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Single-cell recordings reveal subpopulations that grow and generate resistance at bactericidal concentrations of antibiotics

B. Henriques-Normark[a] and S. Normark[a,1]

Antibiotic treatment may select for spontaneous mutations leading to target alterations, decreased uptake, increased efflux, or antibiotic inactivation. Bacterial growth in the presence of the antibiotic is required for resistant mutants to arise. It is well known that antibiotic treatment at subinhibitory levels reduces the growth rate in the population, thereby selecting for fast-growing mutants with decreased susceptibility. Here, the authors present antibiotic perseverance as a new mechanism for selection of resistance even at lethal concentrations of an antibiotic (1). Perseverance implies a heterogenous population not caused by stable genetic changes where a subpopulation of the treated bacteria is not growth-inhibited by the antibiotic but continues to grow and divide for some generations at concentrations beyond the minimal inhibitory concentration. This will result in an expansion of the subpopulation, allowing for a significant increase in the mutation rate, leading to antibiotic resistance.

The authors discovered perseverance using a microfluidic device, allowing time-lapse imaging recordings of the size increase of individual bacteria in the presence of different antibiotics at different concentrations (2). With this single-cell approach, two antibiotics, rifampicin and nitrofurantoin, out of nine tested, gave rise to perseverance. What is not understood is how the state of perseverance is regulated and why it does not occur with antibiotics inhibiting protein synthesis such as chloramphenicol, gentamycin, and tetracycline or DNA replication such as ciprofloxacin (1).

In the presence of rifampicin, about 2% of the *Escherichia coli* population continued to grow and divide but at a 50% reduced growth rate (1). Rifampicin has only one target in the cell, the beta-subunit of DNA-dependent RNA polymerase, inhibiting all RNA synthesis in the cell. So, what could be different in this subpopulation of cells? One possibility is that the bacterial population is heterogenous with respect to the influx rate of rifampicin into the cytoplasm where the target is located. It is known that the outer membrane (OM) of gram-negative enterobacteria constitutes a permeability barrier against some antibiotics, including rifampicin. Lipopolysaccharide (LPS) localized to the outer leaflet of the OM is required for impermeability (3). When LPS biosynthesis is reduced, phospholipids (PL) flip into the outer leaflet and disrupt barrier function. Thus, *E. coli* mutated in the *envA* gene encoding LpxC, the first committed enzymatic step in LPS biosynthesis, are hypersensitive to rifampicin and several other antibiotics (4). It has been demonstrated that the LPS levels are regulated by the YciM/FtsH protease complex, which degrades LpxC (3). Cells also appear to counter the consequences of LPS overproduction by reestablishing the balance with PL biosynthesis. Evidence for this coordination of LPS and PL biosynthesis is a correlation between LpxC and FabZ required for type II fatty acid biosynthesis (5, 6). About 1% of single cells may form a colony on plates containing bactericidal concentrations of rifampicin (7), suggesting that a heterogeneity in OM permeability is present already before exposure to the antibiotic. One may therefore speculate that LpxC and FabZ levels may vary during the cell cycle, thereby affecting the OM barrier and entry rate for antibiotics such as rifampicin.

However, only rifampicin and nitrofurantoin, out of the nine antibiotics tested, showed perseverance (1), suggesting that the mechanisms of action for these two antibiotics mattered. An important finding was that perseverance was associated with a significant reduction in the growth rate and production of thinner cells (1). Bacteria are exposed to environmental stresses during growth and therefore need to respond quickly to such responses to survive. Among such stress-signaling molecules are guanosine tetraphosphate (ppGpp) and guanosine pentaphosphate (pppGpp) that inhibit stable RNA synthesis upon amino acid starvation, a phenomenon known as the stringent response (8, 9). More recently, an inverse correlation between ppGpp levels and growth rate has been found under steady-state conditions (10). These phosphorylated nucleotides interact with a large number of proteins, including RNA polymerase, and DnaA, required for DNA initiation as well as proteins involved in phospholipid biosynthesis. Their biosynthetic enzymes are regulated both at transcriptional and post-transcriptional levels (9). To sort out whether or not bacteria under perseverance contain altered levels of alarmones such as ppGpp and pppGpp, cells could be isolated and analyzed using the microfluidic device used by Gerrit et al. (1).

Author affiliations: [a]Department of Microbiology, Tumor and Cell biology, Karolinska Institutet, SE-171 77, Stockholm, Sweden

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[1]To whom correspondence may be addressed. Email: staffan.normark@ki.se.

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It is possible that the mechanism leading to perseverance against nitrofurantoin is different from that of rifampicin. The mode of action of nitrofurantoin is unusual as the compound needs to be reduced by bacterial nitroreductases to nitroso compounds and then to reactive hydroxylamines that damage a variety of target molecules such as DNA. Resistance to nitrofurantoin occurs by inactivating mutations of NfsA and NsfB, being a major and a minor oxygen-insensitive nitro reductase, respectively. Resistance to nitrofurantoin can also occur by up-regulation of efflux pumps (11, 12). Also, in the case of nitrofurantoin, the subpopulation showing perseverance exhibited a reduced growth rate (1). However, this subpopulation appeared much larger than for rifampicin. Heterogeneities in transcription of NfsA/Nfs B and/or in efflux pumps are possibilities. Nitrofurantoin likely mediates bacterial DNA stress known to elicit various protective stress responses that might be differentially expressed in a treated population, depending on the stage of the cell cycle when hit by the drug.

Perseverance may be of relevance for the development of rifampicin resistance during treatment of tuberculosis. Using a novel steady-state antibiotic exposure system combined with chromosomal barcoding, small *Mycobacterium tuberculosis* subpopulations were found that entered a state of drug tolerance, that were predisposed to develop rifampicin resistance. These subpopulations apparently arose in the culture prior to drug exposure (13). It was suggested that these difficult-to-eradicate subpopulations may explain the need for extended treatment regimens for tuberculosis.

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It is not known whether, but is likely, that subpopulations showing perseverance and drug tolerance are generated by similar mechanisms. Identifying genes and pathways required for these adaptive processes could be possible by single-cell imaging recordings of mutant populations exposed to antibiotics using the microfluidic platform developed by Brandis et al. (1).

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