Skolkovo Institute of Science and Technology

MATURATION AND FUNCTIONAL ANALYSIS OF MICROCIN C-LIKE COMPOUNDS

Doctoral Thesis

by

IULIIA PISKUNOVA

DOCTORAL PROGRAM IN LIFE SCIENCES

Supervisor
Professor Konstantin Severinov

Moscow - 2017

Abstract

The antibiotics are gradually losing their ability to inhibit bacterial pathogens and drug resistance is spreading at an alarming degree. The early antibiotics are no longer that efficient, as bacteria have a set of strategies to escape the antibiotic killing. Taking into account that antibiotics are no longer developed as widely as before, increasing drug resistance of pathogens will inevitably lead to a point of crisis. The exaserabating menace of antibiotic resistance poses an urgent threat for humanity as well as a challenge for the development of new antibiotics.

Shortly after the introduction of penicillin into a widespread clinical practice, it was found to be uncapable to kill all cells in bacterial populations that were sensitive to antibiotic action. The efficency turned out to be low even when the concentrations of antibiotics were significantly increased. The reason of this problem was "persisters"- a small fraction of phenotypically different cells present in the population. However, this phenomenon was not studied until the very end of the 20th century, when the crucial role of persister cells in antibiotic resistance of biofilms was discovered. It became clear that the function of persisters is strongly connected with chronic and recurrent bacterial infections. Persistence is a risk-reducing strategy as it stops some part of population from proliferation and thus secures a fraction of cells providing a reservoir for future growth in case of adverse conditions. Persistence is a widely acknowledged mechanism allowing the bacterial population to protect itself from various stresses, including antibiotic treatment and nutrient-deficient conditions.

In case of stress, i.e. nutrient or oxygen depletion, the group of antimicrobial peptides called microcins is synthetized by ribosomal translation. Microcins have potent antibacterial activity against the closely related bacteria at minimum inhibitory concentrations in the nanomolar range. One of the microcins subclass is microcin C-like compounds, that are encoded by the gene clusters found in diverse gram-negative, gram-positive bacteria, and even cyanobacteria.

Microcin C-like compounds are natural peptide-nucleotide antibiotics that target aspartyl-tRNA synthetase leading to protein synthesis halt and subsequent cessation of cellular division. We show that sublethal concentration of microcin C induces the bacterial persistence with a mechanism similar to that of well-studied intracellular

proteinaceous toxin HipA. We demonstrate that the microcin C-producing cells induce persistence in sensitive cells during co-cultivation. In this work we reveal that the bacterial cell may exploit an additional mechanism to protect itself from being poisoned during microcin C synthesis. We make it evident that the maturation of microcin C from *Y. pseudotuberculosis* IP 32953 requires the leader peptide to be removed immediately prior its export from the producer cell in order to generate bioactive antibiotic. Our findings underscore complex interactions in bacterial communities where an antagonistic compound produced by one community member can benefit other members by increasing their ability to withstand various stresses.

Publications

The thesis-related results were presented at the following scientific conferences and published in international peer-reviewed journals.

List of publications:

1. Peptide-nucleotide Antibiotic Microcin C Is a Potent Inducer of Stringent Response and Persistence in Both Sensitive and Producing Cells. Piskunova J, Maisonneuve E, Germain E, Gerdes K, Severinov K. Mol Microbiol. 2017 Feb 6. DOI: 10.1111/mmi.13640;
2. The Product of *Yersinia pseudotuberculosis mcc* Operon is a peptide-cytidine Antibiotic Activated Inside Producing Cells by the TldD/E protease. Tsibulskaya D, Mokina O, Kulikovsky A, Piskunova J, Severinov K, Serebryakova M, Dubiley S. J.Am.Chem.Soc. 2017 Oct 18. DOI: 10.1021/jacs.7b07118.

Conferences:

1. International conference «Molecular Control of Gene Expression»: Piskunova J., Zuher I. «Influence of expression E.coli mcC operon on hosts proteins' expression», 18-19 June 2015, Moscow;
2. International Conference for Students and Young Scientists "Lomonosov": Piskunova J. “The study of bacterial persistence induction by peptide antibiotic microcin C”, 11-15 April 2016, Moscow;
3. Interdisciplinary School and Conference “Information Technologies and Systems”: Piskunova J, Maisonneuve E, Gerdes K, Severinov K. “Induction of bacterial persistence by peptide antibiotic microcin C”, 25-30 September 2016, St.Petersburg;
4. The 5th Molecular Microbiology Meeting & 4th Midlands Molecular Microbiology Meeting: Mokina O, Tsibulskaya D, Kulikovsky A, Piskunova J, Severinov K, Serebryakova M, and Dubiley S. “New Trojan-Horse Peptide-Cytidylate Antibiotics Target Aspartyl-tRNA Synthetase”, 13-14 September 2017, Birmingham.

Acknowledgements

The author would like to express her thanks to Konstantin Severinov, Kenn Gerdes, Svetlana Dubiley, Darya Tsibulskaya, Etienne Maisonneuve, Elsa Germain, Marina Serebryakova, Anastasia Sharapkova and Sergei Borukhov.

Table of contents

Abbreviations

ATP - adenosine triphosphate

ADP - adenosine diphospate

cAMP - cyclic adenosine monophosphate

Cfx - ciprofloxacin

CFUs - colony forming units

CRP – cAMP receptor protein

GluRS - glutamyl-tRNA synthetase

cxSAM – carboxy-SAM

EDTA – ethylenediaminetetraacetic acid

HPLC - high-performance liquid chromatography

McC^{Eco} - microcin C from *E.coli*

McC^{Yps} - microcin C from *Yersinia pseudotuberculosis* IP 32953

MFS - major facilitator superfamily

MICs - minimum inhibitory concentrations

mRNA - messenger RNA

NMR spectroscopy – nuclear magnetic resonance spectroscopy

PCR - polymerase chain reaction

(p)ppGpp - guanosine (penta-) tetraphospate

RiPP - ribosomally synthesized and post-translationally modified peptides

RNA - ribonucleic acid

ROS - reactive oxygen species

RRE - RiPP precursor peptide recognition element

SAM - S-adenosyl methionine

TA system - toxin-antitoxin system

TCA cycle - tricaboxylic acid cycle

TFA - trifluoroacetic acid

tRNA - transfer RNA

Review of Literature

Microcins

Microcins are gene-encoded antimicrobial peptides synthetized by ribosomal translation, and most of them are post-translationally modified. Microcins are usually produced under conditions of stress, such as nutrient or oxygen depletion and are highly tolerant to heat, extreme pH and protease treatments. Most of them exhibit potent antibacterial activity against the closely related bacteria, with minimum inhibitory concentrations (MICs) within the nanomolar range. The microcin family includes a highly diverse group of antimicrobial peptides. At least 14 types of microcins are now known, but only some of them have been purified and characterized. Class I microcins are encoded by the genes carried on plasmids. The most mature class I microcins are charachetrised by molecular masses of less than 5 kDa. Microcin B, microcin C (McC) and microcin J25 belong to this class. Class II microcins have molecular masses from 5 to 10 kDa, and are divided into 2 subclasses. Most microcins in class IIa do not undergo post-translational modifications, but may have 1-2 disulfide bridges; genes of microcin IIa synthesis are also located on plasmids. Class IIa includes microcin L, microcin V and microcin V. The representatives of class IIb are located in chromosomal DNA including microcin E492, microcin M and microcin H47, linear peptides with C-terminal modifications; genes of IIb microcins.

The genetic organization of microcin clusters is very similar between classes (Figure 1). Microcins are usually synthesized as a peptide precursor that consists of the N-terminal leader (signal) part and C-terminal core part. It has been shown that in some cases the leader part of the peptide is required for the introduction of post-translational modifications and the formation of a mature microcin [1, 2]. In most cases the N-terminal part of the precursor molecule is not present in the mature microcin molecule, being removed during the maturation process or during the export of the molecule from the cell. ABC-transporters are usually responsible for exporting the microcins. These protein transport systems use ATP energy and are typically encoded in microcin clusters [3]. Genes from the microcin cluster are located on one or two operons. Some specific genes present in every microcinogenic cluster include: a structural gene encoding the peptide precursor molecule, genes encoding proteins related to self-immunity/resistance to

microcin produced, and genes encoding the microcin export system. Additionally, the microcin clusters have genes that encode enzymes of post-translational modifications of precursor peptide (Figure 1).

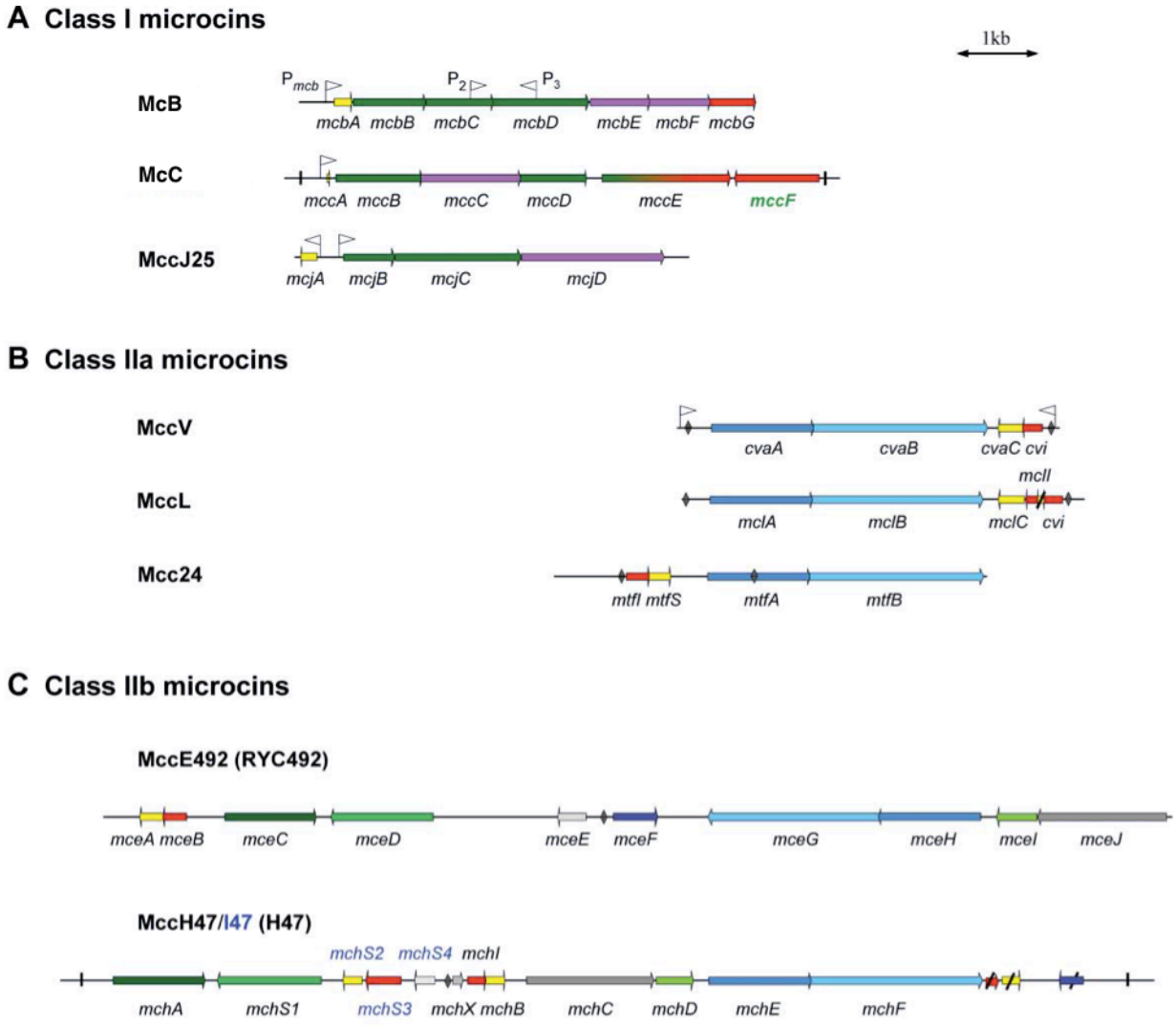


Fig.1. Schemes of genetic organization of microcin gene clusters. Genes are marked with arrows, the direction of which indicates the gene transcription. Genes encoding the precursor peptides are coloured with yellow. Genes required for self-immunity, microcin export and post-translational modifications are shown in red, blue, and green colours respectively. Genes required for both immunity and export are shown in purple. Genes encoding proteins of unknown function are indicated in grey. Promoters, where they are known, are indicated by flags. Class I, IIa and IIb microcins are shown in (A), (B) and (C), respectively [4].

Microcin C

Genetic organization of microcin C cluster

There are 6 genes that are responsible for the synthesis of *E. coli* microcin C [5]. All genes are located on a conjugation plasmid [6, 7]. They are organized in a cluster and five of them are transcribed from one common promoter [8] (Figure 7). The *mccA* gene is the smallest peptide-encoding gene known, with the length of 21 bp; it encodes a ribosomally synthetized McC precursor peptide MRTGNAN [9]. The genes *mccB*, *mccD*, and *mccE* encode proteins that are responsible for post-translational modifications. The product of the *mccC* gene is responsible for exporting a mature McC. MccE and MccF contribute to the self-immunity of microcin-producing cells [9]. Interestingly, the genes of the microcin C cluster are located between 44 bp direct repeats, which may serve as evidence for their horizontal gene transfer. The G+C DNA content of *mcc* genes cluster is different from the G+C content of *E. coli* chromosomal DNA, which may also point at horizontal gene transfer [10].

Structure and post-translational modifications of McC

Microcin C is a peptide-nucleotide antibiotic active against *Enterobacteria*. After the ribosomal synthesis of MccA (MRTGNAN) it is post-translationally modified by the action of MccB, MccE and MccD [11, 12], after that mature McC is formed (Figure 2).

The MccB protein is responsible for the first reaction of the post-translational modification of MccA. Using 2 molecules of ATP it adenylates MccA with the production of MRTGNAD-AMP [11]. MccE is a two-domain protein involved in post-translational modifications of MccA and in self-immunity to McC. The C-terminal domain of MccE is a GNAT family acetyltransferase. It acetylates the α-aminogroup of the processed McC using acetyl-CoA as a donor of the acetyl group. The MccE acetyltransferase protects cells not only against McC, but also against its synthetic analogs and various toxic aminoacyl adenylates [13, 14]. MccD and the N-terminal domain of MccE working together introduce an aminopropyl group during the microcin C maturation process [12]. Aminopropylation is a two-step process. Firstly, MccD transfers 3-amino-3-carboxypropyl from SAM (S-adenosyl methionine) to the oxygen from phosphoric acid of adenylated MccA. This process is not efficient and there is an additional protein, Mtn (5'-methylthioadenosine/S-adenosyl homocysteine nucleosidase),

which catalyzes the reaction. Secondly, the aminopropylation process starts, 3-amino-3-carboxypropyled peptide adenylate is decarboxylated by the N-terminal domain of MccE leading to the production of a mature McC [15]. The major function of MccC is to transport the McC molecules across the plasmatic membrane thus providing for self-immunity [10]. The protein MccC is similar to proteins from the MFS-superfamily. MFS is a wide-ranging family (from bacteria to human) of 12 trans-membrane domains transporters. The proteins of this family transport the substrates across different membranes and take part in various cell processes [17]. MccF is an unusual serine peptidase that ensures immunity of a McC-producing cells through cutting the carboxyamide (N-C) bond, that connencts the peptide and nucleotide parts of McC [18]. MccF recognizes full McC and synthetic analogs of McC with a modification of phosphoric acid to sulfuric acid [16, 18].

Regulation of microcin C biosynthesis

Genes that are responsible for McC biosynthesis are located in one gene cluster, *mccABCDEF*, and genes *mccABCDE* are transcribed from one common promoter, Pmcc, which is located prior to the *mccA* gene [10, 19, 20]. The *mccF* gene is transcribed from its own promoter [10]. The synthesis of McC is not activated during SOS response, but occurs at the stationary phase of cell growth. Besides RpoS (the stationary phase mediator) some global regulators of gene expression have impact on *mcc* transcription. The absence of glucose in media activates McC synthesis at the level of transcription [20, 21]. This occurs because the P*mcc* promoter has a binding site for CRP (cAMP receptor protein) or CAP (catabolite activator protein). CRP in conjunction with cAMP (the level of the latter is increased when the glucose concentration is low) activates transcription of the *mccABCDE* operon [19].

Another transcriptional regulator that influences McC synthesis is H-NS, a small histone-like protein. H-NS binds AT-rich DNA sequences, and inhibits *mccABCDE* operon transcription during the exponential growing phase [19, 20]. Another global *E. coli* transcriptional regulator, the Lrp protein (Leucine-responsive regulatory protein), also inhibits *mccABCDE* operon transcription [19].

To regulate the protein synthesis of *mccABCDE* operon, a special mechanism, based on formation of RNA secondary structures, is applied. It acts at both transcriptional and translational levels. Extensive non-coding region between *mccA* and *mccB* contains

an inverted repeat that functions as a Rho-independent transcription terminator [10]. Due to the function of this terminator, two transcripts of different lengths are synthesized from the Pmcc promotoer: a short terminated *mccA* transcript and the second full-length transcript of the entire operon [22]. The abundance of the short transcript is at least 20 times higher than that of the long one. Deletion of the terminator hairpin leads to the disappearance of the short transcript and triggers a 30-fold decrease in McC production [22]. This structure of the *mcc* operon, with a hairpin separating the precursor gene and genes that encode post-translational modification and self-immunity enzymes, appears to be an evolutionarily conserved feature of *mcc* operons found in diverse bacteria. For effective microcin synthesis the cells need to synthesize a large amount of the precursor peptide and have a relatively small amount of post-translational machinery. The short transcript has a much greater stability than the long one, it causes preferential the short transcript accumulation. The increased stability accounts for the ribosome binding to *mccA* transcript, which protects it from degradation [22].

Immunity of microcin C producer strains

MccC, MccE and MccF are jointly responsible for immunity of McC producer cells [10, 13]. Mature McC is exported from the cell by MccC. The C-end of MccE modifies the processed McC. The presence of *mccC* and *mccE* is sufficient for full cell immunity, but individually these genes provide only partial resistance (about 10 times less than cells with both genes) [5]. MccF makes a microcin C-producing cell immune to microcin C by a degradation connection between the peptide and nucleotide parts of McC [18].

Mechanism of microcin C action

Microcin C (McC) is a peptide-nucleotide antibiotic that acts by means of a Trojan-horse mechanism: it penetrates the cell under the guise of a nutrient substrate and is further cleaved by cytoplasmic proteases to produce a toxin that inhibits cell growth. McC penetrates from the environment across the inner membrane of *E. coli* into the periplasm via OmpC and OmpF porins [23]. McC is transported from the periplasm through the plasma membrane by the YejABEF-transporter. YejABEF is an ATP-dependent oligopeptide permease related to the ABC-transporter superfamily. Deletion of one of the *yejABEF* genes makes *E. coli* resistant to McC action [24]. In both sensitive

and McC-producing cells, aminopeptidases PepA, PepB and PepN then progressively degrade the McC peptide to produce toxic processed McC – non-hydrolysable aminoacyl-adenylate (Figure 2). Cells with deletions of all these peptidases are resistant to McC, because McC is not processed and no toxin is formed [16].

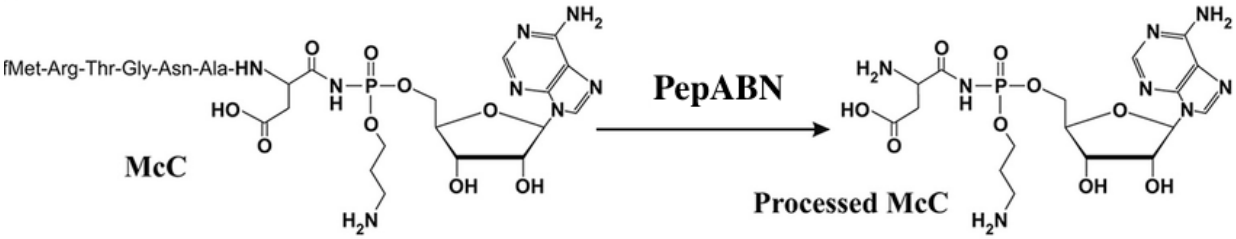


Fig. 2. The structure of mature McC and its processing product [13].

