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REVIEW

Ribosome dependence of persister cell formation and resuscitation

Thomas K. Wood*, Sooyeon Song, and Ryota Yamasaki

Department of Chemical Engineering, Pennsylvania State University, University Park, Pennsylvania, 16802-4400, USA

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Since most bacterial cells are starving, they must enter a resting stage. Persister is the term used for metabolically-dormant cells that are not spores, and these cells arise from stress such as that from antibiotics as well as that from starvation. Because of their lack of metabolism, persister cells survive exposure to multiple stresses without undergoing genetic change; i.e., they have no inherited phenotype and behave as wild-type cells once the stress is removed and nutrients are presented. In contrast, mutations allow *resistant* bacteria to grow in the presence of antibiotics and slow growth allows *tolerant* cells to withstand higher concentrations of antibiotics; hence, there are three closely-related phenotypes: persistent, resistant, and tolerant. In addition, since dormancy is so prevalent, persister cells must have a means for resuscitating (since so many cells should obtain this resting state). In this review, we focus on what is known about the formation and resuscitation of persister cells.

***Keywords*:** persistence, antimicrobial agents, tolerance

Problematic history of persister cells

Persister cells were discovered by Hobby *et al.* (1942) who found that penicillin does not kill about 1% of *Staphylococcus aureus* cells. By reducing the culture temperature to 4°C to stop cell division, they found that penicillin did not kill dormant (non-dividing) cells. Bigger (1944) named these antibiotic-tolerant *S. aureus* cells "persisters" and confirmed that penicillin does not kill dormant (non-dividing) *S. aureus* cells that were formed by reducing the culture temperature to 4°C, by removing nutrients, and by adding boric acid. After a long period of inactivity (62 years), a ribosomal reporter (green fluorescence protein) and cell sorting were used to show persister cells have low metabolic activity (Shah *et al.*, 2006).

*For correspondence. E-mail: twood@engr.psu.edu; Tel.: +1-814-863-4811; Fax: +1-814-865-7846

The persister phenotype is general and is seen in all cells tested to date (Van den Bergh *et al.*, 2017). All pathogens tested to date also form persisters, and when these dormant cells resuscitate, they likely reconstitute infections (Defrain *et al.*, 2018).

One constraint to studying persister cells is that they are usually found at very low concentrations, from 0.0001 to 1% (Van den Bergh *et al.*, 2017). This has caused some groups to make the mistake of studying slowly-growing cells rather than persister cells, such as those created by a nutrient shift (Amato *et al.*, 2013; Amato and Brynildsen, 2014, 2015) and those of the stationary stage (Orman and Brynildsen, 2015). Unfortunately, these studies incorrectly attribute characteristics of slowly-growing (tolerant) cells to persister cells. Another common mistake is adding fresh medium (e.g., during cell washing steps) since persister cells wake immediately in the presence of nutrients (Kim *et al.*, 2018b).

Persistence and viable but non-culturable describe the same dormant state

Most bacteria are starving (Schmidt, 2012); hence, they have created elegant pathways to achieve dormancy. If they were not starving, i.e., if they were growing exponentially, then a single *E. coli* cell, for example, doubling every 20 min, would lead to a population of cells with the mass of the Earth in about 44 h; this situation clearly does not occur, so bacteria usually lack sufficient nutrients and enter a resting state.

Along with persistence, another term used to describe dormant cells is "viable but non-culturable" (VBNC). VBNC was first used to describe *E. coli* and *Vibrio cholerae* cells (in 1982) that form from a lack of nutrients; these cells were reported to not resuscitate on selective and non-selective media (Xu *et al.*, 1982); however, a few stimuli, such as nutrients and temperature shifts, were reported to resuscitate VBNC cells (Li *et al.*, 2014).

The overlap of the phenotypes shared between persisters and VBNC cells is striking. Both persister cells (Mulcahy *et al.*, 2010) and VBNCs (Li *et al.*, 2014) are associated with chronic infections and biofilms (Spoering and Lewis, 2001; Li *et al.*, 2014), and both cell types arise from more than one stress such as oxidative and acid stress (Hong *et al.*, 2012; Li *et al.*, 2014). Hence, these two dormant states have been linked as part of a "dormancy continuum" (Ayrapetyan *et al.*, 2015) with VBNC cells considered more dormant. However, there is little that distinguishes these two resting states.

