AMR or even MDR Bcc strains can occur between or among CF patients, potentially causing untreatable infections in previously naïve patients.

In summary, the recent study by Diaz Caballero *et al*. [1] used WGS to catalogue the adaptative mechanisms employed by *B. multivorans* to survive and persist in its human host for at least a decade. Their study adds to the growing body of literature aimed at unmasking parallel strategies employed by pathogens as they adapt to the CF airways. A consistent finding in these studies is that CF pathogens readily acquire AMR and MDR mechanisms that ensure survival against repeated antibiotic onslaught, which raises questions about the efficacy of current antibiotic treatment strategies. People with CF are critically dependent upon antibiotics to manage their disease, placing them at the greatest risk of acquiring and transmitting AMR and MDR pathogens. These collective findings highlight the need for improving the diagnosis and treatment of respiratory infections and associated airway decline in CF to enhance quality of life and lifespan in this at-risk cohort. The next steps require translation of WGS and RNA sequencing findings to enhance the diagnosis of emerging AMR, to alter current clinical practices to maximize antibiotic stewardship measures and to prevent or delay MDR infections, and to identify targeted alternative treatment strategies (e.g., bacteriophage therapies [10]) to help combat existing AMR and MDR infections.

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Special Issue: Antimicrobial Resistance and Novel Therapeutics

Spotlight

Efflux-Pump Upregulation: From Tolerance to High-level Antibiotic Resistance?

Jakob Frimodt-Møller[1,2,*] and Anders Løbner-Olesen[1,2,*]

A recent study shows that high expression of the efflux-pump AcrAB-TolC, which increases antibiotic tolerance, reduces DNA mismatch repair in *Escherichia coli* to promote spontaneous mutations. Because mutations in target genes can lead to high-level resistance, this highlights how transiently tolerant cells can develop resistance in response to antibiotic treatment.

Antimicrobial resistance is the new challenge of the 21st century [1]. Gram-negative bacteria can adapt to the selective pressure exerted by antimicrobial agents in numerous ways, including (over)expression of efflux pumps. Currently, six families of bacterial efflux pumps that contribute to antibiotic tolerance have been identified, including the resistance-nodulation-cell division (RND) superfamily [2], containing the efflux system AcrAB-TolC. Efflux pumps represent an ancient bacterial defense mechanism to meet and survive the challenge of remaining in environments with toxic compounds and are, for several species, also required for colonization and/or virulence. For instance, lack of AcrAB-TolC renders *Salmonella enterica* serovar Typhimurium nonvirulent in both a mouse infection model and a *Galleria mellonella* infection model [3]. AcrAB-TolC has a diverse set of substrates, including multiple conventional antibiotics, such as β-lactams, chloramphenicol, rifampicin, tetracyclines, and quinolones (for review see [2,4]). The *mar* regulon is the best described regulator of AcrAB-TolC [5]. Here, addition of substrate antibiotics leads to inactivation of the negative regulator MarR, resulting in constitutive expression of the transcriptional activator MarA [5], which, in turn, results in high expression of the AcrA and AcrB proteins and hence a high level of the AcrAB-TolC efflux pump (Figure 1A).