The processed McC is a translation inhibitor [25]. Processed McC is an aspartate whose backbone carboxyl is connected to AMP via a phosphoramidate bond. The molecule looks like the activated aspartyl-adenylate, an intermediate of reaction catalyzed by aspartyl-tRNA synthetase. Processed McC interacts with aspartyl-tRNA synthetase active center and prevents its normal function [26] (Figure 3). Microcin C acts in nanomolar concentrations [27]. It possesses bacteriostatic action against *E. coli* and phylogenetically close strains [25].

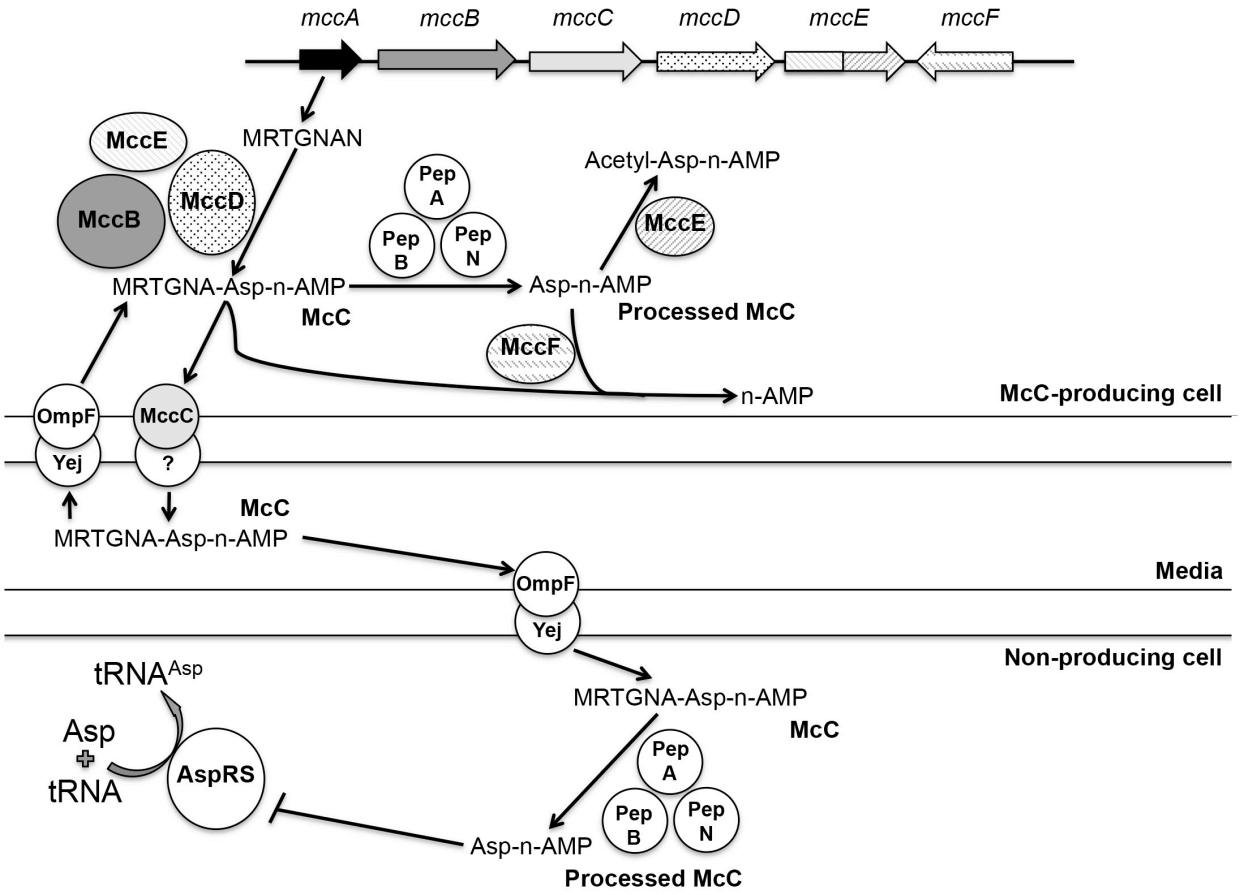


Fig. 3. Mechanism of microcin C synthesis, export, import, and action.

mcc-like gene clusters

The first hypotheses about the existence of potential homologues of mcc genes were put forward for the organism *Helicobacter pylori* [28, 29]. Now it was shown that there are numerous *mcc*-like operons in diverse gram-negative, gram-positive bacteria and even cyanobacteria [30]. *mccB* gene was used for bioinformatics search of these operons as the main and the major player in the McC maturation process. Most of the validated *mcc*-like gene clusters contain only three genes, *mccABC*, which are sufficient for producing a toxic adenylated peptide and exporting it outside the producing cells [31]. The *mccD, mccE*, and *mccF* gene homologs are present in only some of non-*E. coli mcc* clusters. On the other hand, some of them contain additional genes in comparison to the *E. coli* operon [31].

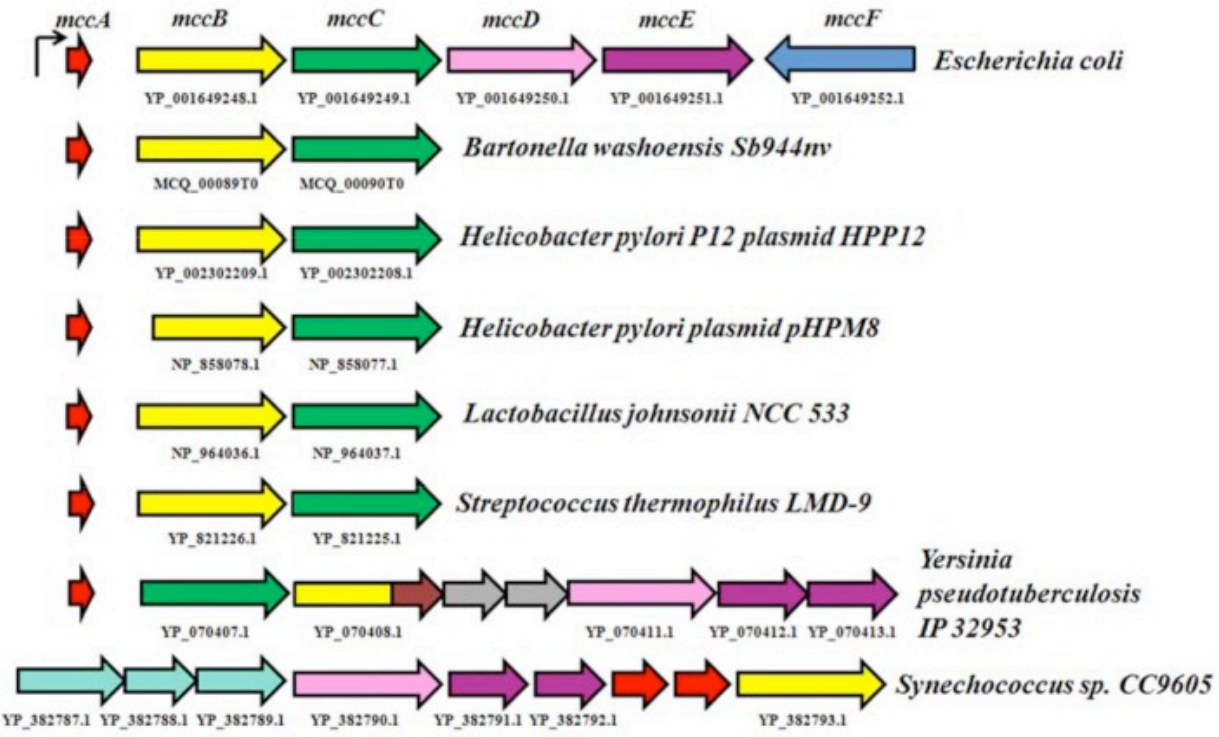


Fig. 4. *mcc*-like gene clusters encoded by *E. coli* and other bacteria. Arrows indicate individual genes: *mccA* - red; *mccB* - yellow; *mccC* - green; *mccD* - pink; *mccE* - purple; *mccF* – blue, genes of ABC-type transporters – cyan, methyltransferase domain of *mccB* – brown [31].

A group of *mcc*-like clusters from *Bacillus amyloliquefaciens* DSM7, *Streptococcus bovis* JB1, and various strains of *Yersinia sp.* and *Serratia sp.* encode MccB homologues extended with a C-terminal methyltransferase domain and also contain an additional gene *mccS*. The N-terminal part of *B. amyloliquefaciens* MccB was shown to perform cytidylation instead of adenylation of the terminal Asn residue of cognate MccA from *B. amyloliquefaciens* [32]. MccS from *B. amyloliquefaciens* is a carboxy-S-adenosyl-L-methionine synthetase. The carboxy-S-adenosyl-L-methionine is used by the C-terminal methyltransferase domain of *B. amyloliquefaciens* MccB as a donor of carboxymethyl group for additional modification of MccA from *B. amyloliquefaciens*. The McC from *B. amyloliquefaciens* inhibits aspartyl-tRNA synthetase as McC from *E. coli*, but the presence of carboxymethyl modification increases the toxicity and inactivates the self-immunity and resistance mechanisms mediated in *E. coli* by MccE [32].

mcc-like gene cluster from *Yersinia pseudotuberclosis* IP 32953

There is a predicted mcc-like gene cluster in *Yersinia pseudotuberculosis* IP 32953, it brings together characteristics of both well-characterised *E. coli* and *B. amyloliquefaciens* clusters (Figure 5).

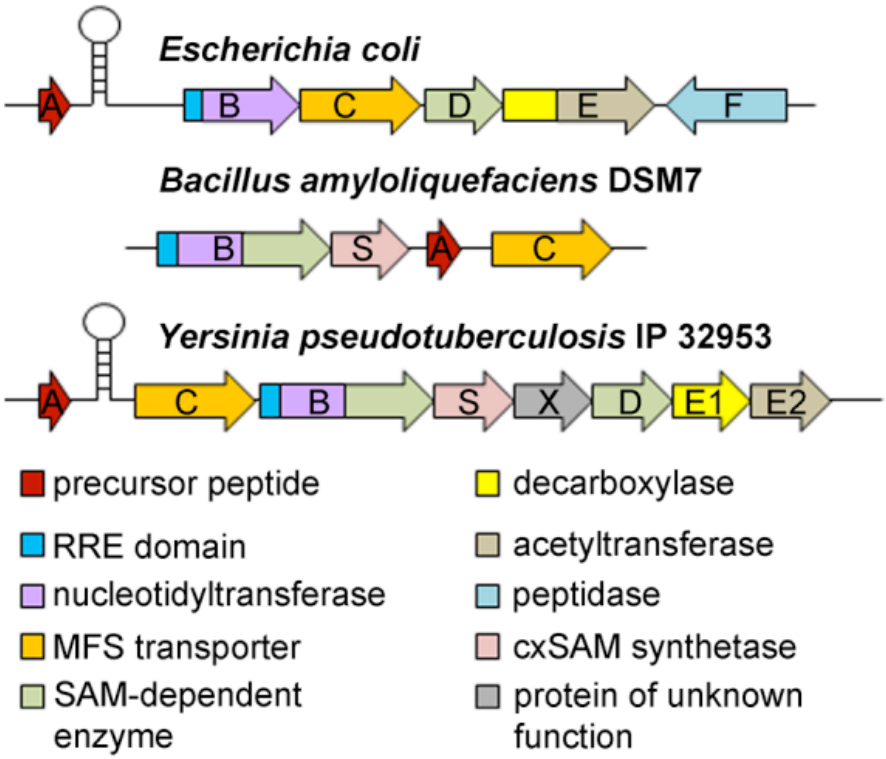


Fig. 5. Comparison of *mcc* gene clusters from *E. coli*, *B. amyloliquefaciens* DSM7, and *Y. pseudotuberculosis* IP 32953. Genes are indicated by arrows, positions of transcription terminators are schematically shown as hairpins. Known (*E. coli* and *B. amyloliquefaciens* DSM7) or predicted (*Y. pseudotuberculosis* IP 32953) *mcc* genes products functions are listed at the bottom.

Gene $mccA^{Yps}$ encodes 42 amino acid precursor molecule that is a 6 time longer peptide in comparison with 7 amino acid $MccA^{Eco}$. $MccA^{Yps}$, as most validated MccA peptides, contains a terminal asparagine. The $mccA^{Yps}$ gene is located upstream of $mccC^{Yps}$, which encodes a predicted export pump of the Major Facilitator Superfamily (YPTB1884). The next gene is $mccB^{Yps}$ (YPTB1885) encoding protein that adenylates $MccA^{Yps}$ *in vitro* [31]. Similar to $MccB^{Bam}$ and unlike $MccB^{Eco}$, $MccB^{Yps}$ contains additional C-terminal SAM-dependent methyltransferase domain. There is $mccS^{Yps}$ gene (YPTB1886) after $mccB^{Yps}$ gene; it is a homologue of $mccS^{Bam}$. The following gene, $mccX^{Yps}$, encodes a protein of unknown function (YPTB1887). $MccD^{Yps}$ (YPTB1888) is

homologous to MccD^{Eco}. *Y. pseudotuberculosis* MccE homolog is split into two separate polypeptides encoded by the adjacent genes. MccE1^{Yps} (YPTB1889), the homolog of MccE-NTD^{Eco}, most probably is a decarboxylase that removes the carboxyl from the carboxylated propylamine transferred by MccD [15]. MccE2^{Yps} (YPTB1890) is a GNAT-type acetyltransferase expected to be involved in self-immunity.

It was previously shown that the microcin encoded by *Y. pseudotuberculosis mcc* operon (McC^{Yps}) is an interesting case of a hybrid molecule: it contains both the terminal carboxymethylated cytosine as previously observed in *B. amyloliquefaciens* microcin C-like compound and propylamine at the phosphate group, one of the features of *E. coli* McC. Uniquely for peptide-nucleotide antibiotics, McC^{Yps} undergoes an additional modification in the production host that is strictly required for biological activity. The 42-aminoacid long MccA^{Yps} peptide part is proteolytically processed inside the producing cell releasing mature McC^{Yps} whose peptide part is only 11 amino acids long.

Upon processing, the compound leaves the producing cell, presumably due to the action of the MccC^{Yps} export pump, and is actively taken up by the YejABEF transporter in sensitive cells as characteristic for *E. coli* McC. The peptide parts of both McC^{Yps} and McC^{Eco} seem to be processed inside sensitive cells by aminopeptidases A, B, and N, with release of toxic warheads that inhibit aspartyl-tRNA synthetase. Aminopropylated aspartamidyl-carboxymethylcytidylate that is produced after processing of McC^{Yps} appears to be a less potent inhibitor of aspartyl-tRNA synthetase than aminopropylated aspartamidyl-adenylate released after processing of McC^{Eco}. McC^{Yps} is active against both *E. coli* and *Y. pseudotuberculosis*.

Irrespective of the antimicrobial activity of McC^{Eco} and McC^{Yps} with the MICs in the nanomolar range, there are still certain problems challenging the application of the majorioty of widely used antibiotics. One of them is the resistance to antibiotic action.

General mechanisms of antibiotic resistance

We are living in a "post-antibiotic era", when we are exposed to a global "antibiotic resistance crisis" [33-36]. The bacterial infections, especially nosocomial infections, continue to spread and account for a significant share of worldwide mortality and morbidity [37, 38]. Antibiotics usually influence essential parts of the bacteria physiology, leading to bacterial cell death or the growth interruption. There are five major antibiotic targets: DNA and RNA synthesis, protein synthesis, the bacterial cell wall, the cell membrane and folic acid metabolism. The antibiotic targets are either absent or are reasonably different for eukaryotic cells, so that antibiotics have little or no effect against eukaryotic cells. For example, the bacterial ribosome that is the target of aminoglycoside and macrolide has structural differences from the eukaryotic ribosome, due to which antibacterial drugs cannot influence eukaryotic cells. Resistance to antibiotics is now being extensively studied. It occurs through general mechanisms: increasing efflux or decreasing influx of antibiotics, modifying the cell or increasing the amount of the target, repairing of the damaged target, protecting the target, inactivating of antibiotics by enzymes, acquiring alternative metabolic pathways, and forming the persister state or biofilm [39-43] (Figure 6). The most specific pathway of antibiotic resistance is the development of special enzymes for recognizing antibiotics and modifying them, making them unable to interact with their targets: for example, β-lactamases split up the β-lactam ring that is essential to antibiotic action. The example of bacterial resistance through altering a metabolic process is the expansion of the *vanA* gene, which renders bacteria resistant to both vancomycin and teicoplanin via alteration of the peptidoglycan synthesis pathway; it is strongly related to chronic infection by vancomycin-resistant *Enterococci* and vancomycin-resistant *Staphylococcus aureus* [44, 45]. The mechanisms of resistance are usually spread by horizontal gene transfer and phage-mediated transfer from bacteria that had originally developed resistance by means of spontaneous mutations or adaptive mutagenesis [46-48].

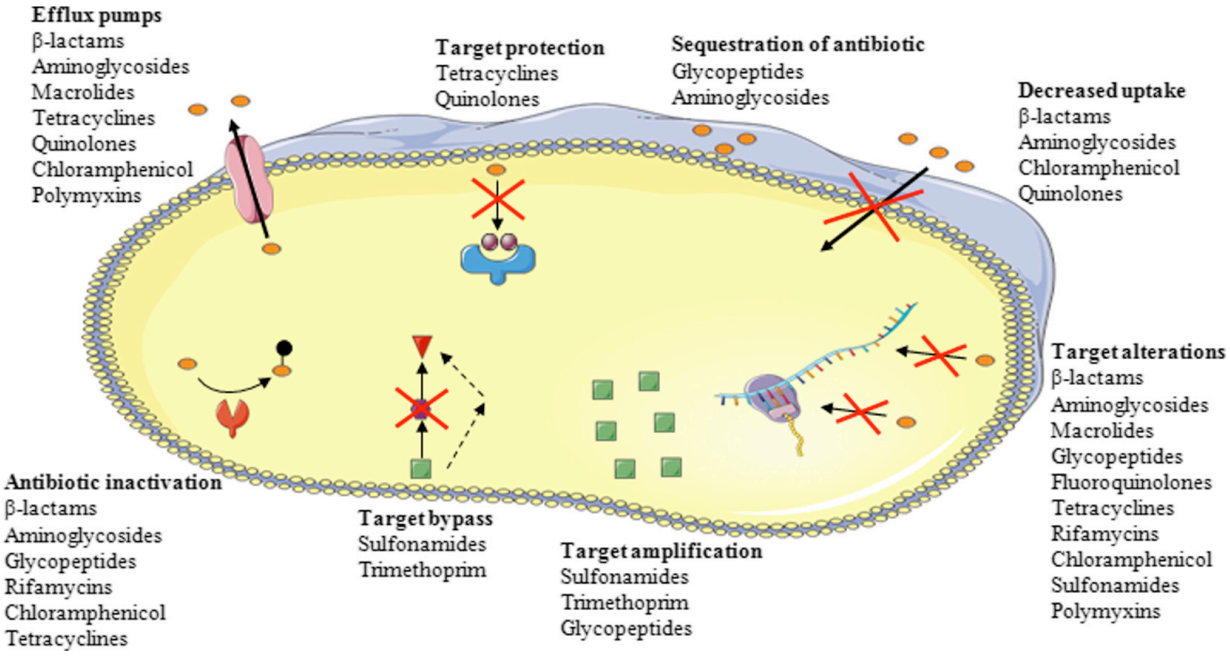


Fig. 6. Major mechanisms of bacterial resistance to different classes of antibiotics [40].

Bacterial persistence

The major role of persistent cells in antibiotic resistance is a recent discovery and it goes a long way towards explaining chronic bacterial infections [49-51]. Persistence is associated with a population's phenotypic heterogeneity [52]. Populations of isogenic bacteria always include a small portion of antibiotic-tolerant phenotypic variants, called "persistent cells", that can restore the initial population [53, 54]. 1944 saw Bigger to show for the first time that an actively growing bacterial population of *S. aureus* includes dormant non-dividing cells. Bigger's paper revealed that the number of persistent cells is increased after cold shock action, removing nutrients from media or boric acid treatment with bacteriostatic concentrations, these actions inhibit bacteria cell growth [55]. Since most antibiotics target the cellular processes being active, they have no effect on dormant cells such as persisters. The experimental evidence of bacterial persistence is a biphasic killing curve experiment (Figure 7), the data for curve are obtained when bacterial culture is treated by a lethal concentration of a bactericidal antibiotic and the number of surviving cells is measured in time-points during the experiment. During the first step of the experiment, the major part of bacterial culture is eliminated, and the number of surviving cells decreases speedily. During the second step, the number of surviving cells does not change significantly, because most remaining cells are highly antibiotic-tolerant persister cells.

