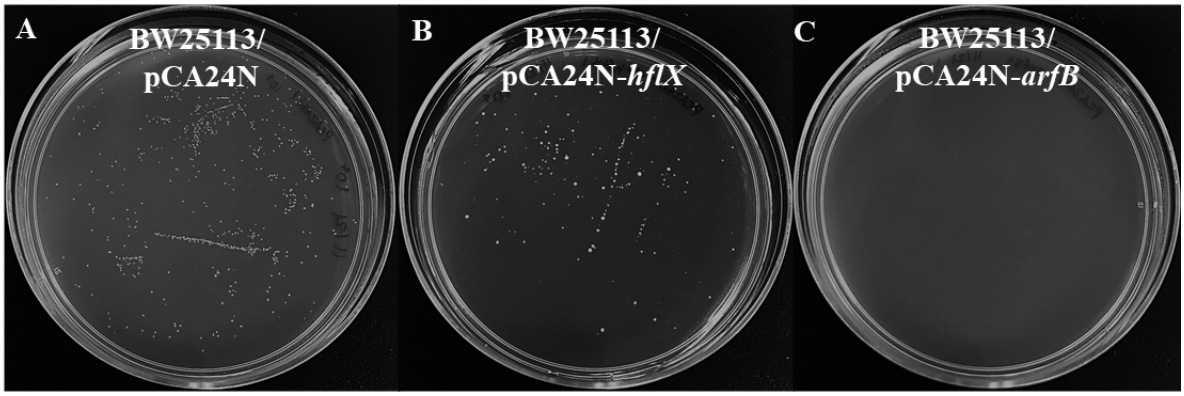


Supplementary Figure 14. Persister cell waking on agar plates with atropine. *E. coli* BW25113 persister cells were incubated at 37 °C on M9 5X Ala agar plates with (**A**) 0 mM, (**B**) 1 mM, and (**C**) 3 mM atropine for 36 hours. One representative plate of two independent cultures is shown.

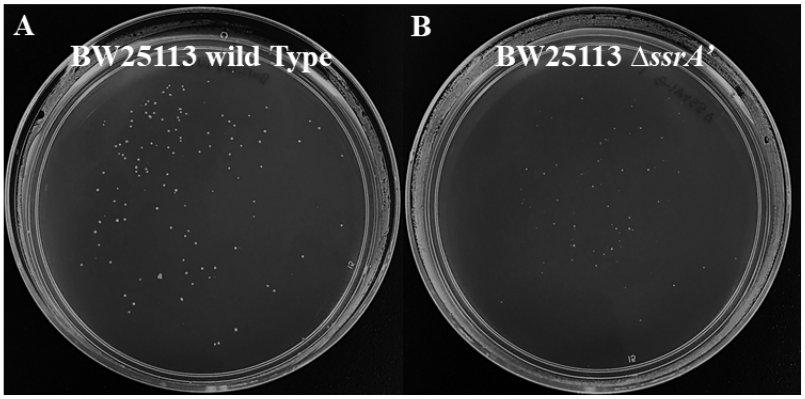


Supplementary Figure 15. Single persister cell waking with atropine. (**I**) Persister cells of wild-type BW25113 waking on M9 5X Ala on agarose gel pads with 3 mM atropine after 6 h at 37°C. Black arrows indicate cells that resuscitate. Scale bar indicates 10 µm. Representative results from two independent cultures are shown. (**II**) Persister cell waking (%) after 6 hours.

