environmental microbiology

Environmental Microbiology (2019) **00**(00), 00–00 doi:10.1111/1462-2920.14828

Persister cells resuscitate via ribosome modification by 23S rRNA pseudouridine synthase RluD

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Summary

Upon a wide range of stress conditions (e.g. nutrient, antibiotic, oxidative), a subpopulation of bacterial cells known as persisters survives by halting metabolism. These cells resuscitate rapidly to reconstitute infections once the stress is removed and nutrients are provided. However, how these dormant cells resuscitate is not understood well but involves reactivating ribosomes. By screening 10,000 compounds directly for stimulating *Escherichia coli* persister cell resuscitation, we identified that 2-{[2-(4-bromophenyl)-2-oxoethyl]thio}-3-ethyl-5,6,7,8-tetrahydro[1]benzothieno[2,3-d]pyrimidin-4(3H)-one (BPOET) stimulates resuscitation. Critically, by screening 4267 *E. coli* proteins, we determined that BPOET activates hibernating ribosomes via 23S rRNA pseudouridine synthase RluD, which increases ribosome activity. Corroborating the increased waking with RluD, production of RluD increased the number of active ribosomes in persister cells. Also, inactivating the small RNA RybB which represses *rluD* led to faster persister resuscitation. Hence, persister cells resuscitate via activation of RluD.

Introduction

Upon myriad stresses such as antibiotic stress, a subpopulation of bacterial cells becomes dormant and multi-stress tolerant (Hobby *et al*., 1942; Bigger, 1944); these cells are known as persisters. The persister phenotype is not due to genetic change, because upon re-growth, persister cells behave the same as the original culture. Persistence is relevant in the environment since almost all cells face starvation (Song and Wood, 2018) and relevant in medicine since recurring infections may be the result of re-growth of persister cells (Van den Bergh *et al*., 2017). The persister subpopulation should be distinguished from slow-growing cells such as those in the stationary phase or those generated by nutrient shifts (Kim and Wood, 2017); these slow-growing cells may be distinguished from persisters because the whole population of slow-growing cells are tolerant to antimicrobials whereas the non-growing persister population is a small subpopulation (less than 1%) (Ronneau and Helaine, 2019). This distinction is critical because tolerant cells utilize alternate sigma factors like RpoS in *Escherichia coli* to redirect gene expression as an active response against stress (Wang *et al*., 2011), whereas persisters cease responding and become dormant (Kim and Wood, 2016, 2017).

To treat persister cell infections, it is important to understand how they form and how they resuscitate. The prevailing view for their formation (Ronneau and Helaine, 2019) is that to reduce metabolism, cells activate toxins of toxin/antitoxin (TA) systems (Wang and Wood, 2011). The best genetic evidence for this is that deletion of toxins MqsR (Kim and Wood, 2010; Luidalepp *et al*., 2011), TisB (Dörr *et al*., 2010) and YafQ (Harrison *et al*., 2009) decreases persistence. Moreover, production of non-TA system toxins also increases persistence (Chowdhury *et al*., 2016). However, as nutrient deprivation also results in persistence (Kim *et al*., 2018a), the subpopulation of cells may become dormant simply by running out of food. In addition, we have proposed a model whereby the alarmone ppGpp (synthesized as a result of myriad stress conditions) directly creates persister cells via ribosome dimerization, without the need of TA systems (Song and Wood, 2019). Regardless of the mechanism, persistence appears to be primarily an elegantly regulated response to an unfavourable environment (Wood *et al*., 2019).

With regard to resuscitating persister cells, little has been determined about the mechanism. It has been suggested that persister cells resuscitate by inactivating toxins such as TacT acetyltransferase via peptidyl-tRNA hydrolase Pth (Cheverton *et al*., 2016), but this has not been demonstrated. It is established that persister cells revive in response primarily to environmental signals, such as fresh nutrients (rather than stochastically) (Kim

Received 26 June, 2019; accepted 7 October, 2019. *For correspondence.E-mail tuw14@psu.edu; Tel. (+)1 814 863 4811; Fax (1) 814 865 7846

et al., 2018b). In addition, persisters revive in a heterogeneous manner, by activating ribosomes; cells increase their ribosome content until a threshold is reached, then they begin to elongate or divide (Kim *et al*., 2018b). For resuscitation, the persisters sense nutrients by chemotaxis and phosphotransferase membrane proteins, reduce cAMP levels to rescue stalled ribosomes, unhybridize 100S ribosomes via HflX and undergo chemotaxis towards fresh nutrients (Yamasaki *et al*., 2019).