Instead of a dormancy continuum, we found that there is

no difference between the *E. coli* VBNC cells that are capable of resuscitation and persister cells; in fact, the cells capable of re-growth in these two groups are the same dormant state (Kim *et al.*, 2018a). We formed *E. coli* VBNC cells through starvation and compared them to persister cells that are associated with antibiotic tolerance and found all the culturable cells in the VBNC-inducing condition were identical to persister cells within 14 days of starvation. Critically, based on antibiotic tolerance, morphology, resuscitation rates, and lack of metabolic activity, the cells capable of growth in the VBNC population were the same as persister cells (Kim *et al.*, 2018a); these are the similar-looking cells with dense cytosol of Fig. 1 for both populations. Surprisingly, most of the VBNC cell particles lack normal cytosol components and are dead; i.e., they are incapable of resuscitating due to their lack of normal cellular material (Fig. 1). These cells that lack normal cytosol components also have intact membranes. Therefore, the Live/Dead stain is not effective with these obviously dead cells because the cells have intact membranes and lack DNA for propidium iodide to stain; i.e., these cells, which are incapable of resuscitation, stain green, rather than red, but they should not be interpreted as live cells (Kim *et al.*, 2018a).

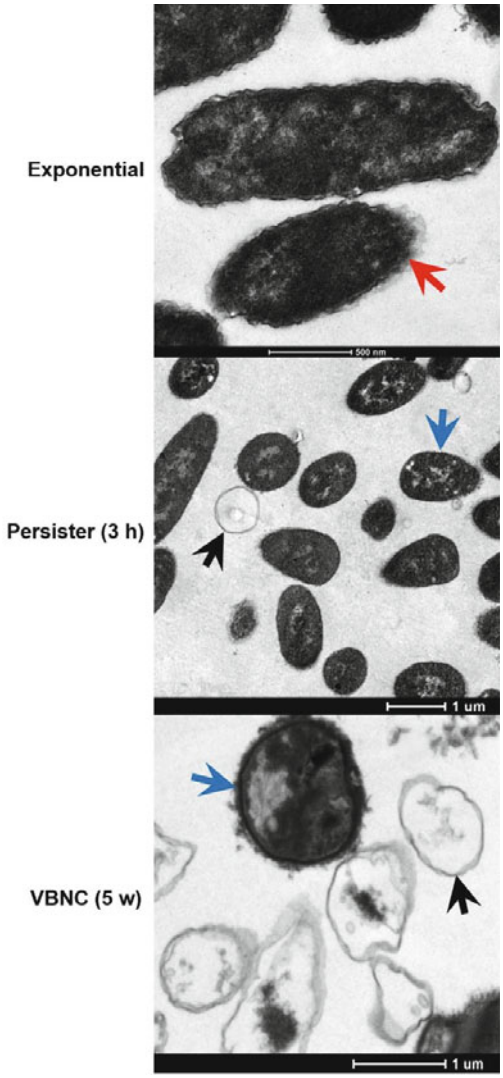

Fig. 1. Most VBNC cells lack normal cytosolic material. Transmission electron microscopy images of exponentially-growing *E. coli* K-12 cells (top), rifampicin-induced persister cells after 3 h of ampicillin treatment (middle), and 5 week (5 w) old VBNC cells in saline solution (bottom). Red arrow shows dense cytosol and intact membranes of exponential cells, blue arrows show material loss, and the black arrow shows dead cells with empty cytosol but intact membranes. Scale bar indicates 500 nm for exponential cells and 1 μm for persister and VBNC cells. Note the persister population has cells that appear like VBNC particles and the VBNC population has cells that appear like persister cells.

The crux is that if both dormant states are the same, persistence and VBNCs, then persister cells are ubiquitous, since most cells in the environment are starving. This includes bacterial cells associated with plants where phytopathogens survive stress and cause losses of over $1 billion dollars worldwide (Martins *et al.*, 2018).

Persister cell formation as a response to environmental stress and dependence on protein production

Rather than primarily a spontaneous or bet-hedging process, persister cells appear to form primarily as an elegantly-regulated response to stress. A simple illustration of this is that stochastically created persisters are rare (Balaban *et al.*, 2004) whereas external stress can convert nearly the whole population into persister cells (Kwan *et al.*, 2013). Also, Lewis *et al.* have shown that most persisters are formed as a result of an environmental stress (contact with antibiotic ciprofloxacin) rather than forming spontaneously and that pretreatment with the natural antibiotic and DNA crosslinker mitomycin C increases persistence (Dörr *et al.*, 2009).