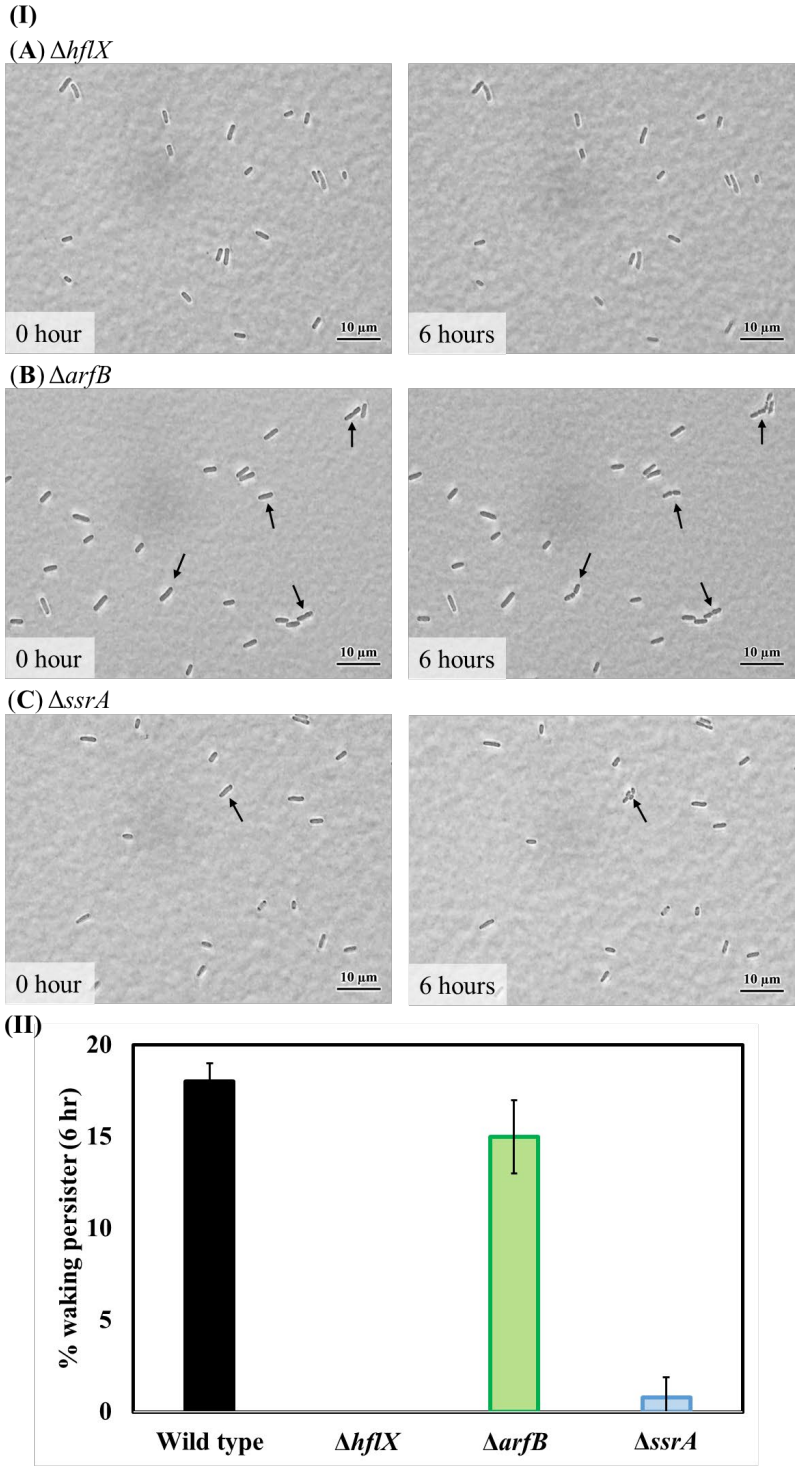
(I)



(II)



Supplementary Figure 16. Persister cells waking on agar plates after producing HflX and ArfB and inactivating SsrA. (**I**) *E. coli* BW25113 persister cells with (**A**) empty plasmid, (**B**) pCA24N_*hflX*, and (**C**) pCA24N_*arfB* were incubated at 37 °C on M9 5X Ala agar plates for 6 days. (**II**) *E. coli* BW25113 persister cells with (**A**) wild type and (**B**) BW25113 ΔssrA'. One representative plate of two independent cultures is shown.



Supplementary Figure 17. Single persister cell waking after inactivating ribosome rescue proteins. (**I**) Persister cells of (**A**) BW25113 Δ*hflX*, (**B**) BW25113 Δ*arfB*, and (**C**) BW25113 Δ*ssrA*, waking on M9 5X Ala agarose gel pads after 6 h at 37°C. Black arrows indicate cells that resuscitate. Scale bar indicates 10 µm. Representative results from two independent cultures are shown. (**II**) Persister cell waking (%) after 6 hours.