In the present study, to discern additional insights into how ribosomes are active as persister cells resuscitate, we converted the complete *E. coli* population into persister cells so that we could screen for the first time compounds that enhance persister resuscitation. From a 10,000 compound library, we identified that 2-{[2-(4-bromophenyl)-2-oxoethyl]thio}-3-ethyl-5,6,7,8-tetrahydro[1]benzothieno [2,3-d]pyrimidin-4(3H)-one (BPOET) stimulates persister cell waking. Critically, we determined that the mechanism by which BPOET resuscitates persisters is via activation of the 23S rRNA pseudouridine synthase RluD, which is important for ribosome activity. Hence, BPOET stimulates persister resuscitation by activating ribosomes via RluD.

Results and discussion

BPOET resuscitates E. coli *persister cells*

To identify compounds that resuscitate *E. coli* persister cells, we first increased by 10^5-fold the persister cell population by pre-treating with rifampicin to cease metabolism by stopping transcription followed by ampicillin treatment to kill any remaining non-persister cells (Kwan *et al*., 2013). In this way, nearly 100% of bacterial cell population was converted into persister cells. Hence, we were able to both screen for compounds that more rapidly resuscitate persister cells as well as confirm our hypotheses via single-cell microscopy. The persister cells generated in this way have been confirmed eight ways (by showing multi-drug tolerance, immediate resuscitation, dormancy based on no cell division when nutrients are absent, dormancy based on metabolic staining/cell sorting, no change in minimum inhibitory concentration, no resistance phenotype, similar morphology to ampicillin-induced persisters and similar resuscitation as ampicillin-induced persisters) (Kim *et al*., 2018b). Furthermore, our method has been used to (i) determine that persister cells wake via ribosome activation (Kim *et al*., 2018b) and (ii) by activating chemotaxis (Yamasaki *et al*., 2019), (iii) show that the cells capable of resuscitation in a viable but not culturable population are equivalent to persister cells (Kim *et al*., 2018a), (iv) identify compounds that kill persister cells (Song *et al*., 2019) and (v) reveal that the alarmone ppGpp directly creates persister cells by stimulating ribosome dimerization (Song and Wood, 2019). In addition, our method to generate a high population of persister cells has been utilized by at least six independent groups (Grassi *et al*., 2017; Cui *et al*., 2018; Narayanaswamy *et al*., 2018; Sulaiman *et al*., 2018; Tkhilaishvili *et al*., 2018; Pu *et al*., 2019).

Using 96-well plates, the persister cells (10 μl) were added to 190 μl of lysogeny broth (LB) containing one each of the 10,000 compounds of the DiverSet library dissolved in dimethylsulphoxide (DMSO) (100 μM final concentration), and growth was monitored via the change in turbidity for up to 48 hr. Starting at a turbidity of 0.05, a 140-fold increase in growth was possible (maximum final turbidity of 0.69). Table 1 shows the 27 compounds that were identified that stimulated persister cell resuscitation relative to the negative control of DMSO; the initial screening results with these compounds were evaluated in LB and M9 buffer (no carbon source) (Table S1A). Confirming the initial results, we found BPOET (100 μM) was most effective in minimal alanine medium and increased persister cell waking by 33-fold in 96-well plates based on the increase in turbidity (Table S1B). By monitoring the resuscitation of 213 individual persister cells, we also found microscopically that BPOET increases waking by fourfold (Fig. 1, Table S2). Hence, we focused on this compound.

BPOET resuscitates E. coli *persister cells by modifying ribosomes*

To determine how BPOET resuscitates persister cells, we pooled the 4267 ASKA clones in which each *E. coli* protein is produced from plasmid pCA24N, produced persister cells carrying these plasmids, contacted with 100 μM BPOET, plated the cells and chose the largest colonies that formed on LB plates from approximately 1000 colonies. Our rationale was that any pathway stimulated by BPOET would be even more active if the number of rate-limiting proteins in that pathway was increased, and cells that wake first would form colonies faster.