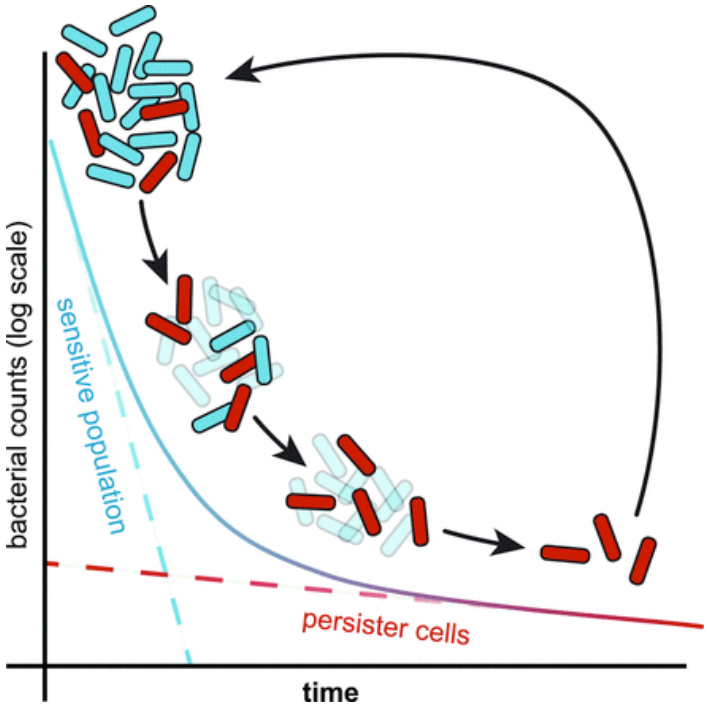


Fig. 7. Biphasic killing kinetics of bactericidal antibiotic treatment [56].

Surviving persister cells are genetically indiscernible from the rest of the bacterial culture. Their re-growth after removal of antibiotic provides a new bacterial culture that is antibiotic-sensitive and has the same level of persister cells as the original culture. The phenomenon of persistence has been observed in every microbial species tested to date, for example: *Pseudomonas aeruginosa, S. aureus* and *Mycobacterium tuberculosis* [57-59].

Persistence is different from resistance: persistent cells are unable to divide during the antibiotic treatment; their antibiotic-tolerant phenotype is not heritable. When the antibiotic treatment is stopped, persister cells lose their antibiotic tolerance and switch to a dividision stage. There are many different mechanisms of antibiotic resistance, but most of them are based on excluding antibiotic-target interaction. By contrast, the antibiotic tolerance of persister cells is especially based on their cellular physiology, which lends protection against antibiotic action [60, 61]. Single-cell observations of persistent cells showed that they are non-growing, which is to say that they are in a dormant state [54, 61]. Analysis of the transcriptome of persisters showed down-regulation of genes that are involved in energy production or some active cellular processes such as flagella synthesis, suggesting that persisters have reduced metabolic activity [57, 58, 62, 63]. Growth inhibition appears to stimulate increased levels of persistence in a population. Some toxin-antitoxin systems were shown to be related to the formation of persistent cells: translation-inhibiting toxins cause cell dormancy and persistence during the inhibition of antitoxin expression [64-66]. The same effect was demonstrated when cells were exposed to rifampicin (transcription inhibitor), tetracycline (translation inhibitor) or carbonyl cyanide m-chlorophenyl hydrazine (ATP synthesis blocking agent) [67]. Persistent cells are resistant to multiple antibiotics and even prophage induction [60, 68-70]. Recent studies focus on heterogeneity of persistent cells in a population: that cells have some differences among themselves that give a varying tolerance to different antibiotics [71-73].

A new hypothesis has recently been elaborated that persistence is due not only to dormant cells [67, 74-77]. The scientific debates are focused on the cases when a population's survival is provided by effective efflux pumps or the catalase-peroxidase enzyme KatG that is activated by an antibiotic. [74, 77]. There are active and passive

mechanisms of persisters formation. The high level of activity of some cellular processes might lead to depletion of energy, to arrest of cell growth, and persister cell formation. So persisters have two strategies at their disposal to survive during antibiotic treatment: passive through formation of persister cells and active through reduction of antibiotics concentrations in cells by intensive efflux work [78].

Factors responsible for spontaneous and induced persistence

The first important study of persistence induction in *E. coli* cells demonstrated that non-growing persister cells form spontaneously before antibiotic exposure and even without any environmental stress [54]. It was therefore concluded that persisters constitute drug-independent and pre-existing variants in a population, so they represent a strategy that provides a competitive advantage during unanticipated changes in the environment [79, 80]. All processes in bacteria cells are naturally noisy and individual cells of a population have fluctuations in macromolecule concentrations [81]. This stochastic noise may generate phenotypic heterogeneity in molecular processes [82]. HipA, one of the well-studied persistence-related protein, induces the persister cell formation as soon as its concentration in cell surpasses a certain threshold level [61, 64, 83-85]. In bacterial population, the concentration levels of HipA fluctuate near the threshold resulting in combined occurrence of growing and persister cells. HipA is a toxin encoded in a toxin-antitoxin (TA) locus. Another toxin, TacT from *Salmonella,* acetylates charged tRNA molecules and therefore inhibits translation and induces persister cell formation [86]. The TA loci have stochastic expression and play a role in induction of bacterial persistence [62, 83]. Resumption of active growth by a population from surviving persisters is also known to occur stochastically [87, 88].

Environmental factors

Spontaneous appearance of persister cells in un-stressed cultures can not explain all cases of persister cells formation. It was shown that persister level depends on several environmental factors. Formation of persister cells is strongly dependent on the phase of growing: the level of persister cells in population is low in the lag- and early-exponential phase, but is increased dramatically from the mid-exponential to stationary phase [58]. It is probably related to nutrient availability. Nutrient starvation has been identified as an important trigger of persister formation [58, 72, 75, 89-92]. Other environmental stimuli,

apart from starvation, can also boost persister levels. Such stimuli include: heat stress, hyperosmotic stress, acid stress, and oxidative stress [93-96]. Treatment by sub-inhibitory levels of antibiotics also increases persistence [67, 97, 98].

Furthermore, chemical signaling is related to the level of persistence. Some quorum sensing molecules increase the level of persisters in *P. aeruginosa, Streptococcus spp.,* and *A. baumannii* [99-101]. For example, indole, the stationary phase signaling molecule, is also involved in persister cells formation [102]. Thus, persistence is like a social signaling event: it can arise from the expression of some cooperative molecule, promote the utilization of antibiotic-degrading enzymes, and can be modeled as a bacterial social phenomenon [102-104].

Another environmental factor that stimulates the dormant state is the formation of biofilms. Bacterial biofilms are surface-associated multicellular communities that develop into a thick extracellular matrix and are prevalent in the environment as well as during infections [105, 106]. The physical structure of biofilms protects the bacterial community from aggressive conditions, including immune responses. Many antibiotics do not penetrate into biofilms [106, 107]. Biofilms usually have higher rates of persister cells than planktonic cultures (100- to 1000-fold more) [106-108]. These high rates of persister formation depend on (p)ppGpp as well as on other signaling molecules and processes, including diverse stress responses such as SOS induction and hypoxia [62, 75, 107, 109]. The insensitivity of biofilms to antibiotic treatment is at least partially explained by large numbers of persisters in the biofilm structure [106-109].

There are also some conditions inducing the decrease of persister levels, so the environmental influence on persistence level is bidirectional. For example, alkaline conditions, hypo-ionic environments and the addition of amino acids or glycolytic nutrients reduce the level of persister cells in the presence of aminoglycoside antibiotics [71, 110-112]. Switching from persisters to actively growing cells is increased under environmental conditions that are convenient for active bacterial growth, for example: addition nutrients to the media [113, 114].

Drug efflux pumps

A recent study has shown that there are higher levels of drug efflux pumps in *E. coli* persister cells than in the rest part of the population [78]. This leads to an increased tolerance to antibiotics (β-lactams and quinolones) by reducing their intracellular concentrations [78]. The role of efflux pumps as elements of bacterial tolerance to antibiotics was also shown for *Mycobacteria* upon infection of host cells and for the system of indole-stimulated persistence induction in *E. coli* [78, 115].

Stress responses

Many genes have been identified as related to persistence [57-63, 116-124]. Most of them are related to stress responses and TA modules, but the exact molecular mechanisms have been identified only for a few cases. Up-regulation of many stress response genes, that involved in SOS or phage-shock response, oxidative stress response, heat- or cold-shock response, stringent responce) was found by transcriptome analysis of samples that were enriched in persister cells [57, 102]. *E. coli* mutants lacking genes related to stress response demonstrated dramatic reduction in persistence level [125]. There is evidence for the relation between cell energy processes and formation of persister cells. Mutations in genes that code protein related to the electron transport chain, the tri-carboxylic acid (TCA) cycle and even glycerol-3-phosphate dehydrogenase increase the level of persistence [63, 120, 124-126]. It has been recently shown that the survival of persister cells in biofilms of *P. aeruginosa* is dependent on ROS (reactive oxygen species) detoxification enzymes controlled by the (p)ppGpp signaling, and the same mechanism may exist in *E. coli* populations [75]. Therefore, these results provide the ground for the important role of stress response in induction of persister cells formation.

(p)ppGpp

Guanosine tetraphosphate (ppGpp) and guanosine pentaphosphate (pppGpp) act as the second messengers or “alarmones” in the stringent response. (p)ppGpp is produced in response to stress signals, it changes metabolic homeostasis for purposes of cell survival [62]. In *E. coli*, (p)ppGpp synthetase RelA is activated by amino acid starvation and heat shock, whereas carbon, nitrogen, phosphate, iron or fatty acid starvations activate the (p)ppGpp synthetase activity of the bi-functional synthetase-hydrolase enzyme SpoT. In

various organisms, mutants that are unable to produce (p)ppGpp show reduced number of persister cells in cultures. For example, *relA-spoT* mutants of *E. coli* and *P. aeruginosa* are defective in persister formation during all steps of growing: exponential growth, stationary phase, and biofilm formation [62, 75, 89, 127]. Bacterial persister cells formation can be induced by stochastic activation of (p)ppGpp signaling pathway in some cells during exponential growing phase and by some environmental stress factors [62].

The ppGpp-mediated stringent response requires DksA, an RNA polymerase binding protein that inhibits transcription of ribosomal RNA genes [128]. DksA plays a role in many cellular processes: cell division, stringent response, quorum sensing in *E. coli*, *P. aeruginosa*, *Salmonella* and *Shigella flexneri* [129-133]. DksA acts as a co-regulator of ppGpp-dependent gene regulation in *E. coli,* and the effect of *dksA* deletion on induction of *rpoS*, the gene that encodes RNA-polymerase sigma factor S (σ^S), is similar to that caused by the absence of ppGpp [129, 134]. Furthermore, DksA was shown to enhance the effect of ppGpp on some negatively and positively regulated genes [135, 136].

Toxin-antitoxin modules

Toxin-antitoxin loci contain two genes, one of which encodes a metabolically stable toxin and the other - an metabolically unstable antitoxin. Bacterial toxin-antitoxin (TA) modules are genetic elements that encode a toxin protein and an antitoxin. Usually toxins inhibit some of essential cellular processes and, therefore, stop bacterial growth; antitoxins prevent or interfere to the functionality of the toxin, antitoxin activity is related to cellular signaling [88, 137]. TA loci were first discovered in bacterial plasmids, because TA loci influence on plasmid maintenance. Toxin and antitoxin have different stability in bacterial cell, therefore, if some cells lose the plasmid, the concentration of antitoxin will rapidly decreased and toxin will inhibit growth of this cell. Then it was shown that TA loci are widespread in bacterial genomes [138]. Presently, all TA loci are split into 6 types based on TA genetic organization and mechanism of action [138] (Figure 8).

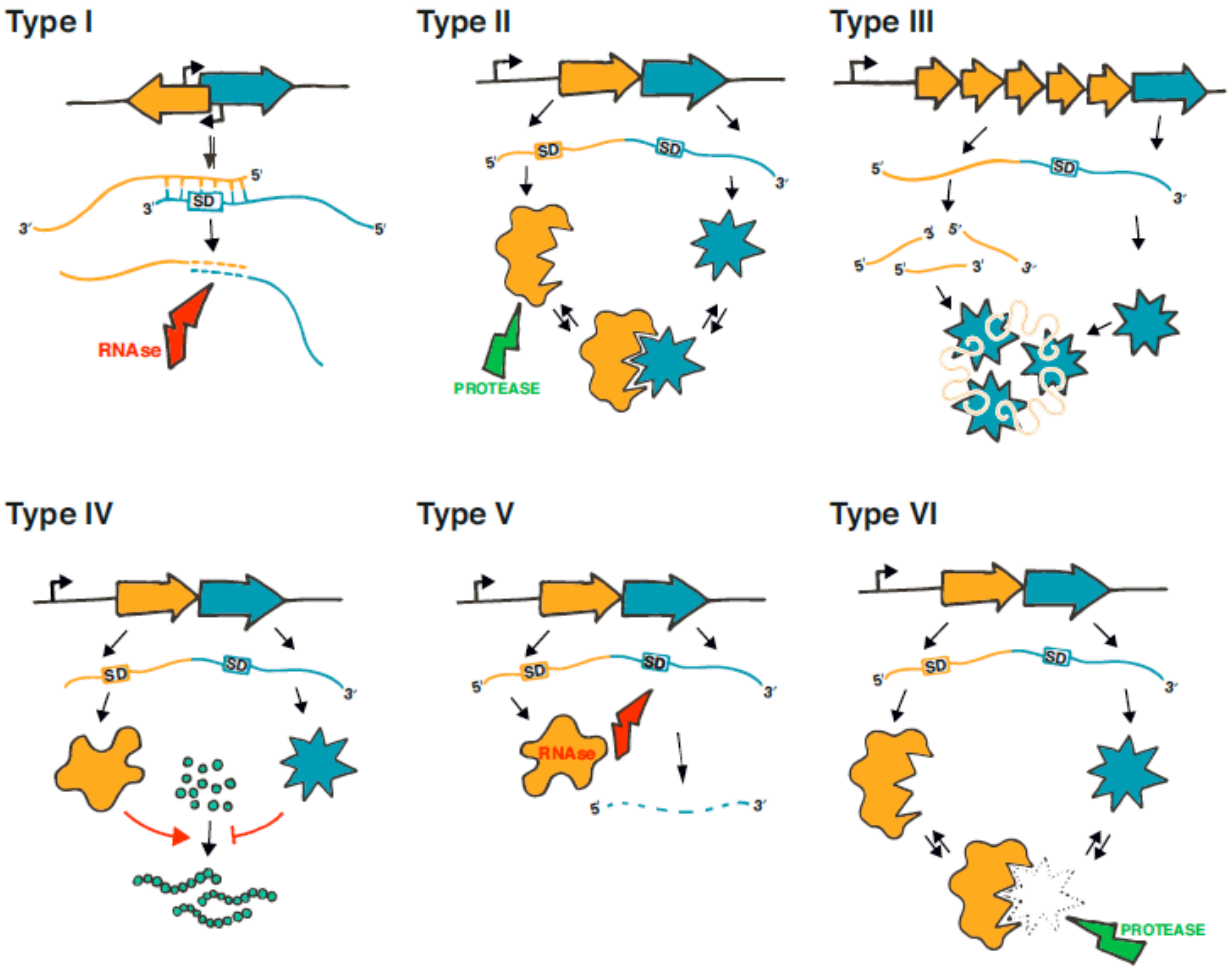


Fig. 8. Mode of action of the six classes of toxin–antitoxin systems. Type I: the toxin translation is prevented by the formation of an duplex between toxin mRNA and antisense RNA antitoxin, which may be degraded by RNAse. Type II: toxin and antitoxin are proteins, which form a complex that inhibits activity of toxin. Type III: a non-coding RNA antitoxin inhibits the function of the toxin by interaction with it. Type IV: antitoxin and toxin are proteins that do not interact directly, but is in competition for the same binding target. Type V: toxin mRNA is degraded by an RNAse antitoxin. Type VI: antitoxin molecules act as a proteolytic adapter, that promotes the degradation of the toxin protein. SD = Shine-Dalgarno ribosome binding site. Toxins are shown in blue, and antitoxins - in yellow [139].

TA modules, especially type I and type II, have been well studied as effectors in persister formation. Type I TA loci usually encode a protein-toxin and a non-coding RNA-antitoxin, that antitoxin interacts with the toxin mRNA and therefore inhibits its translation. Most of the toxins from type I TA loci are small hydrophobic peptides that associate with membranes, as a result, the membrane potential decreases and the cell growth stops. Overexpression of the type I toxins TisB and HokB causes increased persistence [137, 140]. The type II modules are the best studied. Type II antitoxins and toxins are proteins; usually antitoxin binds to toxin and inhibits it. Some of those toxins inhibit translation or replication; the majority of type II toxins have endoribonuclease activity, but a group of them inhibits translation by inactivating glutamyl-tRNA synthetase (GluRS), inactivating translation elongation factors or acetylating charged tRNAs [39, 137]. The first discovered persistence and TA-related gene, *hipA*, is a part of the *hipBA* type II TA operon that belongs to this group [110, 141].

Transcriptome studies showed up-regulation of multiple type II toxins genes in persister cells, especially those coding RNases such as RelE, MazF, DinJ, and MqsR [57, 142]. Moreover, the overexpression of type II toxins induces persister cell formation, but the expression of type II antitoxins reduces the level of persister cells in bacterial cultures, as was shown with *hipBA*, *relBE*, and *mazEF* TA systems [64, 65]. However, the absence of toxin gene in a single TA locus is insufficient to have a detectable difference in the level of persistence; by contrast, deletion of several type II mRNase toxins in *E. coli* causes a reduction in the level of persisters [90]. So, these systems are probably the functional analogs of each other and jointly contribute to persistence.

Persistence formation pathways

The first molecular pathway of persistence induction that has been described consists of the following steps: DNA damage caused by ciprofloxacin initiates the SOS response, activates RecA and enables LexA-mediated transcription inhibition of the *tisB/istR* type I TA module. In *E. coli*, the *tisB/istR*, *symE/symR* and *hokB/sokB* type I TA modules are activated by the SOS response and (p)ppGpp signaling [60, 127, 143]. TisB is a small hydrophobic peptide that interacts with membrane, breaking the proton motive force and therefore halting production of ATP [144]. Together, this blocks the action of most cellular enzymes, so that multidrug tolerance develops [140]. Endogenously expressed TisB contributes only fluoroquinolones resistance, whereas overexpression of TisB causes multidrug tolerance [60] (Figure 9).

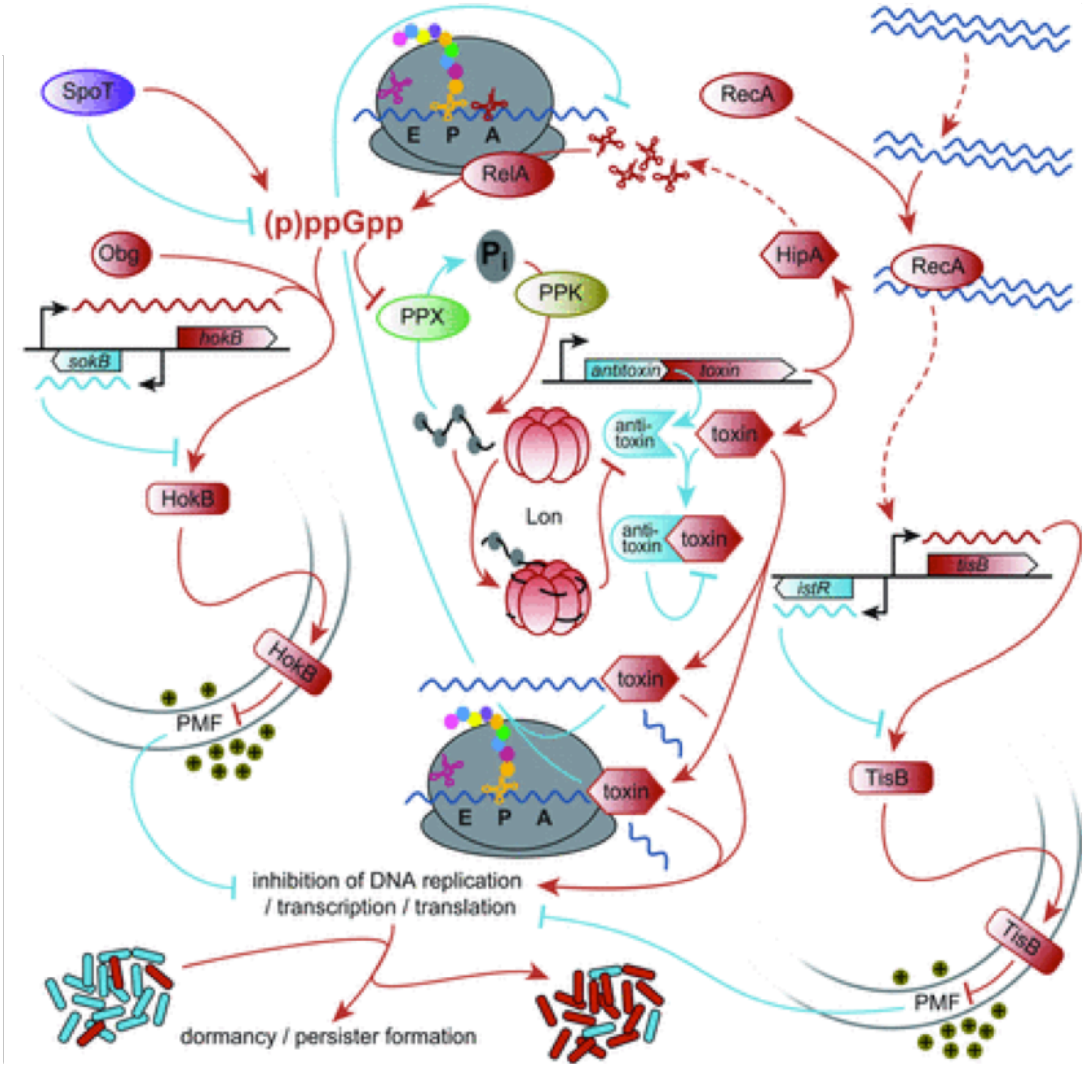


Fig. 9. Influence of toxin-antitoxin modules on persister cell formation in *E. coli* [56].

