Increased tolerance to antimicrobial agents is thought to be an important feature of microbes growing in biofilms. We address the question of how biofilm organization affects antibiotic susceptibility. We established Escherichia coli biofilms with differential structural organization due to the presence of IncF plasmids expressing altered forms of the transfer pili in two different biofilm model systems. The mature biofilms were subsequently treated with two antibiotics with different molecular targets, the peptide antibiotic colistin and the fluoroquinolone ciprofloxacin. The dynamics of microbial killing were monitored by viable count determination, and confocal laser microscopy. Strains forming structurally organized biofilms show an increased bacterial survival when challenged with colistin, compared to strains forming unstructured biofilms. The increased survival is due to genetically regulated tolerant subpopulation formation and not caused by a general biofilm property. No significant difference in survival was detected when the strains were challenged with ciprofloxacin. Our data show that biofilm formation confers increased colistin tolerance to cells within the biofilm structure, but the protection is conditional being dependent on the structural organization of the biofilm, and the induction of specific tolerance mechanisms.