Using this approach, we identified five proteins for which production increased resuscitation: RluD, YjiK, SrlR, Smf and YeeZ. These proteins are related to contacting with BPOET because addition of the diluent DMSO alone and sequencing larger colonies did not identify these five proteins but instead identified TmcA, a $tRNA^{Met}$ cytidine acetyltransferase, which is a general factor required for translation that likely led to larger colony sizes with the diluent. Of the proteins related to BPOET, only RluD (23S rRNA pseudouridine synthase) and SrlR (represses the *gut* operon for glucitol metabolism) have been characterized; we focused on RluD because it is related to ribosomes, and we have shown inactivating ribosomes causes persistence (Kwan *et al*., 2013) and activating ribosomes resuscitates persister cells (Kim *et al*., 2018b; Song and Wood, 2019;

Table 1. Compounds that resuscitate persister cells and their structures that were identified in the initial screen.

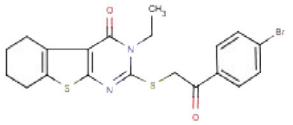
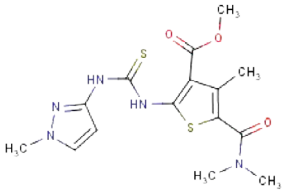
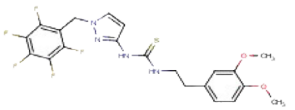
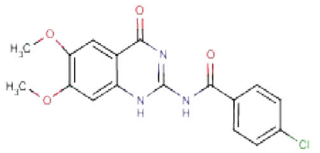
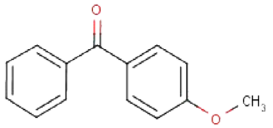
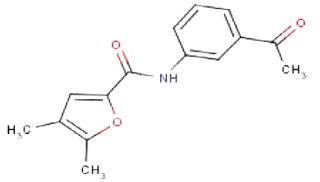
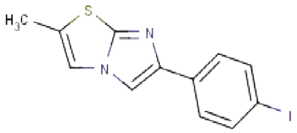
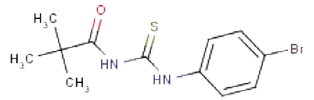
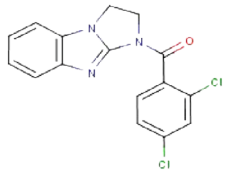
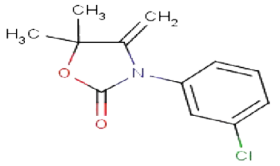
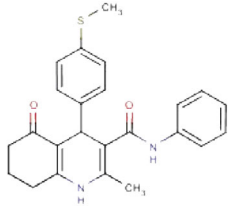
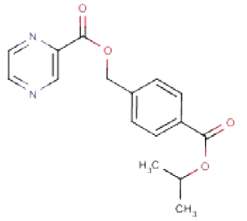
Name	Structure	Name	Structure
2-{[2-(4-Bromophenyl)-2-oxoethyl]thio}-3-ethyl-5,6,7,8-tetrahydro[1]benzothieno[2,3-d]pyrimidin-4(3H)-one (BPOET)		Methyl 5-[(dimethylamino)carbonyl]-4-methyl-2-({[(1-methyl-1H-pyrazol-3-yl)amino]carbonothioyl}amino)-3-thiophenecarboxylate	
N-[2-(3,4-dimethoxyphenyl)ethyl]-*N*′-[1-(pentafluorobenzyl)-1H-pyrazol-3-yl]thiourea		4-Chloro-N-(6,7-dimethoxy-4-oxo-1,4-dihydro-2-quinazolinyl)benzamide	
(4-Methoxyphenyl)(phenyl)methanone		*N*-(3-acetylphenyl)-4,5-dimethyl-2-furamide	
6-(4-Iodophenyl)-2-methylimidazo[2,1-b][1,3]thiazole		N-{[(4-bromophenyl)amino]carbonothioyl}-2,2-dimethylpropanamide	
1-(2,4-Dichlorobenzoyl)-2,3-dihydro-1H-imidazo[1,2-a]benzimidazole		3-(3-Chlorophenyl)-5,5-dimethyl-4-methylene-1,3-oxazolidin-2-one	
2-Methyl-4-[4-(methylthio)phenyl]-5-oxo-*N*-phenyl-1,4,5,6,7,8-hexahydro-3-quinolinecarboxamide		4-(Isopropoxycarbonyl)benzyl 2-pyrazinecarboxylate	