These natural external stresses that create persister cells, like when cells encounter antibiotics, can be understood in terms of reducing protein production. We have shown that persister cells form when protein production is inhibited by stopping transcription with rifampicin, by stopping translation with tetracycline, or by stopping translation by eliminating ATP production with carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (Kwan *et al.*, 2013). Hence, these pretreatment methods may be used to create nearly a 100% population of persister cells (Kwan *et al.*, 2013). Furthermore, these methods produce *bona fide* persister cells as they have been verified eight ways: multi-drug tolerance, immediate change from persister to non-persister in the presence of nutrients, dormancy via flow cytometry with the metabolic dye redox sensor green, dormancy via a lack of resuscitation on gel pads that lack nutrients (some exponential cells divide under these conditions), no change in MIC compared to exponential cells, no resistance phenotype, similar morphology to ampicillin-induced persisters, and similar resuscitation as ampicillin-induced persisters (Kim *et al.*, 2018b). To date, at least five other independent research groups have used our methods to make persister cells groups with *E. coli*, *Pseudomonas aeruginosa*, and *S. aureus* (Grassi *et al.*, 2017; Cui *et al.*, 2018; Narayanaswamy *et al.*, 2018; Sulaiman *et al.*, 2018; Tkhilaishvili *et al.*, 2018); hence, these pretreatment methods are general and relatively facile for creating persister cells so that

they are the dominant phenotype and more easily studied. One caveat is that most strains require optimization of these methods; i.e., pretreatments yield different results in various strains (e.g., chemical, concentration, and time of pretreatment must be optimized).

To aid their study, persister cells may also be formed at high percentages of the population by producing any toxin protein, such as YihS, PntA, YqjE, FocA, and Zur in *E. coli* (Chowdhury *et al*., 2016). As another example, production of toxin MqsR, an endonuclease of the MqsR/MqsA toxin/antitoxin (TA) system (Ren *et al*., 2004; Brown *et al*., 2009; Wang *et al*., 2011, 2013) that cleaves mRNA that contains 5-GCU sequences (Yamaguchi *et al*., 2009), has been used to increase persistence by 14,000-fold for *E. coli* (Hong *et al*., 2012). In addition, pre-treatment with oxidative stress or acid stress increases persistence 12,000-fold (Hong *et al*., 2012). These approaches were used to discern that bacterial persistence increases when the cells are less fit (Hong *et al*., 2012) and to study the persister proteome to find indole decreases persistence in *E. coli* (Hu *et al*., 2015). Based on this insight with indole, halogenated indoles such as 5-iodoindole, 4-fluoroindole, 7-chloroindole, and 7-bromoindole were identified that effectively kill *E. coli* and *S. aureus* persister cells (Lee *et al*., 2016). In addition, fluoroquinolones may be used to induce the SOS response via DNA strand breaks and induce persistence (Dörr *et al*., 2009).

Persister cell formation and toxin/antitoxins

Since ceasing protein production via ribosome activity (Kwan *et al*., 2013) and producing toxic proteins (Hong *et al*., 2012) lead to remarkable increases in persistence, it seems straightforward that cells would use TA systems to become persistent since many TA systems reduce protein production (Harms *et al*., 2016). Other *bona fide* physiological roles of TA systems (Song and Wood, 2018) include (i) inhibiting phage (Pecota and Wood, 1996), (ii) maintaining genetic elements such as plasmids (Ogura and Hiraga, 1983), (iii) reducing metabolism as a response to stress (Gerdes, 2000; Wang *et al*., 2011), and (iv) forming biofilms (González Barrios *et al*., 2006; Kim *et al*., 2009).

The first evidence linking persistence and TA systems was that in which a *hipA7* mutation in the toxin gene of the HipA/HipB TA system increased persistence 1,000 fold (Moyed and Bertrand, 1983). The HipA7 toxin variant (containing the amino acid substitutions G22S and D291A) is produced from a gain-of-function *hipA7* mutation that reduces antitoxin binding (Schumacher *et al*., 2015); although persistence is increased, the HipA7 variant is not toxic (Korch *et al*., 2003), so the link of TA systems to persistence from this work is tenuous.