The stringent response is also related with the persister cells formation. It was demonstrated that induction of persistence in growing *E. coli* cultures is dependent on stochastic expression of type II toxins, and that it is related to high levels of (p)ppGpp [62]. It was found that there is a relation between the (p)ppGpp level and TA induction, and that the main roles in this interaction are played by inorganic polyphosphate accumulation, Lon protease activation, and degradation of type II antitoxins. The resulting increased level of type II toxins (mRNases) induces translation inhibition [62].

The HipA toxin belongs to type II, but unlike other members of this type is not an RNase. It has been shown that persistence induction for cells that have mutant HipA depends on the key elements of stringent response, and that HipA induces persistence induction by affecting the RNase toxins [145] (Figure 9). HipA phosphorylates a conserved serine residue near active site of glutamyl-tRNA synthetase and, therefore, HipA inactivates it [84, 85]. By doing so, it prevents glutamyl-tRNA aminoacylation and generates 'hungry' codons at the ribosomal A site, which is the major trigger for stringent response activation. These bacterial cells increase their (p)ppGpp-mediate persistence level due to activation of other type II toxins. (p)ppGpp is a competitive inhibitor of polyphosphate hydrolase PPX. Inhibition of PPX leads to increased production of polyphosphate by polyphosphate kinase PPK. The increased level of polyphosphate induces Lon protease that degrades type II antitoxins. The activation of 10 cellular RNases from *E. coli* genomic TA modules leads to inhibition of cellular translation and hence induces formation of persister cells [90]. Moreover, Lon also degrades the HipB antitoxin, so the HipA toxin inactivates GltX. This causes RelA-dependent (p)ppGpp synthesis to be activated, probably inducing a positive-feedback loop that leads to activation of the 10 mRNA endonucleases and HipA [56] (Figure 10). On the other hand, type II toxins prevent the entrance of uncharged tRNA into the ribosome by mRNA degradation, so there is control of the (p)ppGpp synthesis via a negative feedback loop [146]. Furthermore, (p)ppGpp itself is a component of a TA module that includes (p)ppGpp and SpoT [92].

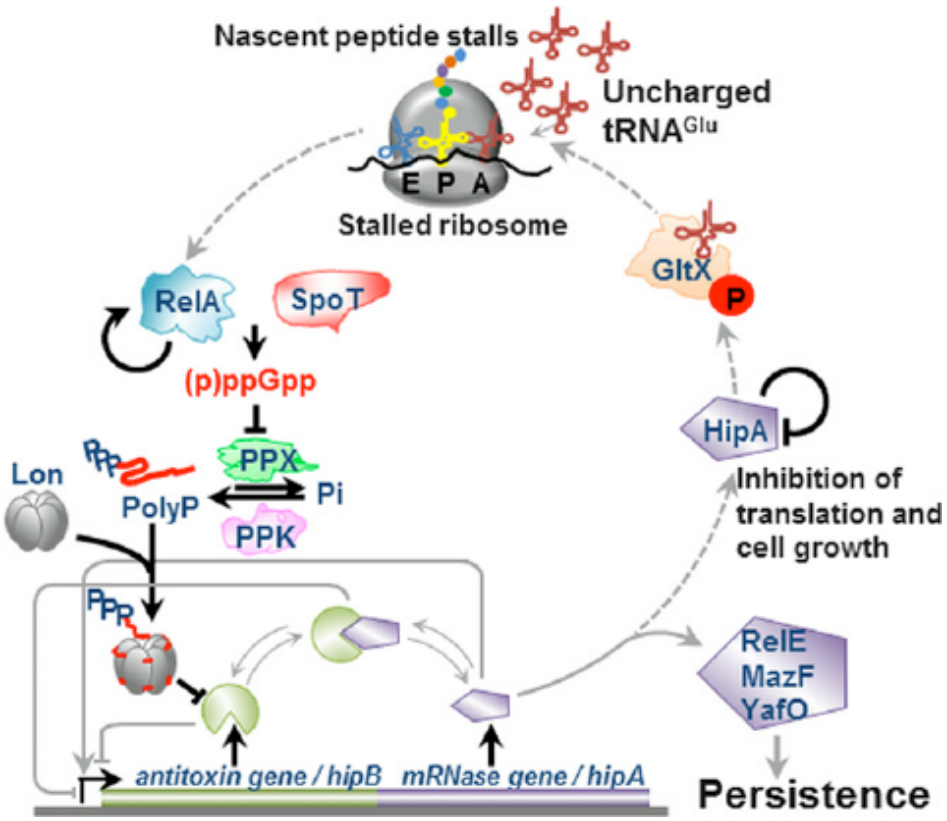


Fig. 10. Molecular model of HipA and toxin-antitoxin loci contribution into (p)ppGpp-mediated persistence induction in *E. coli* [84].

There is another mechanism leading to stringent response mediation of persistence induction in *E. coli* (without Lon and type II TA-modules). The Obg, conserved GTPase, induces persistence by transcription activation of the type I toxin gene *hokB* [127] (Figure 9). HokB is a toxic membrane protein that is a part of plasmid-associated *hok/sok* modules. It is involved in post-segregational killing of plasmid-free cells [147]. HokB induces depolarization of the membrane, formation of the dormancy state and therefore multidrug tolerance, as with the TisB-mediated persistence pathway [127]. (p)ppGpp may interact with Obg, but the mechanism of *hokB* transcriptional activation is not fully established [148].

Clinical implications

For many years the phenomenon of persistence and its clinical significance was overlooked. Yet, the problem of bacterial infection relapse was well recognized. For example, 10% of individuals who was infected with *M. tuberculosis* will have relapse infections [149]. Typhoid fever and urinary tract infection (UTI) caused by uro-pathogenic *E. coli* (UPEC) occur again in 15% of patients after antibiotic treatment [150-152]. In recent years, more evidence about the role of bacterial persisters in the relapse of infections has been accumulated. Monitoring SNPs in bacteria genomes in patients with invasive non-typhoidal salmonellosis showed that 78% of recurrent fever were caused by the same genovar as in the original infection, the same was shown for UPEC strains from patients with UTIs and *S. pyogenes* [153-155]. These facts indicate that bacteria inducing these infections were never fully exterminated during antibiotic treatment. Currently, the relapse is associated with bacterial ability to form persisters and to grow in biofilms. When bacteria live in biofilms, they become imbedded in a extracellular protective polymeric substance. While immune system usually efficiently eradicates non-growing bacteria cells in biofilms are not accessible. As shown in Figure 11, relapsing biofilm-related infections are largely caused by the antibiotic recalcitrance of persisters residing in biofilms [108, 142]: they survive both the antibiotic treatments and the action of immune system and become the founding population that re-establishes infection [107, 114].

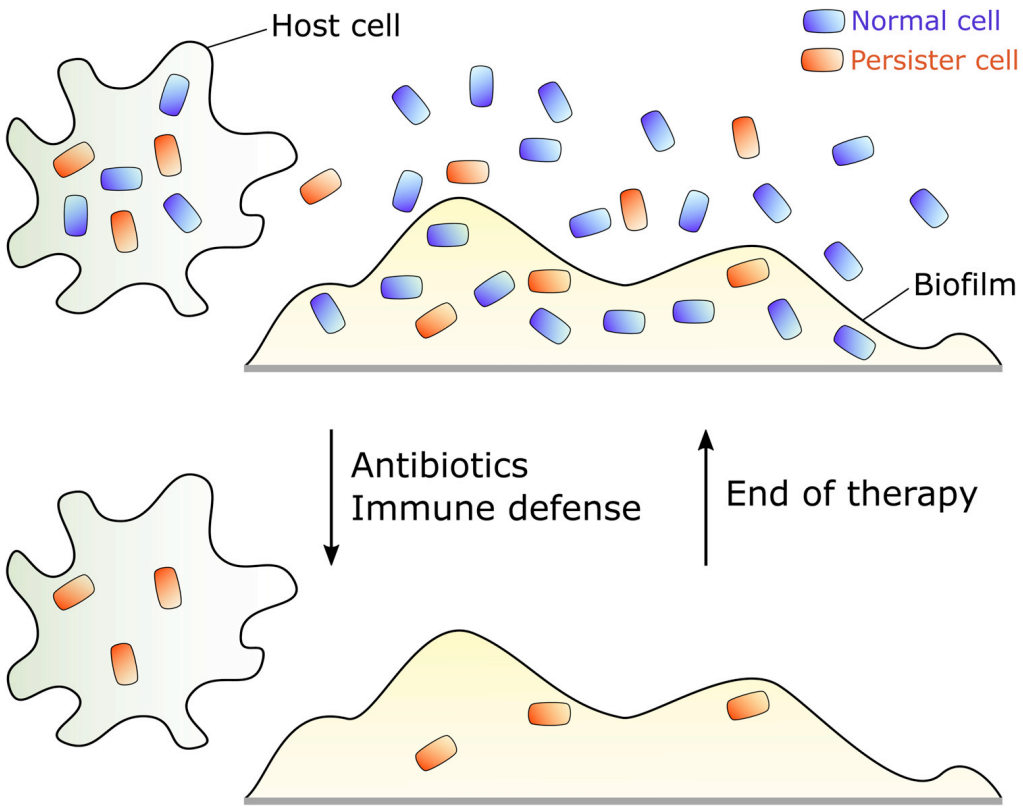


Fig. 11. Bacterial infection relapse model mediated by persister cells. Antibiotic treatment eliminates only planktonic non-persister bacteria, after that the immune system wipes out planktonic persister cells. Persister biofilm-located bacteria are non-sensitive to the action of both the antibiotic and the immune response. Upon termination of antibiotic therapy, that cells are able to resume their growth and cause relapse of infection [156].

There are known high-persistence (*hip*) mutations in bacteria [116]. The *hip* mutants of *E. coli* and *S. aureus* cells can be isolated after treatment with antibiotics *in vitro* [114, 116, 156]. Clinical isolates of *P. aeruginosa* from patients with cystic fibrosis, *E. coli* from patients with UTIs are mostly *hip* mutants of isolates obtained earlier in infection [157, 158]. It was shown that phenomenon of persistence may also be an important factor in the relapse of fungal infections since *Candida albicans* isolates after amphotericin B treatment are enriched by *hip* mutants [159].

Persister cell formation may be an important defense mechanism against antibiotics action before *bona fide* resistance is evolved or acquired. Mathematical modeling can be used to demonstrate that increased resistance can develop through persistence [160]. During the presence of antibiotics, persisters serve as a source of viable cells. Although persisters are non-growing and non-dividing cells, they are stressed and accumulate mutations through stationary-phase, stress-induced, and/or adaptive mutation

mechanisms [161, 162]. Moreover, there is a link between horizontal gene transfer and bacterial stress response. For example, it was shown that SOS response accelerates the horizontal gene transfer of antibiotic resistance genes in *E. coli* and *V. cholerae* [163]. Interestingly, the biofilm environment also increases both mutation rates and horizontal gene transfer [164, 165].

Persistent infections were demonstrated to be associated with about half of all nosocomial infections [152]. Treatment of acute *M. tuberculosis* infection may take several months. Lung infections, which are caused by *P. aeruginosa* and *B. cepacia* become more difficult to treat over time [149, 166]. The long treatment or repetitive usage of antibiotics (because of relapsing infections) have negative effects on patient health (side effects, depletion of the resident microbiota), but also select antibiotic-resistant strains. Therefore, there is an evident need for more effective strategies to treat persistent infections and to understand the persistence phenomenon itself [167, 168].

Project Objectives

Despite enormous advances in developing new antibiotics in the last few decades, many of them become gradually less effective due to antibiotic resistance and bacterial persistence phenomena. One approach overcome the global antibiotic crisis is to investigate the phenomenon of persistence as well as to search for and develop new antibacterial drugs that have a broad spectrum of antimicrobial action without inducing the persistent state.

Microcin C from *E.coli* (McC^{Eco}) is the natural peptide-nucleotide antibiotic that targets an aminoacyl-tRNA synthetase through a unique Trojan-horse mechanism. Many studies have focused on McC^{Eco}: mechanism of biosynthesis, cellular target, and mechanism of action. McC homologs are encoded by gene clusters found in diverse gram-negative, gram-positive bacteria and even cyanobacteria [30]. McC from *Y. pseudotuberculosis* IP 32953 (McC^{Yps}) is unique among this group of compounds since its peptide part needs to be processed inside the producing cell to generate mature McC^{Yps}. In this work the cellular proteases responsible for activation of McC from *Y. pseudotuberculosis* IP 32953 were identified.

Microcin C targets an aminoacyl-tRNA synthetase, and protein HipA, the part of the cellular toxin-antitoxin system and well-studied inducer of bacterial persistence, also targets an aminoacyl-tRNA synthetase. And based on the above-mentioned assumptions, microcin C from *E.coli* was examined as a potent inducer of persistence.

The following tasks were set:

1. Identifying the cellular endoprotease(s) responsible for removal of N-terminal peptide from pro-McC^{Yps}.
2. Experimentally validating the theoretical expectations that microcin C is persistence-inducing agent and determinating of mechanism(s) involved.
3. Characterising the persistence potential of microcin C producing cells, cells sensitive to microcin C, and cells in mixed cultures.

Materials and Methods

Growth media and biological materials

Cells were grown in LB media, MOPS glucose medium supplemented with all amino acids or in M9 minimal medium supplemented with the appropriate carbon source at 37 °C with shaking. Terrific Broth medium is used for protein expression. When required, the medium was supplemented with 10 μg/ml tetracycline, 50 μg/ml ampicillin or kanamicine, 0.3 mM IPTG. Bacterial strains and plasmids are provided in Table 1.

Table 1. Bacterial Strains and Plasmids

Strains/Plasmids	Genotype/Plasmid properties	Source/Reference
MG1655	Wild-type *E. coli* K-12	Laboratory collection
B strain	Wild-type *E. coli* B	Laboratory collection
BL21 (DE3)	*fhuA2 [lon] ompT gal (λ DE3) [dcm]* Δ*hsdS λ DE3 = λ sBamHIo* Δ*EcoRI-B int::(lacI::PlacUV5::T7 gene1) i21* Δ*nin5*	Laboratory collection
DH5a	F$^-$ *endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG* ϕ80d*lacZ* ΔM15 Δ*(lacZYA-argF)U169 hsdR17*(r_K^- m_K^+) λ^-	Laboratory collection
BW25113	*Δ(araD-araB) ΔlacZ4787*(::rrnB-3) λ^- *rph-1 Δ(rhaD-rhaB)568 hsdR514*	Laboratory collection

JW1950-1	F-, *Δ(araD-araB)567*, *ΔlacZ4787*(::rrnB-3), *λ*⁻, *ΔhchA743::kan*, *rph-1*, *Δ(rhaD-rhaB)568*, *hsdR514*	Baba *et al*. (2006)
JW1818-2	F-, *Δ(araD-araB)567*, *ΔlacZ4787*(::rrnB-3), *λ*⁻, *ΔhtpX754::kan*, *rph-1*,*Δ(rhaD-rhaB)568*, *hsdR514*	Baba *et al*. (2006)
JW2903-5	F-, *Δ(araD-araB)567*, *ΔlacZ4787*(::rrnB-3), *λ*⁻, *ΔyggG784::kan*, *rph-1*, *Δ(rhaD-rhaB)568*, *hsdR514*	Baba *et al*. (2006)
JW0909-1	F-, *Δ(araD-araB)567*, *ΔlacZ4787*(::rrnB-3), *λ*⁻, *ΔycbK743::kan*, *rph-1*, *Δ(rhaD-rhaB)568*, *hsdR514*	Baba *et al*. (2006)
JW1489-1	F-, *Δ(araD-araB)567*, *ΔlacZ4787*(::rrnB-3), *λ*⁻, *ΔpqqL787::kan*, *rph-1*, *Δ(rhaD-rhaB)568*, *hsdR514*	Baba *et al*. (2006)
JW2789-1	F-, *Δ(araD-araB)567*, *ΔlacZ4787*(::rrnB-3), *λ*⁻, *ΔptrA746::kan*, *rph-1*,*Δ(rhaD-rhaB)568*, *hsdR514*	Baba *et al*. (2006)
JW2066-5	F-, *Δ(araD-araB)567*, *ΔlacZ4787*(::rrnB-3), *λ*⁻, *ΔyegQ780::kan*, *rph-1*, *Δ(rhaD-rhaB)568*, *hsdR514*	Baba *et al*. (2006)
JW3127-1	F-, *Δ(araD-araB)567*, *ΔlacZ4787*(::rrnB-3), *λ*⁻, *ΔyhbU771::kan*, *rph-1*, *Δ(rhaD-rhaB)568*, *hsdR514*	Baba *et al*. (2006)
JW1590-1	F-, *Δ(araD-araB)567*, *ΔlacZ4787*(::rrnB-3), *λ*⁻, *ΔydgD746::kan*, *rph-1*, *Δ(rhaD-rhaB)568*, *hsdR514*	Baba *et al*. (2006)
JW0157-1	F-, *Δ(araD-araB)567*, *ΔdegP775::kan*, *ΔlacZ4787*(::rrnB-3), *λ*⁻, *rph-1*, *Δ(rhaD-rhaB)568*, *hsdR514*	Baba *et al*. (2006)
JW3203-1	F-, *Δ(araD-araB)567*, *ΔlacZ4787*(::rrnB-3), *λ*⁻, *ΔdegQ760::kan*, *rph-1*, *Δ(rhaD-rhaB)568*, *hsdR514*	Baba *et al*. (2006)

JW3903-5	F-, *Δ(araD-araB)567*, *ΔlacZ4787*(::rrnB-3), *λ*⁻, *rph-1*, *Δ(rhaD-rhaB)568*, *ΔhslV720::kan*, *hsdR514*	Baba *et al*. (2006)
JW3465-1	F-, *Δ(araD-araB)567*, *ΔlacZ4787*(::rrnB-3), *λ*⁻, *ΔprlC754::kan*, *rph-1*,*Δ(rhaD-rhaB)568*, *hsdR514*	Baba *et al*. (2006)
JW2266-1	F-, *Δ(araD-araB)567*, *ΔlacZ4787*(::rrnB-3), *λ*⁻, *ΔyfbL753::kan*, *rph-1*,*Δ(rhaD-rhaB)568*, *hsdR514*	Baba *et al*. (2006)
JW0892-1	F-, *Δ(araD-araB)567*, *ΔlacZ4787*(::rrnB-3), *λ*⁻, *ΔycaL733::kan*, *rph-1*,*Δ(rhaD-rhaB)568*, *hsdR514*	Baba *et al*. (2006)
JW0429-1	F-, *Δ(araD-araB)567*, *ΔlacZ4787*(::rrnB-3), *Δlon-725::kan*, *λ*⁻, *rph-1*,*Δ(rhaD-rhaB)568*, *hsdR514*	Baba *et al*. (2006)
JW0427-1	F-, *Δ(araD-araB)567*, *ΔlacZ4787*(::rrnB-3), *ΔclpP723::kan*, *λ*⁻, *rph-1*,*Δ(rhaD-rhaB)568*, *hsdR514*	Baba *et al*. (2006)
JW5687-1	F-, *Δ(araD-araB)567*, *ΔlacZ4787*(::rrnB-3), *λ*⁻, *ΔglpG757::kan*, *rph-1*, *Δ(rhaD-rhaB)568*, *hsdR514*	Baba *et al*. (2006)
JW2479-1	F-, *Δ(araD-araB)567*, *ΔlacZ4787*(::rrnB-3), *λ*⁻, *ΔyfgC742::kan*, *rph-1*,*Δ(rhaD-rhaB)568*, *hsdR514*	Baba *et al*. (2006)
JW0938-1	F-, *Δ(araD-araB)567*, *ΔlacZ4787*(::rrnB-3), *λ*⁻, *ΔycbZ770::kan*, *rph-1*,*Δ(rhaD-rhaB)568*, *hsdR514*	Baba *et al*. (2006)
JW3495-5	F-, *Δ(araD-araB)567*, *ΔlacZ4787*(::rrnB-3), *λ*⁻, *ΔyhjJ782::kan*, *rph-1*,*Δ(rhaD-rhaB)568*, *hsdR514*	Baba *et al*. (2006)
JW4194-1	F-, *Δ(araD-araB)567*, *ΔlacZ4787*(::rrnB-3), *λ*⁻, *rph-1*, *Δ(rhaD-rhaB)568*, *ΔpmbA782::kan*, *hsdR514*	Baba *et al*. (2006)