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Table 1. Continued

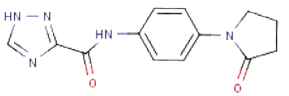
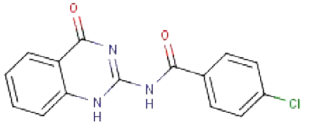
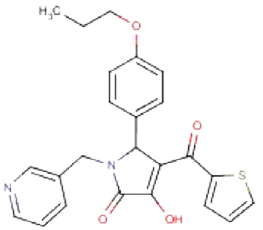
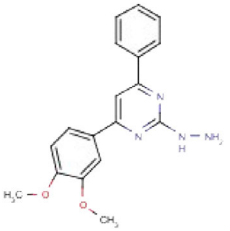
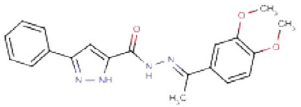
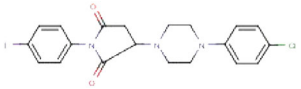
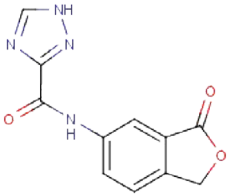
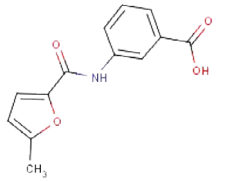
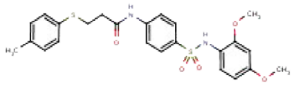
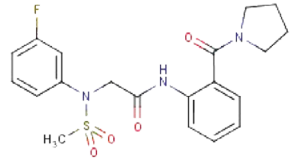
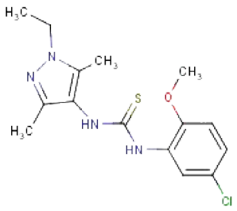
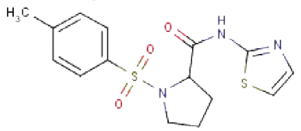
Name	Structure	Name	Structure
N-[4-(2-oxo-1-pyrrolidinyl)phenyl]-1H-1,2,4-triazole-3-carboxamide		4-Chloro-*N*-(4-oxo-1,4-dihydro-2-quinazolinyl)benzamide	
3-Hydroxy-5-(4-propoxyphenyl)-1-(3-pyridinylmethyl)-4-(2-thienylcarbonyl)-1,5-dihydro-2H-pyrrol-2-one		4-(3,4-Dimethoxyphenyl)-2-hydrazino-6-phenylpyrimidine	
N′-[1-(3,4-dimethoxyphenyl)ethylidene]-3-phenyl-1H-pyrazole-5-carbohydrazide		3-[4-(4-Chlorophenyl)-1-piperazinyl]-1-(4-iodophenyl)-2,5-pyrrolidinedione	
N-(3-oxo-1,3-dihydro-2-benzofuran-5-yl)-1H-1,2,4-triazole-3-carboxamide		3-[(5-Methyl-2-furoyl)amino]benzoic acid	
N-(4-{[(2,4-dimethoxyphenyl)amino]sulfonyl}phenyl)-3-[(4-methylphenyl)thio]propanamide		*N*-2-(3-fluorophenyl)-*N*-2-(methylsulfonyl)-*N*-1-[2-(1-pyrrolidinylcarbonyl)phenyl]glycinamide	
N-(5-chloro-2-methoxyphenyl)-*N*′-(1-ethyl-3,5-dimethyl-1H-pyrazol-4-yl)thiourea		1-[(4-Methylphenyl)sulfonyl]-*N*-1,3-thiazol-2-ylprolinamide	

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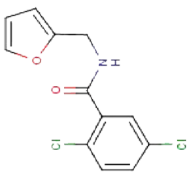
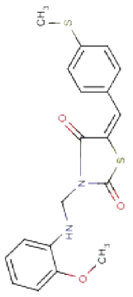
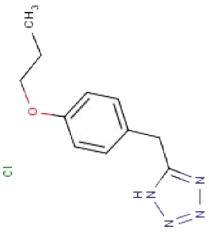
Table 1. Continued

Name	Structure	Name	Structure
2,5-Dichloro-*N*-(2-furylmethyl)benzamide		3-{[(2-Methoxyphenyl)amino]methyl}-5-[4-(Methylthio)benzylidene]-1,3-thiazolidine-2,4-dione	
5-(4-Propoxybenzyl)-1H-tetrazole			

Chemical structures are from ChemBridge. BPOET is indicated in bold and was obtained from ChemBridge (San Diego, CA).