Direct evidence linking persistence and TA systems is that deletion of the single gene that encodes toxin MqsR of the MqsR/MqsA TA system reduces persistence (Kim and Wood, 2010; Luidalepp *et al*., 2011). Also, deletion of the gene encoding toxins TisB of the TisB/IstR-1 TA system (Dörr *et al*., 2010) and YafQ of the YafQ/DinJ TA system YafQ (Harrison *et al*., 2009) reduce persistence. Therefore, it is unnecessary to delete multiple TA systems to change persistence [claims to the contrary, such as the claim that multiple *E. coli* TA systems must be deleted to see an effect have been retracted (Maisonneuve *et al*., 2011)], and TA systems are clearly linked to persister cell formation.

The connection of TA systems to the alarmone guanosine tetraphosphate (ppGpp) came via studies of toxin MazF of the MazF/MazE TA system: ppGpp is required to activate MazF toxicity (Aizenman *et al*., 1996), and ppGpp is generated during starvation and activates the stress response sigma factor for the stationary phase, RpoS, and the stress response sigma factor for misfolded proteins in the periplasm, RpoE (Dalebroux and Swanson, 2012). MazF toxin reduces protein production by cleaving mRNAs and rRNA precursors containing the 5'ACA site (Culviner and Laub, 2018). Hence, the reduction of growth through ppGpp was linked to TA systems that reduce protein production.

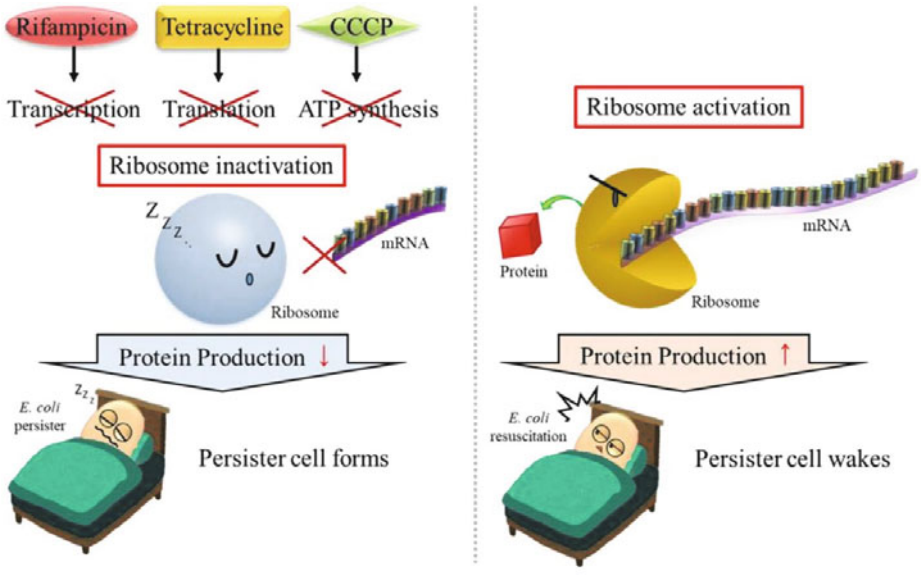

Fig. 2. Schematic showing the ribosome basis of persister cell formation and waking. Left, Inhibition of transcription by rifampicin, inhibition of translation by tetracycline, and inhibition of ATP synthesis by CCCP makes ribosomes inactive, which leads to persister cell formation. Right, In contrast, ribosome activation leads to protein production and persister cell resuscitation.

Next, ppGpp was found to be required for persistence as a result of overproduction of the inactive toxin variant HipA7 of the HipA/HipB TA system (Korch *et al.*, 2003). Similarly, the persistence of *P. aeruginosa* also requires ppGpp (Nguyen *et al.*, 2011).