JW3213-1	F-, *Δ(araD-araB)567*, *ΔlacZ4787*(::rrnB-3), *λ*⁻, *ΔtldD769::kan*, *rph-1*,*Δ(rhaD-rhaB)568*, *hsdR514*	Baba *et al*. (2006)
JW3738-1	F-, *Δ(araD-araB)567*, *ΔlacZ4787*(::rrnB-3), *λ*⁻, *rph-1*, *ΔyifB787::kan*,*Δ(rhaD-rhaB)568*, *hsdR514*	Baba *et al*. (2006)
JW5840-1	F-, *Δ(araD-araB)567*, *ΔlacZ4787*(::rrnB-3), *λ*⁻, *ΔelaD751::kan*, *rph-1*,*Δ(rhaD-rhaB)568*, *hsdR514*	Baba *et al*. (2006)
JW5529-1	F-, *Δ(araD-araB)567*, *ΔlacZ4787*(::rrnB-3), *λ*⁻, *ΔyhbO766::kan*, *rph-1*, *Δ(rhaD-rhaB)568*, *hsdR514*	Baba *et al*. (2006)
Y. pseudotuberculosis #3526	Wild-type *Y. pseudotuberculosis* #3526	Laboratory collection
MGJ5987 (Δ*10TA*)	MG1655 Δ*mazF* *Δ*chpB* Δ*relBE* Δ(*dinJ-yafQ*) Δ(*yefM-yoeB*) Δ*higBA* Δ(*prlF-yhaV*) Δ*yafNO* Δ*mqsRA* Δ*hicAB*	Maisonneuve *et al*. (2011)
MG1655 Δ*lon*	MG1655 Δ*lon::tet*	Winther and Gerdes. (2009)
CF5802 *(ΔppKppX)*	MG1655 Δ*ppk ppx*::kan	Kuroda and Kornberg. (1997)
CF1693 *(ΔrelAspoT)*	MG1655 Δ*relA251::kan* Δ*spoT207*::*cat*	Xiao *et al*. (1991)
Δ*relA*	MG1655 Δ*relA251::kan*	P1 CF1698 x MG1655
MG1655 Δ*dksA*	MG1655 Δ*dksA::kan*	Germain *et al*. (2015)
BW25113 *Δhns ΔtldD ΔtldE*	BW25113 *Δhns ΔtldD ΔtldE*	Tsibulskaya D *et al*. (2017)

BW25113 Δ*tldD*	BW25113 Δ*tldD::kan*	Baba *et al*. (2006)
BW25113 Δ*tldE*	BW25113 Δ*tldE::kan*	Baba *et al*. (2006)
BW25113 Δ*yejA*	BW25113 Δ*yejA::kan*	Novikova *et al*. (2007)
MG1655 Δ*yejA*	MG1655 Δ*yejA*	Laboratory collection
HM21	AT984 zde264::Tn10 dapA6	Moyed and Bertrand. (1983)
MG1655 *zde*	MG1655 *zde264*::Tn*10 dapA6*	P1 HM21 x MG1655
MG1655 RpoS-Mcherry	MG1655 *rpoS-mCherry::frt*	Maisonneuve *et al*. (2013)
pDT1-Ymcc	mcc^{Yps} cluster under natural promoter, Kan^R	Tsibulskaya D *et al*. (2017)
pBad30	Ara promoter, Amp^R	Life Technologies
pp70	pBad30-derived, mcc^{Eco} cluster under natural promoter, AmpR	Zukher *et al*. (2014)
pp70-F	pBad30-derived, mcc^{Eco} cluster under natural promoter, mccFS118A, AmpR	Tikhonov A. *et al*. (2010)
pp70-E	pBad30-derived, mcc^{Eco} cluster under natural promoter, MccE S553A and E572A, Amp^R	Novikova *et al*. (2010)
pET28	T7 promoter, Kan^R	Thermo Fisher Scientific
pET28_ Yer_tldD	T7 promoter, $tldD^{Yrs}$, KanR	This work

pET28_ Yer_tldE	T7 promoter, tldE^{Yrs}, KanR	This work

Preparation and purification of McC^{Eco}

The *E. coli* K-12 strain BW25113 *ΔyejA* harboring McC-producing plasmid pp70 was grown for 18 h at 37 °C in M63 minimum medium containing 1% glycerol, 1 μg/ml thiamine and 100 μg/ml ampicillin. The cells were removed by centrifugation and the cultured medium was loaded onto Sep-Pak C8 cartridge (Waters). The cartridge was washed with water followed by a 0.1% aqueous trifluoroacetic acid wash, and bound material was eluted stepwise with 5, 10, and 20% acetonitrile in 0.1% trifluoroacetic acid. The 10% acetonitrile fraction was concentrated by lyophilization, dissolved in water, and subjected to reverse phase-HPLC (1 ml/min) on a ReproSil-Pur 300 ODS-3 column (5 μm, 250 × 4 mm) using a 0–20% linear gradient of acetonitrile in 0.1% trifluoroacetic acid. The total gradient volume was 50 ml. Pure McC eluted as a single peak; it was lyophilized, dissolved in water, and stored at –20 °C. The yield of chromatographically and mass spectrometrically pure McC ranged from 5 to 10 μg/ml of the cultured medium.

Preparation and purification of pro-McC^{Yps} or McC^{Yps}

E. coli BW25113 *Δhns* transformed with pDT1-Ymcc were used to produce McC^{Yps}. The same plasmid as the one transformed into *E. coli* BW25113 Δ*hns* Δ*tldD* Δ*tldE* was applied to purify pro-McC^{Yps}. The cells were grown in M9 medium supplemented with kanamycin at 37 °C until OD600 reached 0.6 then the culture was shifted to 20 °C for 48h. McC^{Yps} was purified from the conditioned medium. Cell culture was centrifuged at 5.000 *g* for 30 min, the supernatant was filtered through 0.45 μm Millipore filter, supplemented with trifluoroacetic acid (TFA) to 0.1% and loaded on HF Mega Bond Elut-C18 10 g column (Agilent Technologies). McC^{Yps} was eluted with 10% acetonitrile in 0.1% TFA. Dried fraction was re-dissolved in 0.1% TFA and loaded on Eclipse Plus C18 column (4.6 × 250 mm, particle size 5 μm, Agilent Technologies). To extract McC^{Yps} from *E. coli* BW25113 *Δhns* culture medium a 20-min linear gradient (from 5 to 15% acetonitrile in 0.1% TFA) was used. To monitore the elution of substances absorption at 280 nm was applied as well as checking the fractions for

antibacterial activity.

Pro-McC^{Yps} was purified from the cells cultured as described above. The cells were harvested, washed with a buffer containing 150 mM NaCl in 10 mM Tris-HCl pH 7.5 and resuspended in 50% acetonitrile in 0.1% TFA. The cell suspension was sonicated then incubated at 95 °C for 10 min. The precipitate was removed by centrifugation. The soluble fraction was diluted with 2 volumes of 0.1% TFA and loaded on HF Mega Bond Elut-C18 10 g column (Agilent Technologies). Pro-McC^{Yps} was eluted with 40% acetonitrile in 0.1% TFA, dried and re-dissolved in 10% acetonitrile in 0.1% TFA for HPLC chromatography as described above except that from 15 to 50% acetonitrile in 0.1% TFA linear gradient was applied. The fractions were checked for the presence of pro-McC^{Yps} with MALDI-TOF mass-spectrometry.

McC^{Yps} production test

E. coli BW25113 strain Δ*hns* and *E. coli* BW25113 Δ*hns* Δ*tldE* Δ*tldD* harboring pDT1 plasmid and its *mcc*^{Yps}-containing derivatives were grown in 10 mL of LB medium at 37 °C until OD600 reached 0.6. After that, the temperature was changed to 20 °C and the cultures were grown for 20 h. The cells were pelleted, then washed with M9 medium and resuspended in 200 μL of M9 medium. Aliquots (15 μL) of cell suspensions were deposited on freshly prepared lawn of *E. coli* B cells harboring pDT1 plasmid.

Cloning and gene knockouts, protein expression and purification

Coding sequences of *Y. pseudotuberculosis tldD* and *tldE* genes were amplified using Yer_tldD_Nhe_F/Y_tldD_Sal_R (ATAATTGCTAGCATGAGCCTCTCATTTGTCAGTGAG/ATTATAGTCGACTTAC GCTGTGCCGCCCACGGTTAAATTATC) and Yer_tldE_Nhe_F/Yer_tldE_Sal_R (ATAATTGCTAGCATGAAAGTAGTCACTCAAGTTGCAG/ATTATAGTCGACTTA TTCGCCCGCGATTTTCATAACGGGC) primer pairs and resulting PCR products were cloned into pET28 vector between the Nhe I and Sal I restriction sites.

All PCR were performed with Phusion DNA polymerase from New England Biolabs according to the manufacturer's instructions. All restriction digest enzymes were provided by Thermo Fisher Scientific. The extraction of DNA fragments from agarose gel was performed using the GeneJET Gel Extraction Kit (Thermo Fisher scientific) The

ligations were carried out at 22˚C, for 30 minutes using Rapid Ligation kit from Thermo Fisher Scientific as suggested by the manufacturer.

The knockout of *hns* and *tldD* genes in *E. coli* BW25113 Δ*tldE (pmbA)* strain was performed according to Datsenko and Wanner [176] using primers hns_F and hns_R (TGAATTTAAGGCTCTATTATTACCTCAACAAACCACCCCAATATAAGTTTTGTGTAGGCTGGAGCTGCTTCGAA and CTGGCGGGATTTTAAGCAAGTGCAATCTACAAAAGATTATTGCTTGATCAATGAATATCCTCCTTAGTTCCT), tldD_F and tldD_R (AATCCTCTACTGCAGTAACTAACGAGTAGCAAAAACGGTGTAGGCTGGAGCTGCTTC and CGTTCGTGCACGTAGAAAGATTAATTATCCTTCTGAAAATAGTGAAATTAATGGGAATTAGCCATGGTCC).

Bacterial cells transformation

A 50-mL tube containing 10 mL of LB media was inoculated with a single bacterial colony from a fresh plate. The tube was placed in a shaking incubator set at 37 °C, and left overnight.

The next day, 0.5 mL of the overnight culture was used to inoculate 50 mL of LB media into a 250-mL flask. The flasks were incubated at 37 °C with vigorous agitation. The OD600 was measured every twenty minutes. Once the OD600 of the culture reached 0.6, the flasks were cooled in ice-water bath for 15-30 minutes.

The cultures were transferred to ice-cold centrifuge tubes. The cells were harvested by centrifugation at 3 000 rcf for 15 minutes at 4 oC. The supernatant was decanted and the cell pellet was resuspended in 50 mL of ice-cold 10% glycerol. The washing step was repeated three times then the cells were re-suspended in 1 mL of ice-cold 10% glycerol, aliquoted and stored at -80 °C.

MicroPulser electroporator (Bio-Rad Laboratories) was used for cell transformation. DNA samples were pipetted to the electrocompetent cells and transferred to a cold 1mm wide electroporation cuvette. After the 1.8 kV was pulsed, 0.9 mL of warm SOC medium was added and cells were incubated for 1h at 37 C. The cells were cultivated on plates containing appropriate antibiotics.

Protein expression and purification

The BL21 (DE3) cells transformed with pET28_Yer_tldD or pET28_Yer_tldE were grown at 37°C on 200 ml Terrific Broth medium supplemented with necessary antibiotics until the OD_{600} reached 0.6. Then the medium was supplemented by 0.3 mM IPTG. The cells were grown for 20 h at 18°C with vigorous agitation. The cells were harvested and resuspended in 8 ml of loading buffer (20 mM Tris-HCl [pH 8.0], 500 mM NaCl, 10 mM $MgCl_2$) and disrupted by sonication. The lysates were cleared by centrifugation at 30,000 × g for 30 min at +4°C. To the supernatants, 300 µl of His Bind resin (Novagen) equilibrated in the same buffer was added, and proteins were allowed to bind for 4 h at 4°C with gentle agitation. The resin was settled by gravity, washed with 15 ml of wash buffer (20 mM Tris-HCl [pH 8.0], 500 mM NaCl, 10 mM $MgCl_2$, 50 mM imidazole), and bound proteins were eluted with 0.5 ml of elution buffer (20 mM Tris-HCl [pH 8.0], 500 mM NaCl, 10 mM $MgCl_2$, 200 mM imidazole). Four consecutive elutions were performed with each resin sample. Proteins were at least 90% pure as judged by visual inspection of overloaded Coomassie-stained SDS gels. Then proteins were purified by Mono Q ion-exchange chromatography.

Biochemical reaction

For TldD/E digestion, 100 µM $MccA^{Yps}$-CMP was incubated in 10 mM Tris-HCl pH 7.5 buffer containing 20 mM NaCl, and 2 µM of $TldD^{Yps}$, $TldE^{Yps}$, both $TldD^{Eco}$ and $TldE^{Yps}$, or $TldD^{Eco}$ and $TldE^{Eco}$ subunits) at room temperature for 10 min.

$tRNA^{Asp}$ aminoacylatio reaction

E. coli BL21 cells were grown in 50 mL of LB medium at 37°C until OD600 reached 0.8. The cells were pelleted by centrifugation, washed with 20 mM Tris-HCl, pH 8.0, 10 mM MgCl2, 100 mM KCl and resuspended in 1 mL of the same buffer supplemented with 1 mM DTT. To prepare S30 cell lysate, the bacterial cells were disrupted by sonication and fractionated by centrifugation at 30 000 *g*. 10 µL of 250 µM inhibitor solution was combined with 30 µL of S30 cell lysate (3 mg/mL final protein concentration) and reactions were incubated at 25 °C to allow processing of peptide parts of microcins. At various times, 4 µl of S30 extract-inhibitor mixture aliquots were combined with 16 µL of the aminoacylation mixture (30 mM Tris-HCl, pH 8.0, 30 mM KCl, 8 mM MgCl2, 1 mM DTT, 3 mM ATP, 5 mg/mL bulk *E. coli* tRNA, 23 µM 14C-

Asp). Reactions were incubated at 25 °C to allow tRNAAsp aminoacylation. The reactions were terminated by applying on Whatman 3MM paper filters and drying. After washing with ice-cold 10% trichloroacetic acid, the filters were additionally washed with acetone and dried. Incorporation of radioactivity into insoluble fraction was determined by Cherenkov counting.

Bacterial growth experiments

Overnight cultures of MG1655 were diluted 100-fold in 50 ml of fresh LB medium and incubated for 2 h at 37 °C with shaking. 5-ml aliquots were transferred to 50 ml Falcon tubes and McC was added in different concentrations (0-5 μM). The cultures were allowed to continue growth and optical density at OD_{600} was monitored.

Persistence assay

The level of persister cells in bacterial culture was determined by measuring the number of colony forming units (CFUs) per 1 ml upon treatment by 1 μg/ml ciprofloxacin. For that purpose, the overnight cultures were diluted 100-fold in 10 ml of fresh LB medium and incubated for 2 h (otherwise indicated) at 37°C with shaking. Then aliquots of 5 ml were transferred to Falcon 50ml tubes and ciprofloxacin was added. The tubes were incubated with shaking at 37 °C for 4 hours. For determination of CFUs, 1 ml aliquots were removed every hour during the experiment, the cells harvested, then resuspended in 1 ml of fresh LB medium, then serially diluted and plated on LB plates without antibiotics (otherwise indicated). The level of persister cells in bacterial culture was calculated by dividing the number of CFU/ml in the culture after 4 h of incubation with ciprofloxacin by the number of CFU/ml in the culture before adding the antibiotic.

McC and persistence

To determine the level of persister cells in bacterial culture that was experiencing pretreatment with McC, overnight cultures were diluted 100-fold in 10 ml of fresh LB medium and then were grown for 1.5 h (OD_{600} ~ 0.3), then various concentrations of McC were added and the cells were grown for additional 30 min. Then aliquots were exposed to 1 μg/ml ciprofloxacin and the level of persister cells in bacterial culture was determined as described above.

In vivo (p)ppGpp measurements

To determine the (p)ppGpp content level formed by cells after McC treatment, overnight cultures were diluted 100-fold in 10 ml of MOPS glucose minimal medium supplemented with all amino acids as previously described [168] and incubated at 37 °C with shaking. At OD_{600}~0.05, cells were labeled with $H_3{}^{32}PO_4$ (100 μCi/ml). After 2–3 generations (OD_{600}~0.2–0.3), 3 μM McC were added. Samples were withdrawn 0, 10, 30 and 60 min after the addition of McC. Reactions were stopped by the addition of 10 μl of 2 M formic acid. Aliquots (10 μl) of each reaction were separated on PEI Cellulose TLC plates (GE Healthcare) at room temperature for 1 hour. 1.5 M KH_2PO_4 (pH 3.4) was used as the chromatographic solvent. The chromatographic products were revealed by PhosphoImaging (GE Healthcare).

Microscopy

For fluorescence and phase microscopy, cells were grown in LB to mid-exponential phase at 37 °C, centrifuged, resuspended in fresh LB, diluted 10-times in M9 medium, and mounted on pre-warmed microscope slides covered with a thin film of 1.2% agarose (in a 10-times diluted LB in M9 medium). Images were obtained using a Zyla 5.5 CMOS camera attached to a Nikon Eclipse Ti-E microscope. The images were acquired and analyzed using NIS-ELEMENTS.

Co-cultivation assay

Cells transformed with pp70 (or empty vector) and cells marked with a chromosomal tetracycline resistance gene with empty vector (i.e McC sensitive strains) were co-cultured. The overnight cultures were mixed in a 1:1 ratio and diluted 200-fold in 10 ml of fresh LB medium and incubated for 6 h at 37 °C with shaking. At select time points OD_{600} and CFUs were measured on solid LB medium to enumerate the total CFU number of the co-culture or on LB medium with tetracycline to enumerate the number of CFUs from the McC-sensitive cells population. The persistence assay for co-cultivated cultures was slightly modified: the co-cultures were cultivated for 3 hours before the addition of ciprofloxacin (Cfx).

MALDI-MS and MS-MS analyses

MALDI-TOF MS analysis was performed on UltrafleXetreme MALDI-TOF/TOF mass spectrometer (Bruker Daltonik, Germany) equipped with Nd laser. Samples for the analysis were prepared as described earlier2. The MH+ molecular ions of crude cell lysates were measured in linear mode; the accuracy of average mass peak measurement was within 1 Da. The MH+ molecular ions of HPLC fractions were measured in reflector mode; the accuracy of monoisotopic mass peak measurement was within 30 ppm. Spectra of fragmentation were obtained in LIFT mode, the accuracy of daughter ions measurement was within 1 Da range. Mass-spectra were processed with the use of FlexAnalysis 3.2 software (Bruker Daltonik, Germany) and analyzed manually.

High-resolution mass spectra were recorded on a Fourier Transform Ion Cyclotron Resonance Mass Spectrometer (Varian 902-MS) equipped with a 9.4 T magnet (FTMS) in positive MALDI mode. Calibration was performed using a ProteoMass Peptide MALDI-MS Calibration Kit (Sigma-Aldrich).