Yamasaki *et al*., 2019). RluD is involved in the synthesis and assembly of 70S ribosomes as well as their function based on its post-transcriptional modification of 23S rRNA to form three pseudouridine (5-ribosyl-uracil) nucelosides at positions 1911, 1915, and 1917 (Gutgsell *et al*., 2005). In pseudouridine, uracil is attached via a carbon–carbon bond to the sugar base rather than through a carbon-nitrogen bond. The 23S rRNA pseudouridines increase the stability of the tertiary structure of 23S rRNA and are located in a stem loop structure that is involved in peptidyltransferase and interacts with mRNA, tRNA, 16S rRNA, and ribosome release factor. Hence, RluD is responsible for efficient ribosome function (Gutgsell *et al*., 2005).

RluD enhances persister cell resuscitation

To explore further the role of RluD and persister resuscitation, we utilized single-cell studies because persister cells are heterogeneous (Kim *et al*., 2018b) and wake with different frequencies (which would be missed if we monitored planktonic populations). We found that deleting *rulD* reduces the frequency of single-cell persister resuscitation dramatically (−10-fold) compared with the isogenic wild-type strain on minimal glucose agarose gel pads (Figs 1A and S1, Table S2). In addition, no colonies were found on M9 glucose agar plates after inactivating RluD (Fig. S2), confirming that persister cells are severely challenged in resuscitation without RluD. Corroborating these two results with the *rluD* mutant, production of RluD increased the frequency of single-cell waking by 11-fold on glucose medium in the wild-type background (Figs 1A and S1, Table S2), and persister cell resuscitation in the *rluD* mutant could be restored to wild-type levels by producing RluD from pCA24N (Fig. S2). In addition, producing RluD via pCA24N-*rluD* had little impact on cell growth (Table S3), the *rluD* deletion has no effect on persister cell formation (Fig. S2) and the *rluD* mutation had little effect on cell growth in LB medium and M9 glucose (Table S3). Hence, RluD increases persister cell resuscitation but does not affect persister formation.

To confirm BPOET stimulates persister cell resuscitation through RluD, we tested whether BPOET increases waking when RluD is overproduced (i.e. BW25113/pCA24N-*rluD* + 100 μM BPOET vs. BW25113/pCA24N + 100 μM BPOET) and found twofold greater waking with overproduced RluD and BPOET (Figs 1A and S1, Table S2). Furthermore, the addition of 100 μM BPOET had no effect BW25113 *rluD* single-cell resuscitation (Figs 1A and S1, Table S2). Therefore, BPOET stimulates persister cell waking through its interaction with RluD.