Unfortunately, the mechanism from increasing ppGpp to the activation of TA systems has not been discerned. The model proposed by the Gerdes group, that Lon protease is activated by polyphosphate (which builds up as ppGpp is activated) and then degrades antitoxins to activate toxins, has been retracted (Maisonneuve *et al.*, 2013, 2018) due to contrary evidence presented (Van Melderen and Wood, 2017). First, polyphosphate inactivates Lon protease *in vitro*, rather than activates Lon activity (Osbourne *et al.*, 2014). Also, several labs found little connection between Lon and persistence (Shan *et al.*, 2015; Chowdhury *et al.*, 2016). Critically, a former member of the Gerdes group showed that for the YoeB/YefM TA system, one of the TA systems upon which the polyphosphate model is based (Maisonneuve *et al.*, 2013), degradation of YefM antitoxin is actually independent of ppGpp and polyphosphate (Ramisetty *et al.*, 2016). The model also disagrees with other prior results from the Gerdes lab showing that the degradation of antitoxins RelB and MazE is independent of ppGpp (Van Melderen and Wood, 2017). Finally, ppGpp is not absolutely necessary for persistence (Chowdhury *et al.*, 2016), and the model is incapable of explaining persistence from RNA-based antitoxins.

It is also notable that comprehensive mutation studies have not provided significant insights into the genetic basis persistence cell formation. For example, transposon-sequencing (Shan *et al.*, 2015), protein expression (Spoering *et al.*, 2006), and gene knockouts (Hu and Coates, 2005; Hansen *et al.*, 2008) have not revealed much information about how persister cells form. Therefore, although TA systems are indubitably related to persister cell formation, further experimentation is required to determine how cells elegantly respond to stress and become dormant. For this future work, it is important to use physiologically-relevant levels of TA systems, since as we have indicated (Song and Wood, 2018), non-physiological levels have led to the mistakes connecting toxins of TA systems to cell death for phage inhibition (Pecota and Wood, 1996; Blower *et al.*, 2011), plasmid stabilization (Gerdes *et al.*, 1986), and programmed cell death (Kolodkin-Gal *et al.*, 2007; Nariya and Inouye, 2008).

Persister cell resuscitation relies on ribosomal content

Just as persister cell formation is primarily a response to stress rather than a spontaneous process (Dörr *et al.*, 2009; Kwan *et al.*, 2013), resuscitation is also an elegant response to better environmental conditions rather than primarily a spontaneous process. Since dormancy is arguably the most prominent bacterial phenotype, it makes sense that cells must encode elegant pathways for resuscitation. Furthermore, persister resuscitation depends on ribosome activity.

In regard to waking, persister cells, made from producing the inactive HipA7 toxin variant were found to wake in about an hour (Balaban *et al.*, 2004). Using stationary-phase cells, the Tenson group also estimated persisters wake in about one to two hours after contact with fresh medium (Joers *et al.*, 2010). To more accurately study persister cell waking, we first converted to bacterial population to predominantly persister cells and then studied the resuscitation of single cells. Upon removal of stress and addition of fresh nutrients, we have found, by watching *E. coli* single cells on complex medium agar pads, that persister cells wake five ways: (i) immediate division, (ii) immediate elongation followed by division, (iii) immediate elongation but no division, (iv) delayed elongation/division, and (v) no growth (Kim *et al.*, 2018b). In addition, when persister cells begin dividing, they grow at the same rate as exponential cells so they are fully-recovered once cell division commences (Kim *et al.*, 2018b). Since (i) there is heterogeneity in waking, (ii) the cell composition is roughly 50% protein on a dry basis, and (iii) ceasing ribosome activity leads to persistence, we reasoned that differing ribosome levels dictate persister waking (Kim *et al.*, 2018b). In support of this hypothesis, by using a validated green fluorescence protein reporter of ribosome levels, we observed heterogeneity in the level of ribosomes in persister cells (Kim *et al.*, 2018b). Critically, we found that higher ribosome levels result in faster waking (Kim *et al.*, 2018b) i.e., as cells became persistent, they had varying amounts of ribosomes, and the persister cells with high ribosome levels woke first and those with low levels of ribosome had to increase their ribosome levels to a threshold value, then they began to wake (Kim *et al.*, 2018b). Furthermore, persister cells that had high residual ampicillin antibiotic levels failed to resuscitate (Kim *et al.*, 2018b). Hence, persister cell resuscitation depends on protein production via activating ribosomes and is primarily a response to changing environmental conditions.

In support of the model of persister cells requiring ribosomes to resuscitate, the Lewis group found the gene for the ribosome modulation factor (RMF) is one of the most induced genes in persister cells formed from producing the inactive HipA7 toxin variant (Keren *et al.*, 2004). RMF creates 100S ribosome dimers during starvation (Shimada *et al.*, 2013).