Determination of antibiotic activity of McC variants

E. coli B strain were grown in LB medium. The overnight culture was diluted 1000-fold with soft agar containing M9 medium supplemented. 5-µL drops of microcins with tested concentration were deposited on the surface of the solidified agar and allowed to dry. Plates were incubated for 16 h at 30 °C to form a lawn.

Results and Discussion

Part 1: Antibacterial activity of McC^{Yps} depends on proteolytic cleavage of N-terminal leader peptide from pro-McC^{Yps} by the TldD/E protease action

As it was previously demonstrated, McC^{Yps} undergoes an additional modification in the production host that is strictly required for biological activity. In course of this modification, the 42-amino acid long MccA^{Yps} peptide part is proteolytically processed inside the producing cell releasing mature McC^{Yps} with a peptide part of only 11 amino acids long. It is not typical event for peptide-nucleotide antibiotics, but it is common for RiPPs. Most RiPP-related precursor peptides contain specific leader peptide, that is responsible for peptide recognition by post-translational machinery, and after completing all modifications the leader peptide is cut by a protease. For most of the RiPPs, some cellular protease is responsible for the leader peptide cleavage. We decided to find out which protease is responsible for McC^{Yps} maturation. In the following section, this question is addressed.

The accumulation of N-terminal peptide of pro-McC^{Yps} inside the producing cells

The finding that active McC^{Yps} is different from the compound that accumulates in the producing cells raises a question about the mechanism by which the active compound with shorter peptide part is generated. Inspection of mass spectra of wild-type *E. coli* cells carrying pDT1-Ymcc, containing the entire *mcc*-cluster from *Y. pseudotuberculosis* under the control of native promoters, revealed a prominent peak with an average *m/z* 3777 that corresponded to N-terminal 31-amino acid fragment of MccA^{Yps} (Figure 12). This fragment is the most likely to result from a proteolytic event that generates mature McC^{Yps}. The same was shown for Δ*hns E. coli* cells, in these cells the level of McC^{Yps} production is higher than in E. coli BW25113 cells or *Y. pseudotuberculosis* 3526.

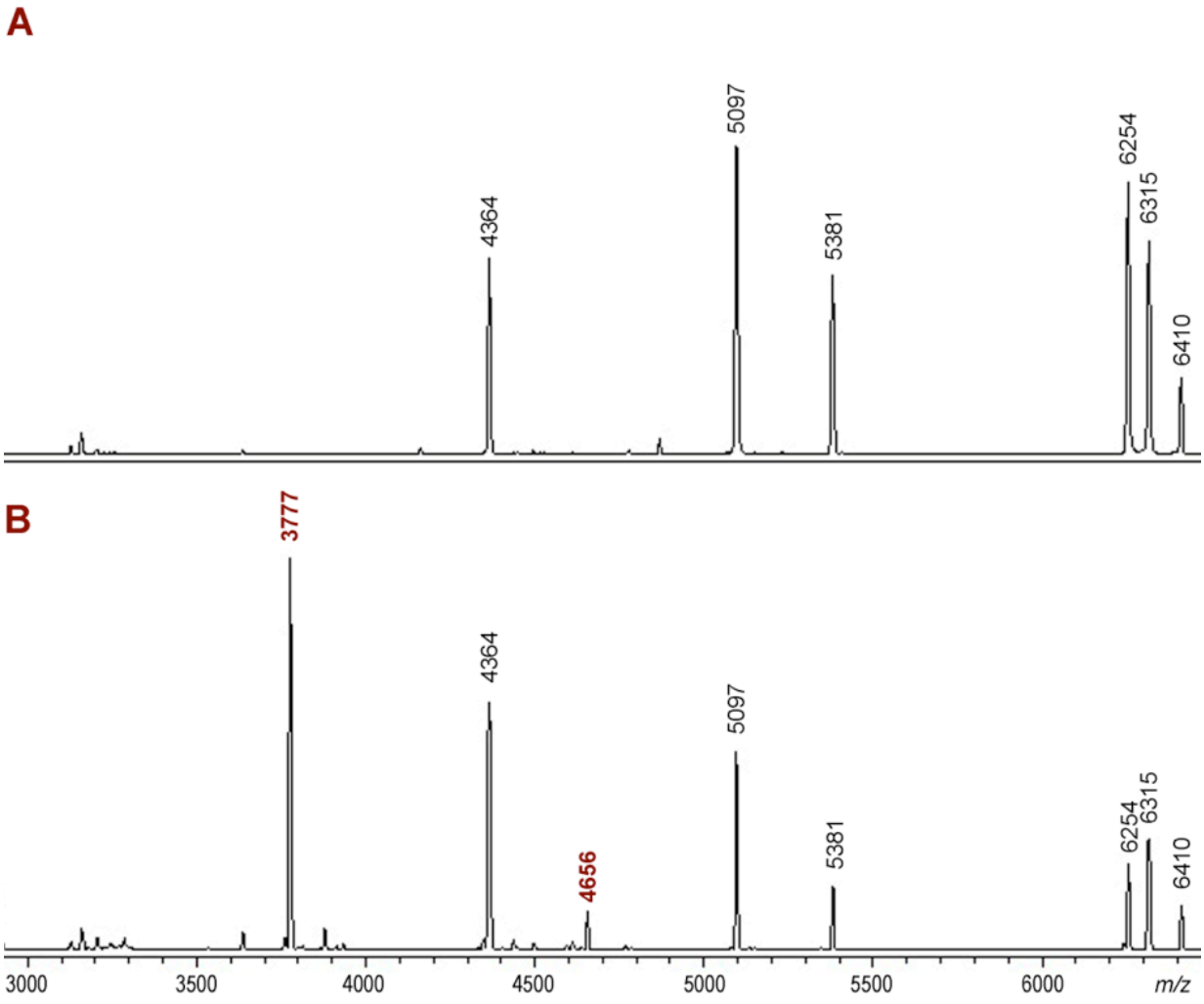


Fig. 12. MALDI-TOF analysis of E. coli BW25113 cells harboring the pDT1 vector (A) or the pDT1-Ymcc plasmid (B) carrying *Y. pseudotuberculosis* IP 32953 *mcc* cluster. The

m/z values of different peaks present in the panel B spectrum and absent from the spectrum in panel A are highlighted by red color font; they correspond to full-length MccA^{Yps} peptide (*m/z* 4656) and its N-terminal proteolytic fragment (MccA^{Yps}1-31, *m/z* 3777). Mass-peaks of *E. coli* proteins that are present in both control and experimental cells can correspond to ribosomal proteins RL36 (m/z_{calc} 4364), RS22 (m/z_{calc} 5097), RL34 (m/z_{calc} 5381), RL33 (m/z_{calc} 6254), RL32 (m/z_{calc} 6315), and RL30 (m/z_{calc} 6410).

The TldD/E protease is responsible for endoproteolytic processing of pro-McC^{Yps} inside producing cells

To identify a protease(s) involved in MccA^{Yps} cleavage, a set of mutants in the known and putative *E. coli* protease genes present in the Keio collection of single-gene *E. coli* mutants [176] was transformed with the pDT1-based plasmid harboring the *mccACB* genes from *Y. pseudotuberculosis* and screened for the presence of *m/z* 3777 mass-ion (N-terminal proteolytic fragment (MccA^{Yps}1-31)).

Table 2. Bacterial Strains from the Keio collection and Data about *E.coli* cellular proteases

Strains	**Gene of known or putative *E.coli* protease**	**Information about known or putative *E.coli* protease**
JW1950-1	*hchA*	Cysteine-type protease peptidase family
JW1818-2	*htpX*	Zinc-dependent endoprotease, member of the membrane-localized proteolytic system in *E. coli*
JW2903-5	*yggG*	Low osmolarity induced metalloprotease

JW0909-1	*ycbK*	M15A protease-related family, periplasmic conserved protein
JW1489-1	*pqqL*	M16 peptidase family, putative periplasmic zinc-dependent metalloendopeptidase
JW2789-1	*ptrA*	M16 peptidase family, endopeptidase, may have preference for small peptides
JW2066-5	*yegQ*	U32 peptidase family, uncharacterized protease
JW3127-1	*yhbU*	U32 peptidase family, predicted protease
JW1590-1	*ydgD*	S2B peptidase family, putative precursor protease
JW0157-1	*degP*	S2C peptidase family, serine protease, required at high temperature, virulence factor, involved in degradation of damaged proteins
JW3203-1	*degQ*	S2C peptidase family, periplasmic pH – dependent serine protease

JW3903-5	*hslV*	T1B peptidase family, the heat shock Thr-Protease
JW3465-1	*prlC*	M3 peptidase family, oligopeptidase, may play a specific role in the degradation of signal peptides after they are released from precursor forms of secreted proteins
JW2266-1	*yfbL*	M28A peptidase family, aminopeptidase
JW0892-1	*ycaL*	M48 peptidase family, homolog of YggG of E. coli, putative Zn-dependent protease
JW0429-1	*lon*	S16 peptidase family, ATP-dependent Ser-protease, broad substrate specificity
JW0427-1	*clpP*	S14 Peptidase family, ATP-dependent protease, chymotrypsin-like activity, major role in the degradation of misfolded proteins

JW5687-1	*glpG*	S54 peptidase family, putative membrane rhomboid-type serine protease that catalyzes intramembrane proteolysis
JW2479-1	*yfgC*	Beta-barrel assembly-enhancing metalloprotease, maintains the integrity of the outer membrane by promoting either the assembly or the elimination of outer membrane proteins
JW0938-1	*ycbZ*	S16 peptidase family, putative ATP-dependent Lon protease homolog
JW3495-5	*yhjJ*	M16 peptidase family, zinc-dependent protease
JW4194-1	*pmbA*	Zinc-dependent protease required for the production of the antibiotic peptide MccB17

JW3213-1	*tldD*	Zinc-dependent protease required for the production of the antibiotic peptide MccB17
JW3738-1	*yifB*	ATP-dependent protease
JW5840-1	*elaD*	SUMO/Ulp1_C peptidase family, cysteine-type protease
JW5529-1	*yhbO*	C40 peptidase family, conserved stress-related putative cysteine-type protease

Since only the cells lacking either *tldD* or *tldE* genes coding the subunits of TldD/E protease [177] did not contain the N-terminal $MccA^{Yps}$ fragment, TldD/E may be propsed to be involved in McC^{Yps} maturation.

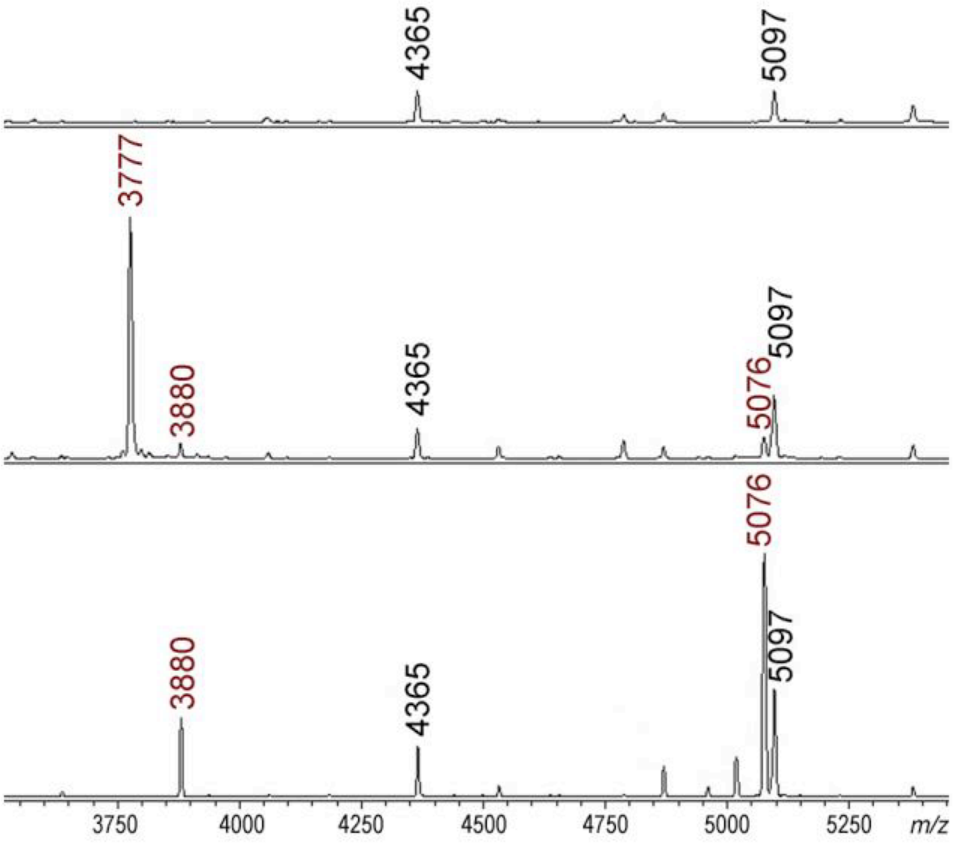


Fig. 13. MALDI-MS spectra of *E. coli* Δ*hns* cells harboring empty pDT1 vector (top) or the pDT1-Ymcc plasmid (middle), and Δ*hns E. coli* Δ*tldDtldE* harboring pDT1-Ymcc (bottom). The peaks of full-length $MccA^{Yps}$ modified peptide (*m/z* 5076) and its N-terminal proteolytic fragments 1-31(*m/z* 3777) and 1-32 (*m/z* 3880) are marked with red-color font. Mass-peaks present in control and experimental cells match *E. coli* ribosomal proteins RL36 (*m/zcalc* 4364) and RS22 (*m/zcalc* 5097).

Mass spectrometric analysis of intracellular contents of Δ*tldD ΔtldE* Δ*hns* triple mutant transformed with pDT1-Ymcc revealed, compared to *Δhns* mutant cells, *m/z* 5076 signal was strongly increased. It corresponds to full-sized $MccA^{Yps}$ with C-terminal modifications. The *m/z* 3777 peak corresponding to N-terminal product of cleavage that generates bioactive McC^{Yps} was absent from the spectra of *ΔtldD ΔtldE Δhns* triple mutant cells (Figure 13).

To test whether the cells carrying deletions of Δ*tldD ΔtldE* produce active McC^{Yps}, the cells carrying deletions of *tld* genes and *hns* were transformed with pDT1-Ymcc and tested for bioactivity by depositing aliquots (15 μL) of the cell suspensions under scrutiny on the freshly prepared lawn of *E. coli* B cells harboring pDT1 plasmid.

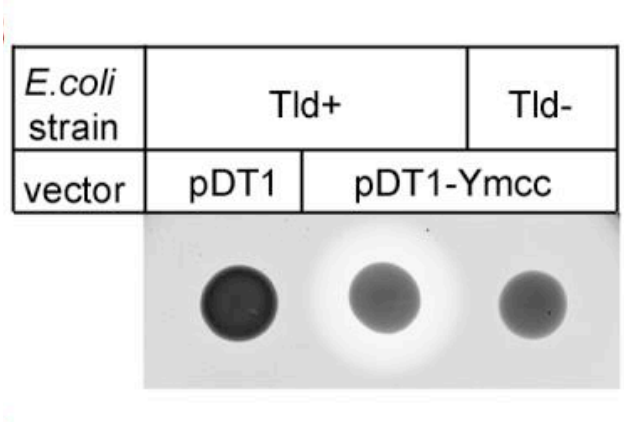


Fig. 14. The *tld* genes are required for active McC^{Yps} production. Growth inhibition zones on the lawn of *E. coli* B cells were formed around patches of Tld+ (*E. coli* Δ*hns*) and Tld- (*E. coli* Δ*hns* Δ*tldE* Δ*tldE*) strains harboring pDT1-control or pDT1-Ymcc plasmids. The cells were pelleted, then washed with M9 medium and resuspended in 200 μL of M9 medium. Aliquots (15 μL) of the cell suspensions were deposited on the freshly prepared lawn of *E. coli* B cells harboring pDT1 plasmid.

Therefore, we conclude that the TldD/E protease is an essential component of McC^{Yps} production. A minor *m/z* 3880 peak was seen in Δ*hns* cells with or without *tld* mutations. This peak corresponds to the N-terminal leader fragment of MccA^{Yps} cleaved at position 32 (Figure 13). The intensity of this peak is increased in *tld* mutants suggesting that there are additional proteases capable of off-target cleavage that does not result in the biologically active compound.

The TldD/E protease is sufficient for endoproteolytic processing of pro-McC^{Yps}

The TldD/E proteins are highly conserved in eubacteria and *Y. pseudotuberculosis* TldD and TldE are 85 and 83% identical to *E. coli* counterparts. Taking this into account, we decided to check if *Y. pseudotuberculosis* TldD and TldE are responsible for the endoproteolytic processing of pro-McC^{Yps}.

Recombinant *Y. pseudotuberculosis* TldD and TldE were purified by Ni-NTA chromatography and MonoQ chromatography, after that *in vitro* reactions with MccA^{Yps}-CMP were performed. Reactions with recombinant *E. coli* TldD/E were used as a control.

Recombinant *Y. pseudotuberculosis* TldD and TldE when present together and combined with MccA^{Yps}-CMP cause accumulation of a *m/z* 3777 mass-ion. This corresponds to N-terminal leader fragment present in *E. coli* cells with intact *tld* genes. No corresponding C-terminal peptide-nucleotide fragment was detected suggesting that it was rapidly degraded. A similar result was obtained by the recombinant *E. coli* TldD/E.

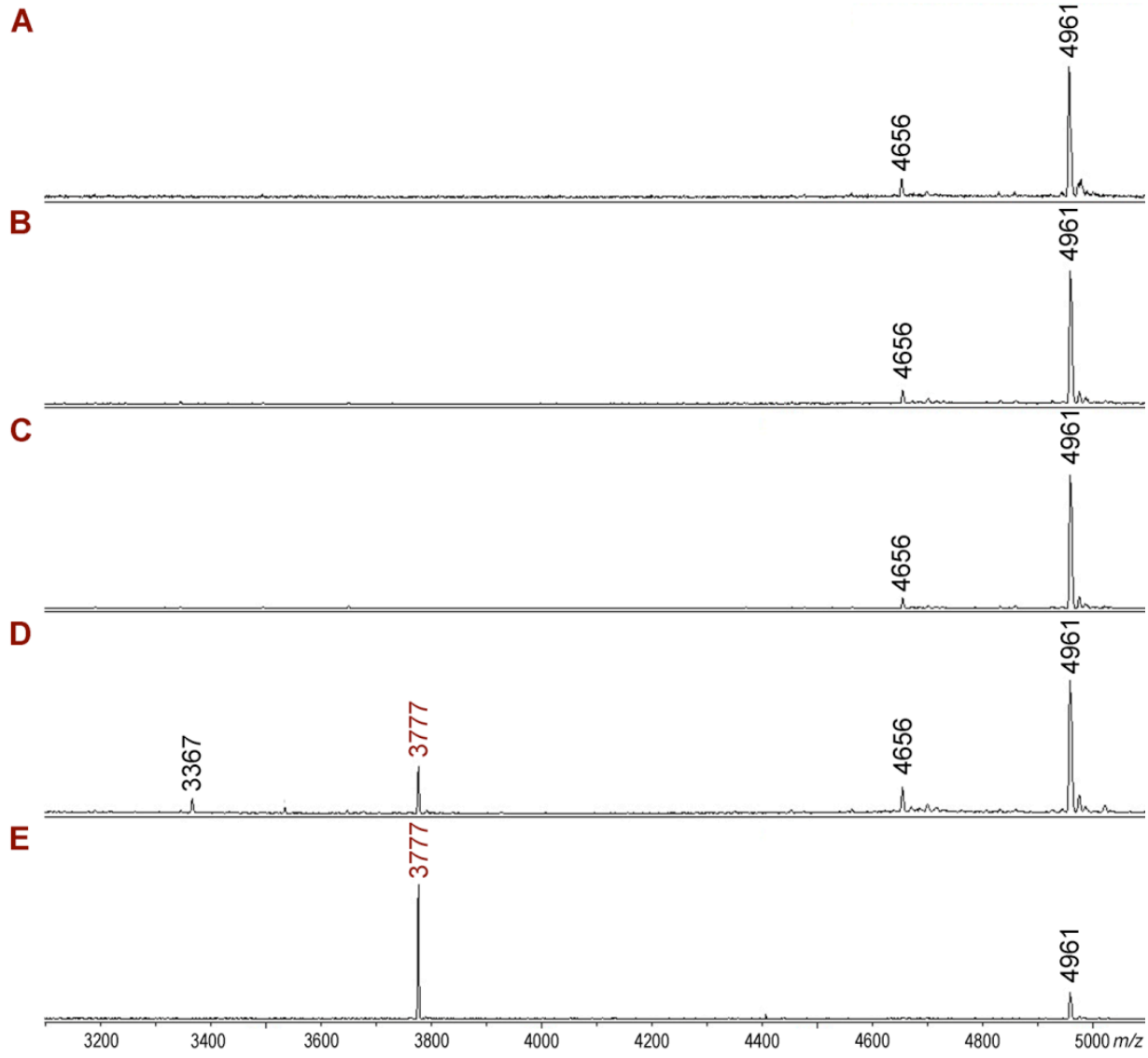


Fig. 15. MALDI-MS analysis of the products of *in vitro* proteolysis by TldD/E. Cytidylated MccA^{Yps} (*m/z 4961*) was treated with recombinant TldD^{Yps} (B), TldE^{Yps} (C), a mixture of TldD^{Yps} and TldE^{Yps} (D), or *E. coli* TldD/E enzyme (E). The *m/z* 3777 mass-peak that appears in the presence of TldD^{Yps} and TldE^{Yps} (D), or *E. coli* TldD/E (marked

by red-color font *m/z* value) corresponds to the major MccA^{Yps} proteolytic peptide accumulated in *E. coli* cells expressing the *mcc*^{Yps} gene cluster.