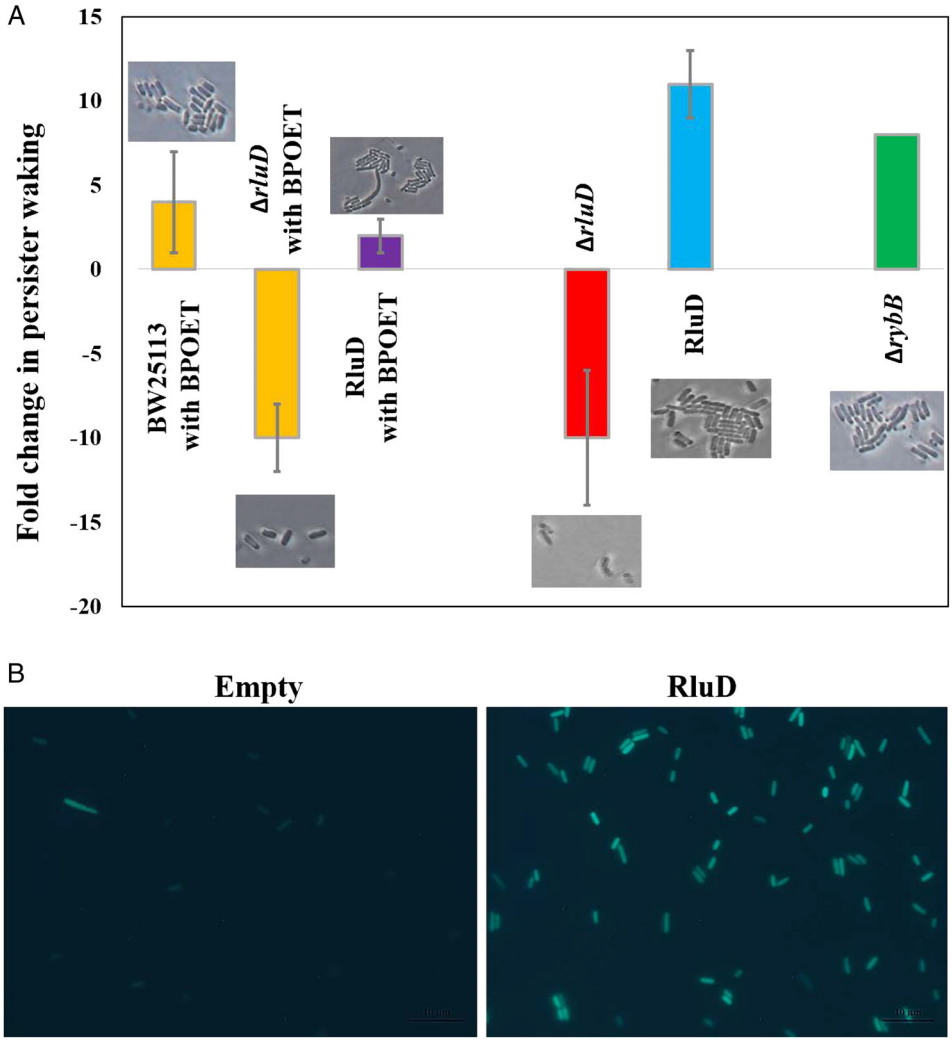


Fig. 1. RluD increases persister resuscitation by increasing ribosomes for resuscitation.
A. Single-cell persister resuscitation as determined using light microscopy (Zeiss Axio Scope.A1). The total and waking number of persister cells are shown in Supplementary Table S2. Microscope images for waking cells are shown in Supplementary Fig. S1. The fold-changes in resuscitation are relative to BW25113 with DMSO for BW25113 with BPOET, relative to BW25113 with BPOET for Δ*rluD* with BPOET, relative to BW25113 for the Δ*rluD* mutant, relative to BW25113/pCA24N for the strain producing RluD from pCA24N plasmid in BW25113, and relative to MG1655 for MG1655 Δ*rybB*. M9 glucose (0.4%) (Rodriguez and Tait, 1983) agarose gel pads were used for all the strains except BW25113, Δ*rluD*, BW25113/pCA24N, and BW25113/pCA24N-*rluD* with BPOET where M9 5X alanine (4.2 mM) agarose gel pads including 100 μM of BPOET or DMSO were used. The results are the combined observations from two independent experiments after 6 hr for the BW25113 and mutants with BPOET, after 16 hr for the BW25113/pCA24N and BW25113/pCA24N/rluD with BPOET, after 4 h for BW25113 and its deletion mutants (no BPOET) and after 6 hr for cells harbouring pCA24N and its derivatives (no BPOET) as well as for MG1655 and MG1655 Δ*rybB* (see Supplementary Table S2 for details). Error bars indicate standard deviations.
B. Active 70S ribosomes in single persister cells for MG1655-ASV/pCA24N-rluD ('RluD') versus MG1655-ASV/pCA24N ('Empty'). Cells are shown on agarose gel pads at time 0 for resuscitation; that is, after the formation of persister cells. Representative results from three independent cultures are shown. The persister cells were generated as described previously (Kwan *et al*., 2013; Kim *et al*., 2018b) by treating exponentially growing cells (turbidity of 0.8 at 600 nm) with rifampicin (100 μg/ml) for 30 min to stop transcription, centrifuging, and adding LB with ampicillin (100 μg/ml) for 3 hr to lyse any non-persister cells. Cells pellets were washed twice with 0.85% NaCl then re-suspended in 0.85% NaCl. The green fluorescent protein (GFP) signal of the resuscitating persisters of *Escherichia coli* K-12 MG1655-ASVGFP (Shah *et al*., 2006) with RluD was monitored using a fluorescence microscope (Zeiss Axioscope.A1, bl_ph channel at 1000 ms exposure and GFP channel at 10,000 ms exposure). *E. coli* K-12 MG1655-ASVGFP produces an unstable variant of GFP (half-life less than 1 hr) under the control of the 16S rRNA ribosomal promoter *rrnb*P1 (Shah *et al*., 2006). To identify proteins responsible for resuscitation, all 4267 ASKA clones (GFP–) (Kitagawa *et al*., 2005) were combined, grown together 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). The pooled ASKA plasmids (1 μl containing 30 ng DNA) were 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 to maintain the plasmids, and the cells were incubated at 250 rpm to a turbidity of 0.8. Rifampicin followed by ampicillin was added to make persister cells, then the persister cells were washed twice with 1× PBS buffer, contacted with 100 μM BPOET for 2 hr in M9 medium that lacked a carbons source, and plated on LB (Cm) agar plates. Faster colony appearance indicated faster persister resuscitation. Plasmids were isolated from the colonies and sequenced using primer pCA24N_F: 5'-GCCCTTTCGTCTTCACCTCG.