After our initial publication showing the importance of ribosomes for cell resuscitation (Kim *et al.*, 2018b), a second group, Pu *et al.* (2019), used microscopy and single cells to confirm our original results by showing that persisters are heterogeneous in their waking (Kim *et al.*, 2018b). Whereas we showed waking is a function of ribosome content (Kim *et al.*, 2018b), Pu *et al.* (2019) claimed persisters wake as a function of removing protein aggregation. Unfortunately, the protein aggregation they found appears to be primarily the result of cell death and not an indicator of persister resuscitation. For example, Pu *et al.* (2019) showed pre-treating cells with chloramphenicol to stop protein translation, prior to antibiotic treatment to kill non-persister cells, reduced the formation of cells that failed to resuscitate, whereas no pre-treatment with chloramphenicol increased dramatically the number of cells with protein aggregation (these cells never resuscitated). Strikingly, the cells they formed with protein aggregation look similar to the dead cells that lack a normal cytosol (Fig. 1) that we found with transmission electron microscopy (Kim *et al.*, 2018a). Also, pre-treating with chloramphenicol is analogous to the methods we used to stop protein production and convert the majority of the cells to the

persister state seven years earlier (Kwan *et al*., 2013), so it is expected that pre-treating with chloramphenicol should increase the number of persister cells, as Pu *et al.* (2019) found. Hence, in the work of Pu *et al.* (2019), stopping protein production again increased the persistence of *E. coli*, and their protein aggregation, which is was non-specific and included nearly a thousand different proteins, is an indicator of cell death, not an indicator of deep dormancy (unless one considers cell death as the ultimate dormant state). It is also difficult to imagine an enzyme that could re-nature a thousand different proteins and bring cells back to life. The main cause of this error is that Pu *et al.* (2019) used propidium iodide staining to try and enumerate what they called VBNC cells (i.e., cells that did not wake that were "most dormant"); we have shown the propidium iodide stain does not indicate correctly the viability of the majority of the remaining dead cell particles, which have intact cell membranes (Kim *et al*., 2018a). Indeed, as pointed out above, the resuscitating (non-dead) fraction of nutrient-starved cells (i.e., the population previously known as VBNCs by other authors) are the same as persister cells (Kim *et al*., 2018a). Therefore, the hypothesis of dormancy depth of Pu *et al.* (2019) is appropriate where it overlaps with the concept of heterogeneity of waking of persister cells (due to a difference in ribosome level) but is invalid in its application to cells which will never wake because they are dead. Surprisingly, these are also some of the same authors (Pu *et al*., 2016) that argued before (incorrectly) that persister cells are not dormant and can actively efflux antibiotics (Kim and Wood, 2016).

It has been proposed in the scout model (Epstein, 2009), without providing data, that persister cell waking is spontaneous, with cells waking periodically to see if it is safe to grow. Using our methods to create a nearly uniform population of *E. coli* persister cells, we have found that persisters do not wake primarily spontaneously (Yamasaki *et al*., 2018). Also, our results showing rapid cell wake-up once the antibiotic stress is removed and fresh nutrients provided (Kim *et al*., 2018b) indicates another aspect of the scout model that does not match our data: cells do not stay dormant and "invisible" since they wake rapidly when presented with nutrients and the stress is removed.

Perspectives

The prominent role of ribosome levels in persister cell formation and waking is clear (Fig. 2). If persister cell waking is not primarily spontaneous, what remains to be determined is what receptors enable persister cells to sense nutrients, how this information is propagated to the cytosol from the exterior of the cell, and how ribosome and protein synthesis is reconstituted. In our opinion, there is an elegant (i.e., highly-regulated) and conserved pathway that enables cells to resuscitate in the presence of nutrients. In other words, just as persister cells form more readily as an elegant response to environmental stress rather than as a bet-hedging process, they also resuscitate in a highly-regulated fashion. This view about the elegant regulation of persister cells mirrors the evolving views scientists had about biofilm formation: initially researchers wondered if there was a genetic basis for biofilm formation (O'Toole *et al*., 2000) whereas now genetic pathways exist for many strains, and biofilm formation is usually considered a highly-regulated cell response to its environment, involving common features such as the secondary messenger cyclic diguanylate (Ha and O'Toole, 2015).

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