The results may imply that *in vivo* McC^{Yps} is removed from the producing cells immediately after processing to prevent further degradation. Consequently, the TldD/E protease is responsible for proteolytic maturation of pro-McC^{Yps} in the natural host *Y. pseudotuberculosis*.

Antibacterial activity of McC^{Yps} depends on proteolytic cleavage of N-terminal leader peptide from pro-McC^{Yps}

Accumulation of unprocessed microcin C-like compound in *E. coli Δhns ΔtldD ΔtldE* strain harboring pDT1-Ymcc allowed us to purify pro-McC^{Yps} and full length modified MccA^{Yps}, as well as to test its activity against *E. coli* B cells. For that 5 μl drops with equal concentrations of McC from *E.coli* and *Y. pseudotuberculosis* were deposited on freshly prepared lawns of *E. coli* B strain. After an overnight culturing at 30 ^{o}C, the inhibition zones on lawns of tester cells were visible. The compound was found to be inactive at concentrations when McC^{Yps} produced robust growth inhibition zones on lawns of wild-type *E. coli*.

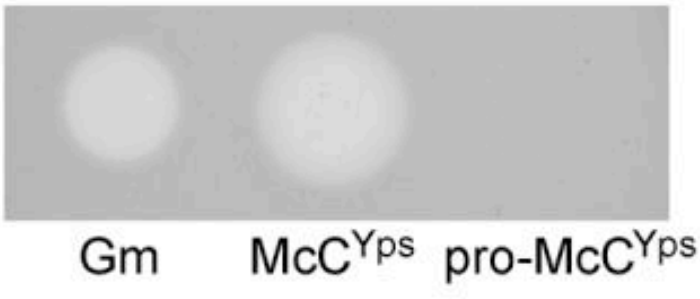


Fig. 16. Comparison of inhibitory activity of pro-McC^{Yps} and McC^{Yps} against *E. coli*. 5-μL drops of 40 μM solutions of McC^{Yps} and pro-McC^{Yps} purified from *tld- E. coli* were deposited on a fresh lawn of *E. coli* B cells. Growth inhibition zones appearing after 16 h incubation at 30 °C are shown. A 5-μL drop of 0.5 mg/ml gentamycin (Gm) was used as a positive control.

Summary 1

1. The TldD/E protease is responsible for the endoproteolytic processing of pro-McC^{Yps} inside producing cells.
2. Antibacterialy active form of McC^{Yps} does not contain N-terminal leader peptide.

Overall, our data demonstrate that the conservative cellular proteases TldD/E play a crucial role in McC^{Yps} maturation process. The processing of modified MccA^{Yps} inside the producing cell is carried out by TldD/E. The exact function of this highly evolutionary conservative endoprotease remains obscure. Recent structural works indicate that it may be a general-purpose enzyme capable of degrading unstructured polypeptides [169, 170]. In *E. coli*, TldD/E is essential to produce microcin B, a thiazole-oxazole containing gyrase inhibitor that is unrelated to McC. During the microcin B synthesis, TldD/E removes the leader part of pro-microcin B molecule after post-translational modifications required for the activity to be introduced by the microcin B synthase [170, 171]. There is evidence that TldD/E cleavage of pro-microcin B is associated with its export outside the producing cell [170, 171]. The situation may be similar to the case of McC^{Yps} for two reasons because we fail to detect mature McC^{Yps} inside the producing cells. Solely the N-terminal MccA^{Yps} fragment is detected during *in vitro* digestion while the McC^{Yps} is degraded. The existence of long N-terminal peptide in pro-McC^{Yps} preventing antibacterial activity of that molecule may allow producing cells to avoid self-intoxication. The presence of stable N-terminal 31-amino acid MccA^{Yps} TldD/E cleavage fragment inside the producing cells suggests that this peptide is structured and may have a function of its own, for example in regulating the *mcc^{Yps}* expression.

Part 2: Study of microcin C-induced persistence

As it was previously shown, one of the ways to induce persistence involves HipA, a serine-threonine kinase that inhibits cell growth. It phosphorylates serine residue near the active center of glutamyl-tRNA synthetase leading to inhibition of $tRNA^{Glu}$ aminoacylation [84, 85]. The absence of glutamyl-$tRNA^{Glu}$ stimulates generation of "hungry" codons at the ribosomal A site, which in turn triggers the synthesis of (p)ppGpp and, therefore, induces persistence. McC from *E.coli* and *Y. pseudotuberculosis* is known to interact with aspartyl-tRNA synthetase and prevent aminoacylation of $tRNA^{Asp}$ *in vitro* [26]. Since McC from *E.coli* and *Y. pseudotuberculosis* target an enzyme of the same class as HipA, a question most naturally raises: can McC from *E.coli* and *Y. pseudotuberculosis* induce persistent cells formation as well. In the following section, this question is addressed.

McC from *E.coli* and *Y. pseudotuberculosis* target aspartyl-tRNA synthetase

Both McC from *E.coli* and *Y. pseudotuberculosis* have antimicrobial activity against *E.coli* B cells. In order to determine that aspartyl-tRNA synthetase is a cellular target of McC from *Y. pseudotuberculosis* as McC^{Eco}, 12.5 μM of McC^{Eco} or McC^{Yps} was added to *E. coli* S30 extracts and, after incubation for various times, aminoacylation reactions of $tRNA^{Asp}$ were carried out (Figure 17). Aminoacylation levels remained unchanged after the first 5 minutes of incubation compared to control extracts (no microcin added), then decreased from 5 to 10 minutes of incubation and remained stable afterwards. The kinetics of inhibition suggest that processing of peptide parts of both microcins proceeds at similar rates. While the reactions containing McC^{Eco} retained only 5% of aminoacylation activity after processing was complete, the reactions containing McC^{Yps} were inhibited by 50%. Thus, as expected, the McC^{Yp}s warhead, an aspartamide attached to aminopropylated carboxymethyl CMP, is a worse mimic of aspartyl adenylate compared to aminopropylated aspartamidyl-AMP produced after processing of McC^{Eco}.

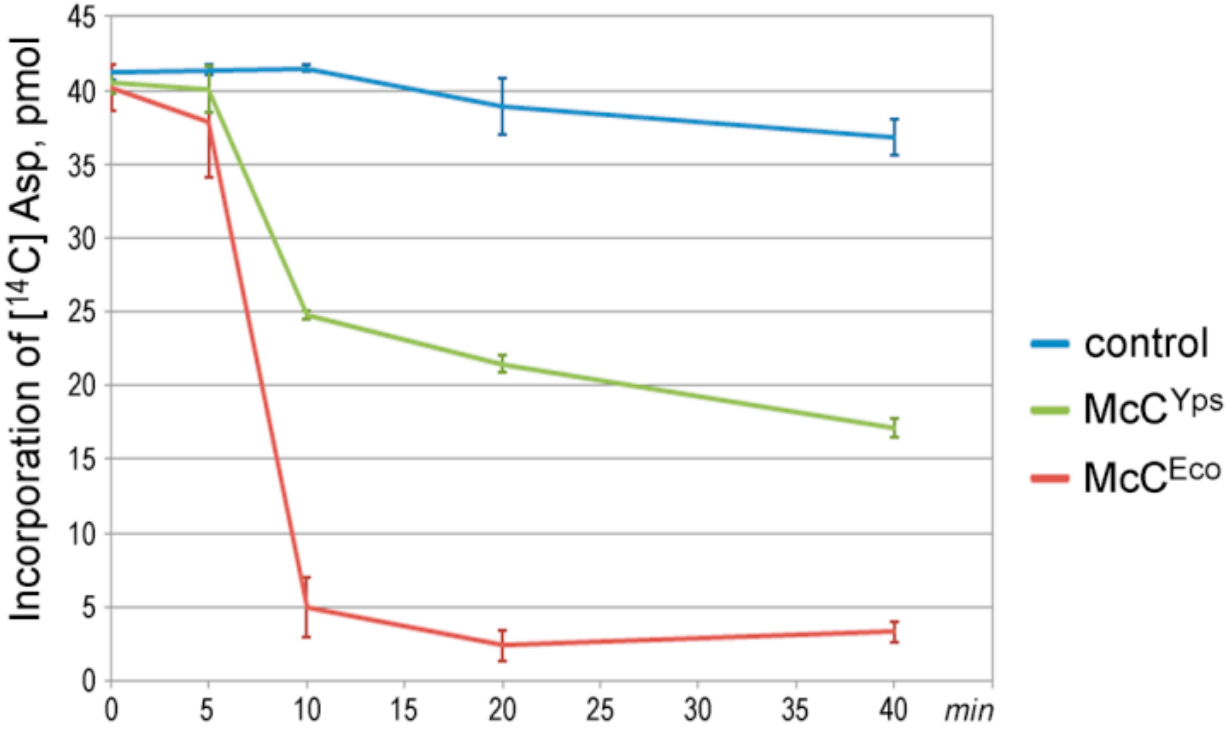


Fig. 17. In vitro inhibition of aminoacylation of $tRNA^{Asp}$ by McC^{Yps} and McC^{Eco}.
S30 extracts prepared from wild-type *E. coli* B cells were supplied with HPLC-purified compounds to final concentration 12.5 μM. After indicated times of incubation at 37°C, and $tRNA^{Asp}$ aminoacylation was measured by determining incorporation of [^{14}C]

aspartate into the acid-insoluble fraction. Water was used as a blank control. Error bars show standard deviations calculated from three independent measurements.

Despite McC from *E.coli* and *Y. pseudotuberculosis* have similar activity against aspartyl-tRNA synthetase, McC from *E.coli* is produced and purified with better yield in our system As a result, we decided to concentrate our experiments on McC from *E.coli.*

Antimicrobial activity of McC from *E.coli* in liquid medium

The growth of McC^{Eco}-sensitive wild-type *E. coli* liquid cultures supplemented with various concentrations of McC^{Eco} was measured. Growth was significantly inhibited by McC^{Eco} when present at concentrations of 5 μM or higher (Figure 18A and B).

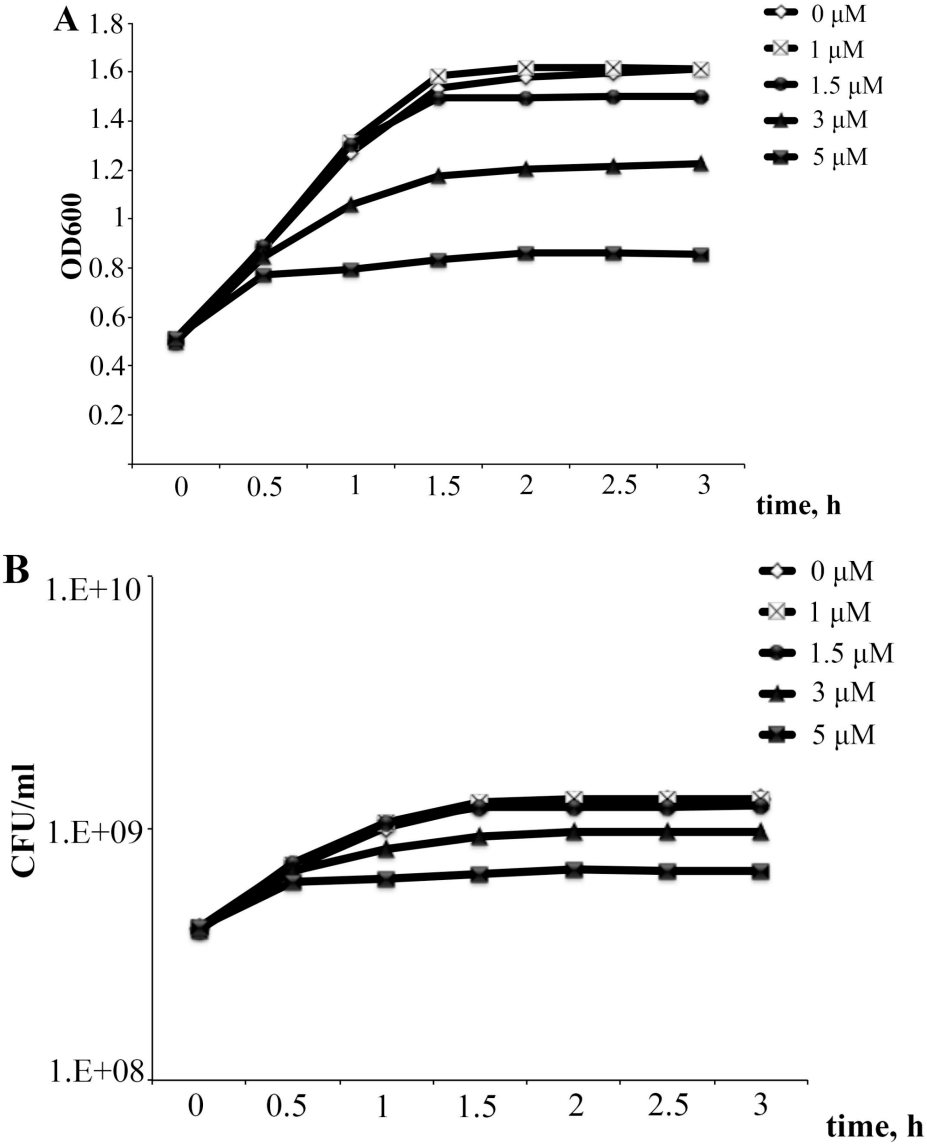


Fig. 18. Growth curves and CFU analysis of cells after McC^{Eco} treatment. (A) Growth curves of MG1655 (wt) strain after McC^{Eco} treatment. The overnight culture was diluted

100-fold in LB and growth was allowed to continue at 37 ^{o}C. When OD_{600} reached 0.5, the indicated concentrations of McC^{Eco} were added to culture aliquots and further growth was monitored by following OD_{600} at indicated time points. (B) As in (A), but showing the number of colony forming units present in cultures as assayed on LB agar plates at various time points after the McC^{Eco} addition.

Microcin C from *E.coli* dramatically increases persistence

In order to determine whether McC^{Eco} is able to induce persister cells formation, cultures that had been supplemented with McC^{Eco} (and untreated control cultures) were treated with lethal concentrations of ciprofloxacin. At various times after adding Cfx, culture aliquots were removed; the cells were washed and plated on LB agar plates without antibiotics to determine the number of surviving colonies (CFUs). In the absence of McC^{Eco}, the frequency of cells that survived the Cfx treatment was ~10^{-4} (0.01% surviving cells, Figure 19A and B). The addition of McC^{Eco} increased survival in a concentration-dependent manner. At the highest tested concentration of 5 μM, when McC^{Eco} strongly inhibited cell growth, 10% of cells in the culture survived the Cfx treatment. In the presence of 1.5 μM McC^{Eco}, when its effect on culture growth was hardly perceptible, up to 1% of the cells survived the Cfx treatment, which is a 100-fold increase compared to control cultures without McC^{Eco}. Therefore, McC^{Eco} induces persister cell formation when added to a growing culture of *E. coli* cells.

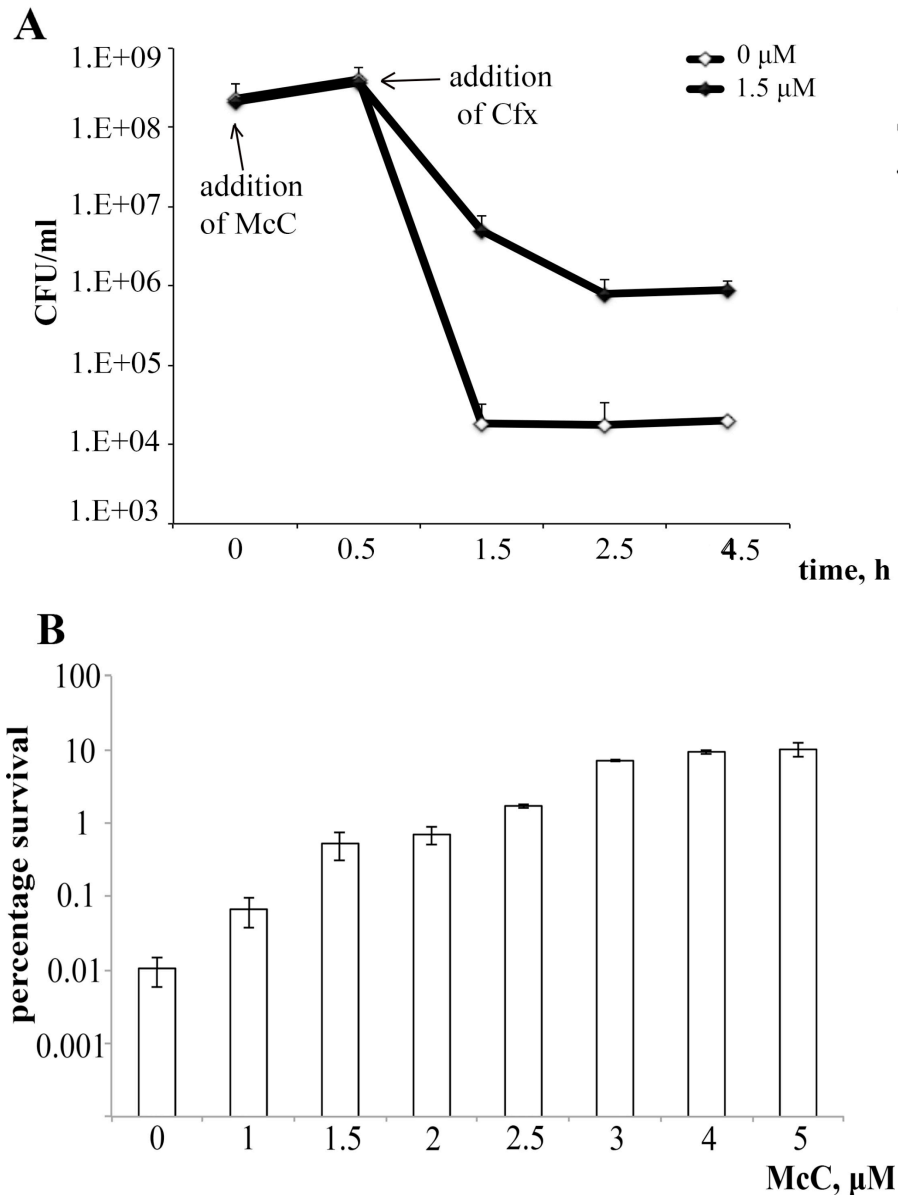


Fig. 19. Microcin C from *E.coli* induces persistence in growing *E. coli* cultures. (A) An example of killing curves obtained after ciprofloxacin treatment of MG1655 (wt) culture with or without McC^{Eco} (1.5 μM). The overnight culture was diluted 100-fold in LB and growth was allowed to continue at 37°C. When OD_{600} reached 0.5, the indicated concentrations of McC^{Eco} were added to culture aliquots. After 30 min incubation with McC^{Eco}, 1 μg/ml Cfx was added. The incubation was continued and culture aliquots were removed at various time points followed by CFU determination. Mean values and standard deviation obtained from three independent experiments are shown. (B) For each killing curve obtained with or without McC^{Eco}, the percentage of surviving cells was calculated after 4-hour incubation in the presence of Cfx. Error bars indicate the standard deviations of averages of at least 3 independent experiments, p-value < 0,001 (two-tailed Student's t-test) compared with wild-type.