Accumulating evidence, especially from the *E. coli* AcrAB-TolC efflux system,

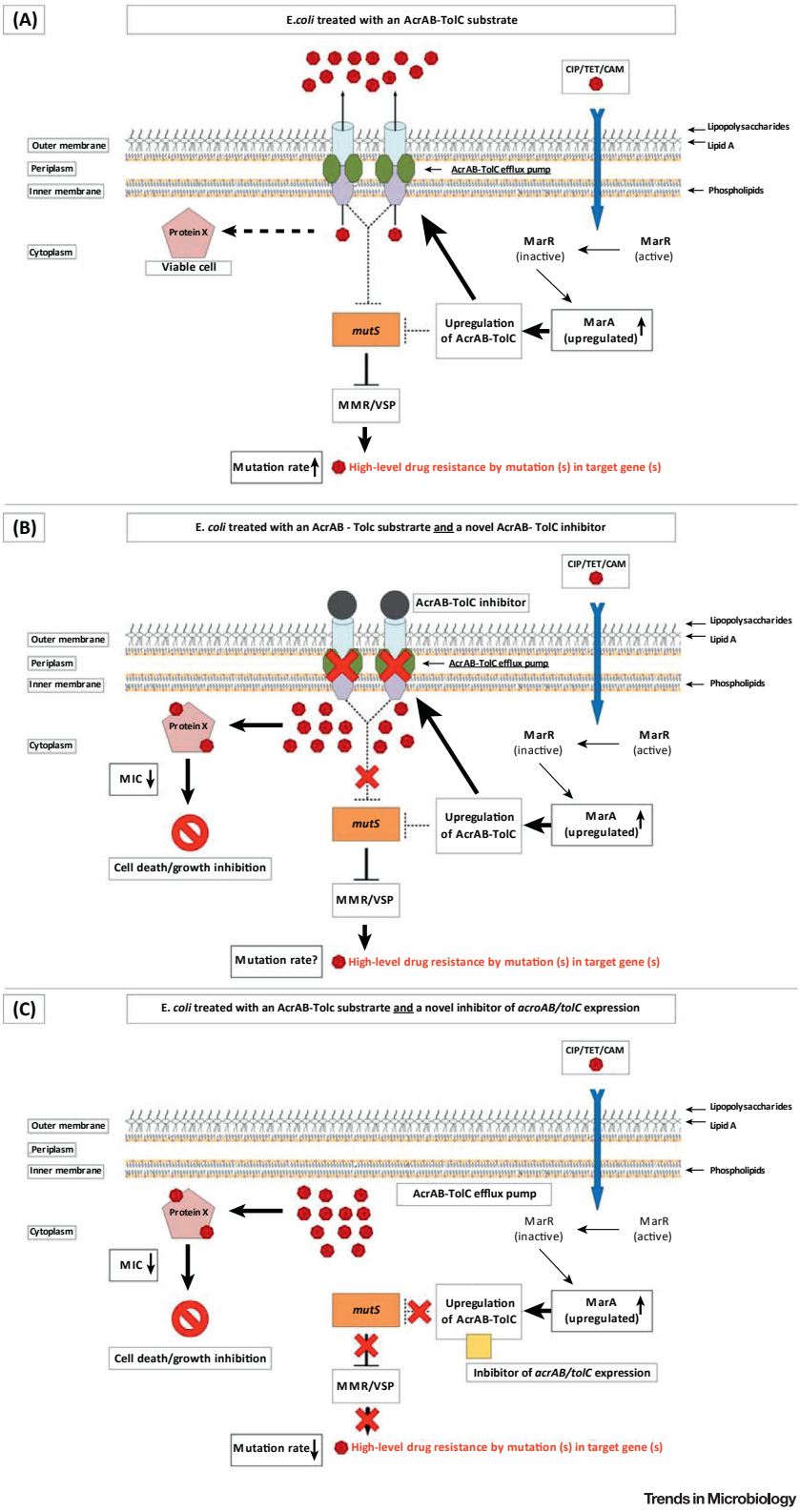

Figure 1. The Connection between Upregulated AcrAB-TolC, Downregulation of MutS, and Antibiotic Survival. (A) When *Escherichia coli* is treated with a low-level AcrAB-TolC substrate, such as ciprofloxacin (CIP), tetracycline (TET), or chloramphenicol (CAM) (dark red hexagon), MarR is inactivated, which increases expression of the *acrAB* activator, MarA. MarA, in turn, upregulates *acrA* and *acrB* genes, resulting in an increased level of the efflux pump AcrAB-TolC. AcrB is located in the inner membrane (purple hexagon) and is connected to TolC located in the outer membrane (light blue can) by AcrA (green hexagon). An elevated AcrAB-TolC level leads to downregulation of *mutS*, resulting in reduced functioning of the mismatch repair (MMR) and very short patch (VSP) repair systems producing a mutator phenotype, but it remains unknown whether *mutS* is regulated directly by AcrAB-TolC or coregulated with *acrAB*, or both (indicated by dashed lines). The deficiency in MMR/VSP repair may lead to high-level drug resistance by mutation(s) in target gene(s). (B) In the presence of a putative AcrAB-TolC inhibitor (dark gray circles), the antibiotic accumulates within the cell, resulting in increased cell death/growth inhibition by binding to target protein X, thus lowering the minimum inhibitory concentration (MIC). It is not known whether inhibition of the AcrAB-TolC efflux pump affects *mutS* expression and the ability to promote high-level antibiotic resistance through target-gene mutations. (C) A putative inhibitor of *acrAB* or *tolC* expression (yellow square) that reduces the levels of AcrAB-TolC efflux-pump structural components results in a reduced MIC and prevents downregulation of *mutS*. This lowers the risk of introducing mutation(s) in target gene(s).