Table 2. *E. coli* bacterial strains and plasmids used in this study.

	Features	Source
Strains		
BW25113	*lacI*^{q} *rrnB*$_{T14}$ Δ*lacZ*$_{WJ16}$ *hsdR514* Δ*araBAD*$_{AH33}$ Δ*rhaBAD*$_{LD78}$	Baba *et al*. (2006)
BW25113 Δ*rluD*	Δ*rluD* Km^{R}	Baba *et al*. (2006)
MG1655	F^{-} lambda^{-} *ilvG* *rfb-50 rph-1*	Guyer *et al*. (1981)
MG1655 Δ*rybB*	Δ*rybB* Km^{R}	Hobbs *et al*. (2010)
MG1655-ASV	*rrnb*P1::GFP[ASV]	Shah *et al*. (2006)
Plasmids		
pCA24N	Cm^{R}; *lacI*^{q}	Kitagawa *et al*. (2005)
pCA24N_*rluD*	Cm^{R}; *lacI*^{q}, P$_{T5\text{-}lac}$::*rluD*$^{+}$	Kitagawa *et al*. (2005)

Km^{R} and Cm^{R} indicate kanamycin and chloramphenicol resistance respectively. The strains were grown routinely in lysogeny broth (Bertani, 1951) at 37°C.

RluD increases active ribosomes for resuscitation

Using a GFP reporter that indicates the number of 70S ribosomes in individual persister cells (Kim *et al*., 2018b), we found that overproducing RluD from pCA24N before making persister cells (i.e. before rifampicin treatment) makes 85% ± 6% of the cells have high ribosome fractions compared with 22% ± 2% of the cells that do not overproduce RluD (Fig. 1B, Table S4). The GFP reporter indicates transcription of *rrsB* (16S rRNA), *gltT* (tRNA-glu), *rrlB* (23S rRNA) and *rrfB* (5S rRNA); hence, it indicates production of the three major rRNA building blocks. Although this is not a direct observation of 70S ribosomes, this method is a suitable proxy for the number of active ribosomes based on the measurement of rRNA concentrations and has been used frequently (Lu *et al*., 2009; Piques *et al*., 2009; Burger *et al*., 2010; Kim *et al*., 2018b), and we have verified its use by isolating ribosomes and comparing GFP fluorescence (Kim *et al*., 2018b). Hence, the increased persister cell resuscitation with RluD is directly due to the increase in active (70S) ribosomes of persister cells since persisters wake via ribosome activation (Kim *et al*., 2018b).

RybB antagonizes persister cell resuscitation

Because the small RNA RybB reduces RluD (Gogol *et al*., 2011), we investigated its impact on persister resuscitation. As expected, we found that the deletion of *rybB* increases the frequency of persister cell waking by eightfold (Figs 1A and Fig S1, Table S2).

In summary, the results presented here demonstrate that ribosomes may be activated for recovery from dormancy, corroborating our model for the ribosome dependence of persister cell formation (Wood *et al*., 2019). Specifically, by screening for compounds for the first time that enhance persister cell resuscitation, we have (i) determined that ribosomes are modified by RluD as cells resuscitate and resume ribosome activity, (ii) identified a novel compound, BPOET, that activates persister cells and (iii) linked small RNAs to persistence. Hence, these results extend our understanding of how persister cells are activated which has a far-reaching impact in that all bacteria cope with nutrient stress and become dormant.

Acknowledgements

This work was supported by funds derived from the Biotechnology Endowed Professorship at the Pennsylvania State University. We are grateful for the *rybB* mutant provided by Dr. Gisela Storz.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1: Supporting Information