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Functional significance of leuS mutations in Escherichia coli resistance to ciprofloxacin

Linnéa Garoff*[1], Diarmaid Hughes[2]

[1]*Uppsala Univeristy; Department of Medical Biochemistry and Microbiology*

[2]*Uppsala University; Department of Medical Biochemistry and Microbiology; Biomedical Center, Box 583*

Background: When experimentally evolving *Escherichia coli* for increased resistance ciprofloxacin, we observed that mutations arose frequently in aminoacyl-tRNA synthetase genes. Resistance to ciprofloxacin requires multiple genetic alterations, usually affecting drug targets and efflux regulation. We sought to explain why mutations in tRNA synthetase genes would be selected for in resistance development to ciprofloxacin, and chose to study two independently selected mutations in *leuS*.

Material/methods: *E. coli* was experimentally evolved *in vitro* to increased resistance to ciprofloxacin, followed by whole genome sequencing. Methods of λ-red recombination and P1 phage transduction were used to create isogenic strains. PCR assays and local sequencing confirmed all constructs. Total RNA was isolated from the isogenic strains and subjected to transcriptome analysis. Susceptibility to ciprofloxacin was evaluated by minimal inhibitory concentration (MIC) measurements using antibiotic strips, and by competition assays with fluorescently labelled cells measured in a magnetic associated cell sorter (MACS).

Results: Repairing the *leuS* mutations in evolved strains significantly reduced the resistance to ciprofloxacin, while introducing the *leuS* mutations into strains carrying only target and efflux mutations increased the MIC 4-fold. Competitive fitness as a function of ciprofloxacin concentration also increased markedly with the *leuS* mutations. The increase in drug resistance was entirely dependent on RelA, supporting the hypothesis that the mechanism of reduced susceptibility was via induction of the stringent response. RNA sequencing of strains carrying *leuS* mutations revealed global changes in transcription, which overlapped with RelA-dependent activation of transcription associated with the

stringent response to amino acid starvation. In addition, increased expression of genes encoding efflux pump components were found: the MATE-efflux pump MdtK, the AcrAB-TolC associated protein AcrZ and a putative efflux pump YdhJK. Deletion of *mdtK* revealed that this pump accounted for about 20% of the resistance associated with the *leuS* mutations, whereas a double knockout of *mdtK* and *acrZ* almost entirely reduced the resistance associated with one of the *leuS* mutations. Further investigations are ongoing to elucidate the role of the putative YdhJK efflux pump.

Conclusions: In this study we identified *leuS* as a novel mutational target in ciprofloxacin resistance development in *E. coli*. The studied *leuS* mutations induced a RelA-dependent global change in transcriptional patterns resulting in a 4-fold increase in MIC for ciprofloxacin. Our working hypothesis is that these *leuS* mutations were selected because they caused increased expression of efflux pump components. If this explanation holds also the other aminoacyl-tRNA synthetase mutations observed in *in vitro* evolution, it suggests that there exists a novel and potentially large mutational target for *E. coli* resistance development to ciprofloxacin.