suggests that efflux pumps have much broader functional roles, beyond transport of toxic compounds. Two recent publications report the involvement of efflux pumps in antibiotic survival by mechanisms that may be unrelated to efflux. First, a number of genes encoding efflux pump components, including *tolC*, *acrA*, and *acrB*, were reported to be more highly expressed in dormant *E. coli*, so-called persisters [6], thus implicating a connection between efflux systems and persister formation. Second, an increased level of the efflux system AcrAB-TolC of *E. coli* results in lower expression of the DNA mismatch repair gene *mutS* [7]. Both mismatch repair (MMR) and the very short patch (VSP) repair system (which removes T–G mismatches created by the deamination of 5-methylcytosine to thymine) [8] are defective without MutS. Thus, MutS-deficient cells have a mutator phenotype [8]. Furthermore, MutS-deficient cells have an increased recombination in interspecies crosses due to the ability of MutS to suppress RecA-mediated strand transfer [8]. This recent discovery by El Meouche and Dunlop highlights an elaborate route for *E. coli* to survive antibiotic treatment by initially pumping out the antibiotic through an increased level of the AcrAB-TolC efflux pump to ensure short-term survival, and at the same time increasing the overall mutation rate by limiting MMR, and presumably also VSP repair, through lowered *mutS* expression [7]. Therefore, low-level resistance to one or more antibiotics (shown for ciprofloxacin, tetracycline, and chloramphenicol [7]), may promote the subsequent acquisition of beneficial single-nucleotide mutations in antibiotic target genes, that is, creating an evolutionary opportunity for the increased AcrAB-TolC subpopulations to evolve high-level resistance/or persistence (Figure 1A). It is currently unknown whether *mutS* is regulated directly by AcrAB-TolC, or is coregulated with *acrAB*, or both (Figure 1A). If upregulation of RND efflux in *Pseudomonas aeruginosa* also results in MMR deficiency, this could in part explain the 'success' this opportunistic pathogen enjoys during genomic adaptation to the harsh environment in the lungs of cystic fibrosis patients. For *P. aeruginosa*, some of the most frequently mutated genes are negative regulators of RND efflux systems, with *mexZ* (negative regulator of MexXY-OprM) being the most frequent [9]. Interestingly, *mexZ*-deficient *P. aeruginosa* has recently been proposed to provide an evolutionary opportunity for mutant populations to evolve high-level resistance [10].

Putative inhibitors of the AcrAB-TolC efflux pump and/or its expression are highly interesting and could be used in a combinatorial treatment along with ciprofloxacin, tetracycline, or chloramphenicol. Such inhibitors could either sterically block efflux of the antibiotic (Figure 1B) or block expression of *acrAB* (or *tolC*) (Figure 1C). Both types of inhibitor should, in principle, result in a better clinical output by relieving the bacteria of an intrinsic antibiotic defense mechanism, that is, lowering the minimum inhibitory concentration for the antibiotic. However, as the mechanism behind low *mutS* expression in response to AcrAB-TolC upregulation remains unknown, it is not clear whether both groups of inhibitors restore mutation rate or whether this applies only to those lowering the AcrAB TolC level (Figure 1B, C). El Meouche and Dunlop [7] highlight the extraordinary ability of *E. coli* to adapt to and overcome antibiotic therapy. Thus, treatment of *E. coli*, and possibly other bacterial infections, may in the future include a combination of conventional antibiotics and novel AcrAB-TolC-inhibiting compounds. This could increase the chances of successful clearance and diminish the risk of high-level resistance development.

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