McC^{Eco} induces stringent response

Since McC^{Eco} inhibits aspartyl-tRNA synthetase, it should cause accumulation of uncharged $tRNA^{Asp}$, which in turn should lead to production of (p)ppGpp and stringent response. To test this hypothesis, two cultures: a wild-type and a strain with deletion of *relA,* a gene that encodes ribosome-dependent (p)ppGpp synthetase, were subjected to McC^{Eco} treatment. The overnight cultures were diluted 100-fold in MOPS glucose minimal medium supplemented with all amino acids and incubated at 37 °C with shaking. At OD_{600}~0.05, cells were supplemented with $H_3{}^{32}PO_4$. After 2–3 generations, 3 μM McC were added to the growing cultures. Samples were collected 0, 10, 30 and 60 min after the addition of McC^{Eco} and aliquots of cell extracts were separated by PEI Cellulose TLC. As can be seen from Figure 20A, McC^{Eco} induced strong accumulation of ppGpp. The (p)ppGpp synthesis was dependent on the presence of RelA (Figure 20A). To determine if McC^{Eco}-induced persistence is mediated by (p)ppGpp accumulation, the persistence assay was performed with mutant cells lacking the *relA* gene. Cells lacking both *relA* and *spoT* were also tested. Mutant cells lacking *dksA* were tested in a persistence assay along with (p)ppGpp synthetase-deficient cells. The results, shown in Figure 20B, demonstrate that the fraction of cells surviving the Cfx treatment in the absence of McC^{Eco} decreased in the order wt > Δ*relA* > Δ*relAspoT*, as reported previously for HipA [62, 84, 172]. In the presence of McC^{Eco}, survival of *relA* and *relAspoT* mutants was increased 10-fold, whereas the survival of wild-type cells increased 100-fold in these conditions. By contrast, deletion of *dksA* had no such effect (Figure 20B), suggesting that McC^{Eco}-mediated persistence does not require DksA. Therefore, McC^{Eco}-mediated persistence has a clear (p)ppGpp-dependent component with the contribution of other mechanisms.

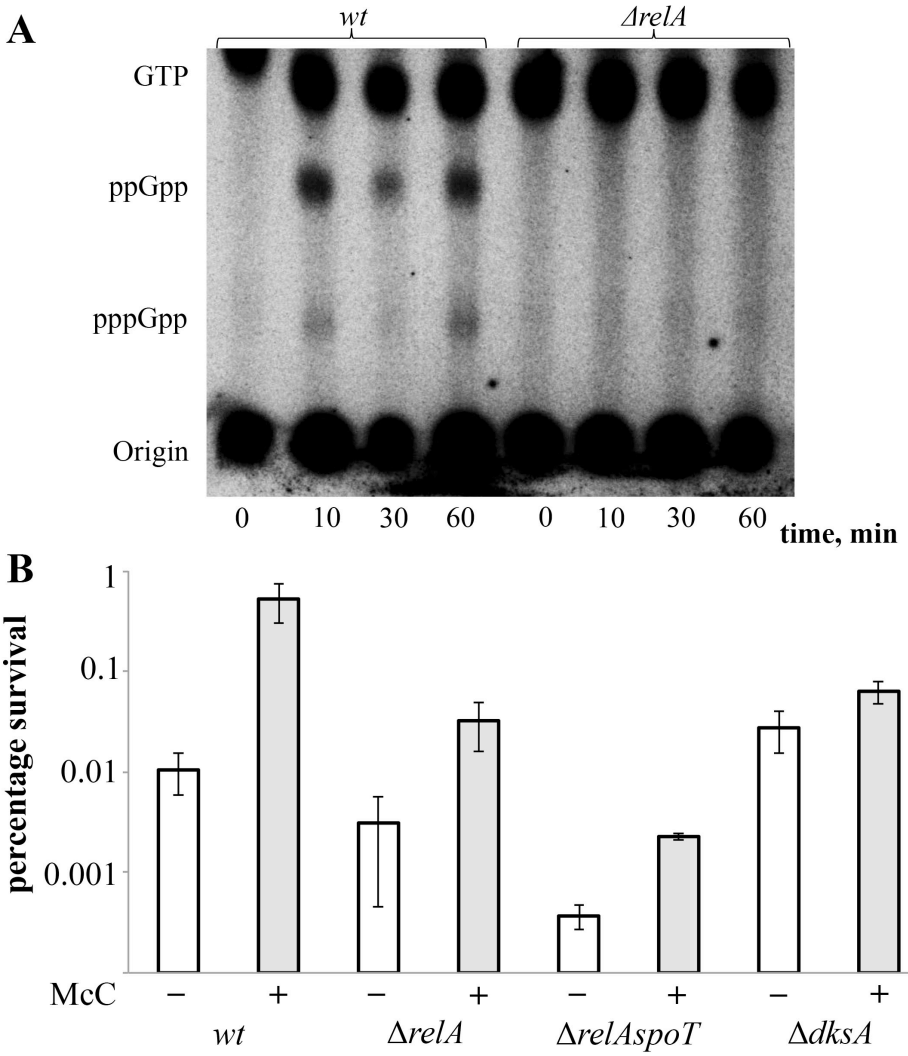


Fig. 20. McC^{Eco} treatment leads to (p)ppGpp production, which contributes to persister formation. (A) Accumulation of (p)ppGpp following McC^{Eco} addition. MG1655 and the Δ*relA* isogenic deletion strain were grown exponentially in a low-phosphate MOPS minimal medium. At OD_{600}~0.05, they were labeled with $H_3{}^{32}PO_4$. After 2–3 generations, 3 μM McC^{Eco} were added. Samples were collected before and 10, 30 and 60 minutes after McC^{Eco} addition (3 μM), separated by thin-layer chromatography (TLC), and (p)ppGpp was identified using chemically synthesized standards. A representative autoradiograph of a thin-layer chromatography plate is shown. (B) Exponentially growing cells of MG1655 and isogenic Δ*relA*, Δ*relAspoT* and Δ*dksA* deletion strains, untreated (white bars) or pretreated with 1.5 μM McC^{Eco} (grey bars), were treated to 1 μg/ml of ciprofloxacin. The percentage of survival after 4 h of antibiotic treatment is shown (log scale). Error bars indicate the standard deviations of averages of at least 3 independent experiments, p-value < 0,05 (two-tailed Student's t-test) compared with and without McC^{Eco}.

McC^{Eco} induced persistence depends on TA modules, Lon and polyphosphate

Previous analysis showed that the high (p)ppGpp level caused by HipA expression trans-activated the Toxin-Antitoxin(TA)-encoded RNases, which in turn led to high persistence [62, 84, 172]. In addition, HipA-mediated trans-activation of RNases depended hierarchically on (p)ppGpp, polyphosphate (Poly(P)) and Lon protease [84, 173]. (p)ppGpp being an important component for McC^{Eco}-dependent persistence increase, predicted that such increase should also depend on Poly(P), Lon and TAs. Consistently, when cultures of *E. coli* cells lacking all 10 Type II TA systems encoding RNases were treated with McC^{Eco}, a more than 10-fold drop in the percentage of surviving cells compared to the wild-type control was observed (Figure 21). Moreover, deletion of *ppk-ppx,* encoding the poly(P) synthesis/degradation system, had a similar effect. Finally, deletion of *lon* abolished McC^{Eco}-dependent increase in persisters. Similar trends were observed in cells overproducing HipA [84, 173].

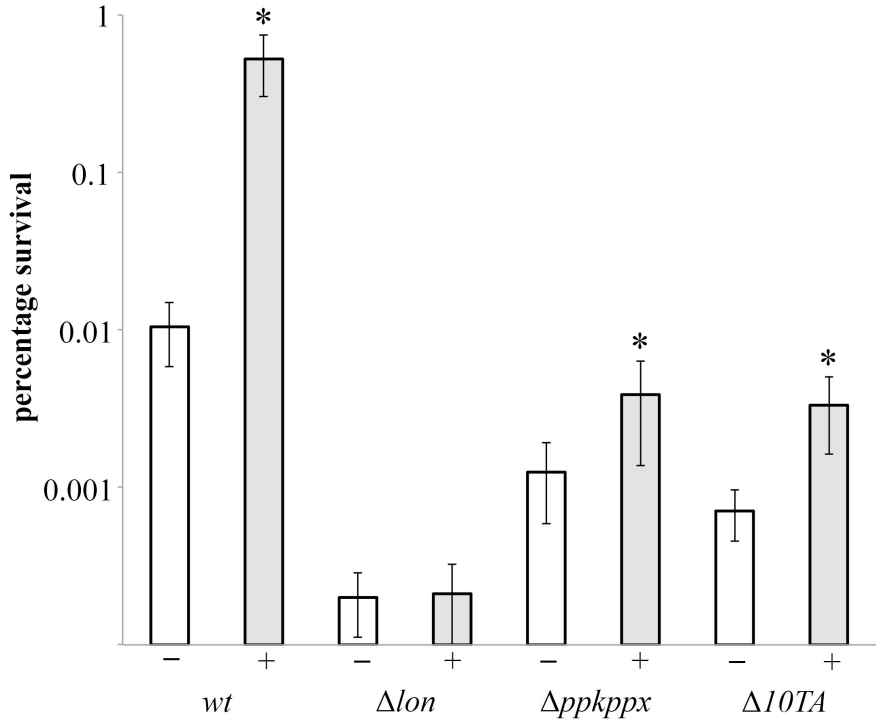


Fig. 21. Persistence induction by McC^{Eco} depends on Lon protease, inorganic polyphosphate and toxin-antitoxin modules. Exponentially growing cells MG1655 and isogenic deletion strains Δ*lon,* Δ(*ppkppx*) and Δ*10TA* cultures, untreated (white bars) or pretreated with 1.5 μM McC^{Eco} (grey bars), were treated to 1 μg/ml of ciprofloxacin. Percentage of survival after 4 h of antibiotic treatment is shown (log scale). Error bars

indicate the standard deviations of averages at least 3 independent experiments, where * indicates p-value < 0,05 (two-tailed Student's t-test) compared with and without McC^{Eco}.

McC^{Eco}-producing cells have increased levels of persistence

While McC^{Eco}-producing cultures are growing, they themselves could be affected by the McC^{Eco} they produce in the medium and thus have an increased fraction of persister cells. The previously described MG1655 *rpoS::mCherry E. coli* strain was used to directly test this hypothesis and visualize persisters at the single cell level by fluorescence microscopy. The level of *rpoS::mCherry* translational fusion (based on the activation of stationary-phase RNA polymerase σ^S factor) is related to the (p)ppGpp level and thus allows to identify individual persister cells [62]. MG1655 *rpoS::mCherry* cells transformed with the pp70 plasmid, which carries the entire *mcc*^{Eco}-cluster under the control of native promoters [22], or control empty vector were grown until the late logarithmic phase, when McC^{Eco} production begins, and examined microscopically. Statistical analysis of more than 120,000 cells indicated that in cells carrying pp70 the frequency of mCherry-positive cells was increased almost 30-fold compared to control cells (Figures 22A and B).

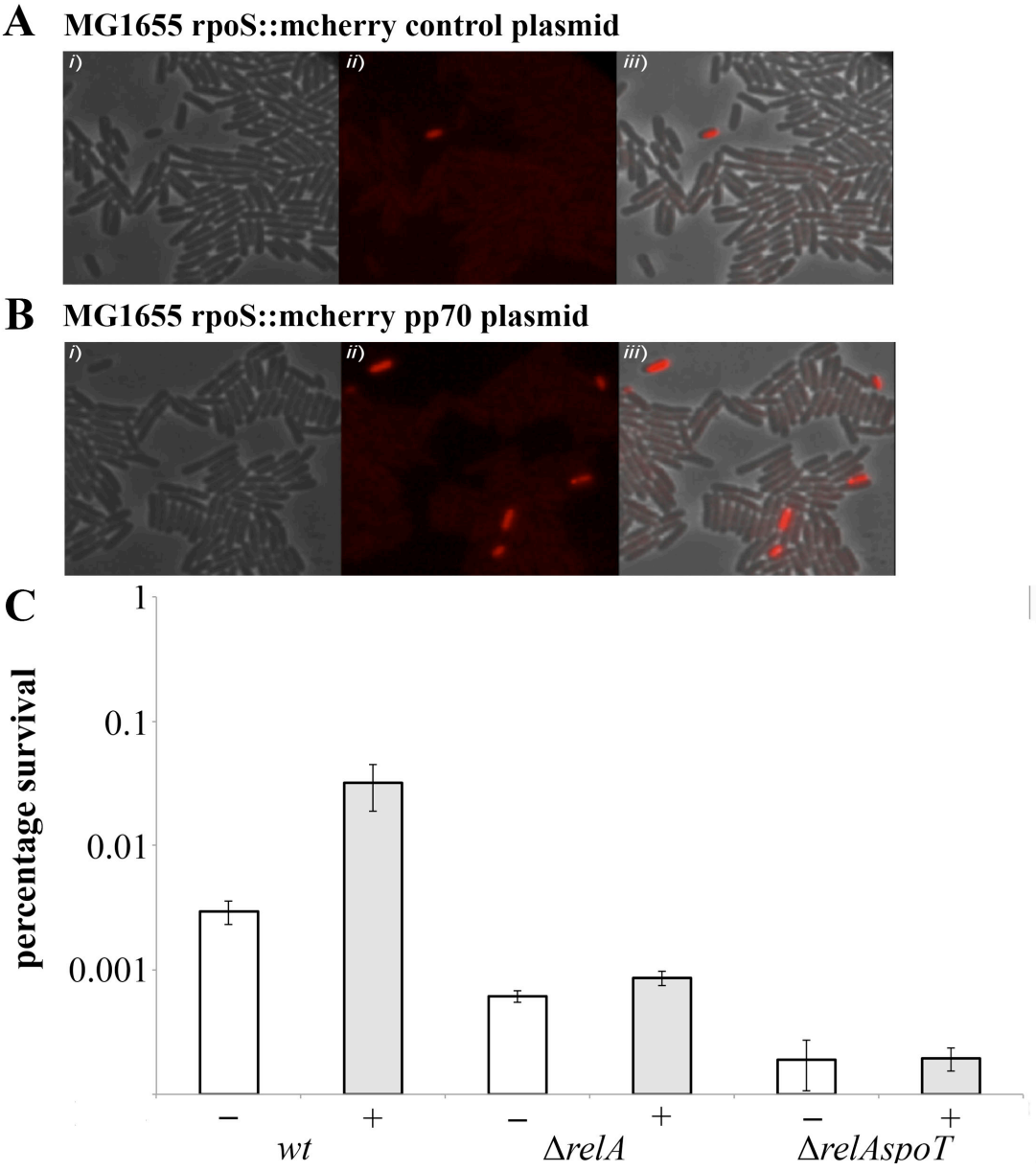


Fig. 22. McC^{Eco}-producing cells have increased frequency of (p)ppGpp "ON" cells and persister cells. (A) Snap-shot of exponentially growing cells of MG1655 (top panel), and MG1655 harboring the pp70 plasmid (carries the entire McC^{Eco} cluster *mccABCDEF* under a natural promoter) (bottom panel), carrying an *rpoS::mcherry* translational fusion. (i) Phase contrast, (ii) RpoS-mCherry fluorescence, (iii) overlay of (i) and (ii). (B) Exponentially growing cells of MG1655 and isogenic deletion strains Δ*relA* and Δ*relAspoT* with (grey bars) the pp70 plasmid were treated by 1 μg/ml of ciprofloxacin. The percentage of survival after 4 h of antibiotic treatment was compared to that of the control strains carrying the empty vector plasmid (white bars) (log scale). Error bars indicate the standard deviation of averages of at least 3 independent experiments, p-value < 0,05 (two-tailed Student's t-test) compared with and without McC^{Eco}.

In agreement with microscopic observations, at least 10-times more colonies surviving the Cfx treatment were observed in *E. coli* MG1655 cultures transformed with pp70 than in control culture transformed with empty vector (Figure 22C). Importantly, cultures of *relA* and *spoT* mutants harboring pp70 produced McC^{Eco} normally but did not show increased levels of persisters compared to isogenic non-producing controls (Figure 22C and 23). Thus, cells in McC^{Eco} producing wild-type cultures must have elevated levels of (p)ppGpp that lead to increased levels of persisters.

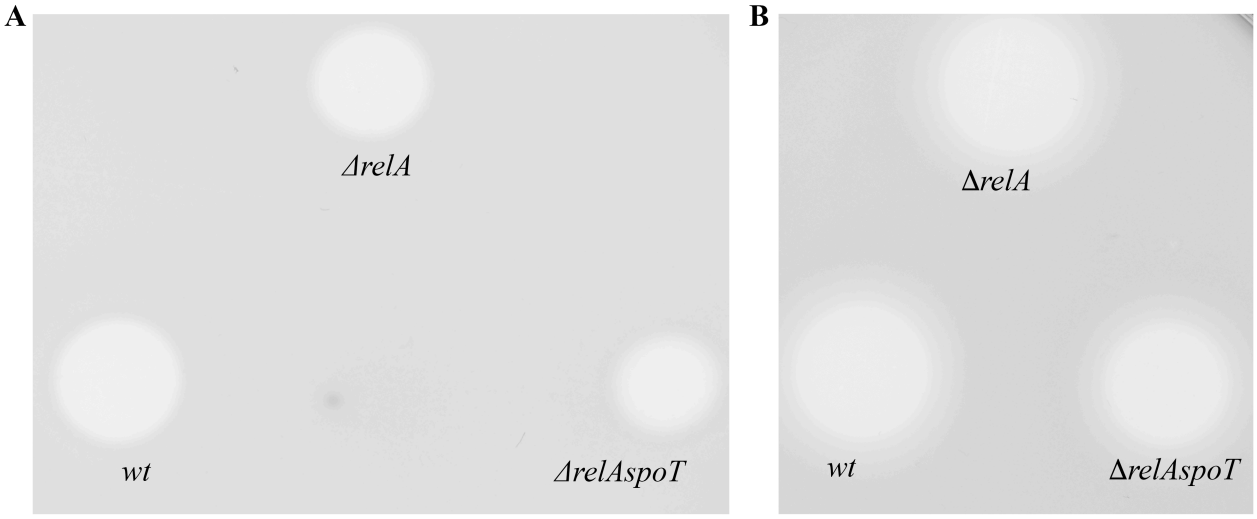


Fig. 23. Antibacterial activity of McC^{Eco} produced by wild-type (wt) or isogenic Δ*relA* or Δ*relAspoT* strains. Aliquots of the cultured media after 2 (A) or 4 (B) hours of growth of indicated strains carrying the *mcc* cluster plasmid were placed over a growing lawn of McC^{Eco} -sensitive *E. coli* tester cells. Growth inhibition zones are seen as clear circles on the turbid surface of the cell lawn.

To determine whether increased persistence in McC^{Eco} producers is due to endogenously produced or imported McC^{Eco}, persister levels were determined in cultures of McC^{Eco}-resistant *yejA* mutants (McC^{Eco} transport inside these cells is abolished) transformed with pp70. Though the mutant cultures produced the same amounts of McC^{Eco} as the wild-type cultures, the frequency of persisters decreased by more than tenfold (Figure 24). Thus, import of previously produced McC^{Eco} from the outside followed by processing clearly contributes to increased persistence levels of McC^{Eco} producing cultures. However, the percentage of *yejA* cells surviving the Cfx treatment in cultures transformed with pp70 was still ~10 times higher than in cultures transformed

with control vector plasmid (Figure 24). Thus, endogenous McC^{Eco} that accumulates in producing cells must also contribute to increased persistence levels.

The *mcc*^{Eco} operon encodes, in addition to the $MccC^{Eco}$ export pump, two proteins that contribute to autoimmunity of producing cells. The C-terminal domain of $MccE^{Eco}$ acetylates and inactivates processed McC^{Eco} [13]. $MccF^{Eco}$ is a hydrolase that cleaves the nucleotide moiety from either intact or processed McC^{Eco} [18]. Both proteins can lead to McC^{Eco} resistance when overexpressed. However, their contribution to auto-immunity of producing cells is not known. The cells that harbored pp70 derivatives encoding $MccE^{Eco}$ with inactivated acetyltransferase or non-functional $MccF^{Eco}$ were tested for persistence levels. Both kinds of cells produced McC^{Eco} normally. The cells lacking the $MccE^{Eco}$ acetyltransferase had the same levels of persisters as the cells harboring parental pp70 (Figure 24). By contrast, the cells lacking functional $MccF^{Eco}$ showed ~10-fold increase in persistence levels. It therefore follows that $MccF^{Eco}$ is a more active contributor to McC^{Eco} detoxification in producing cells. The same result was obtained in Δ*yejA* producers (Figure 24), indicating that $MccF^{Eco}$, but not $MccE^{Eco}$ detoxify endogenous McC^{Eco} that enters producing cells from the cultured medium.

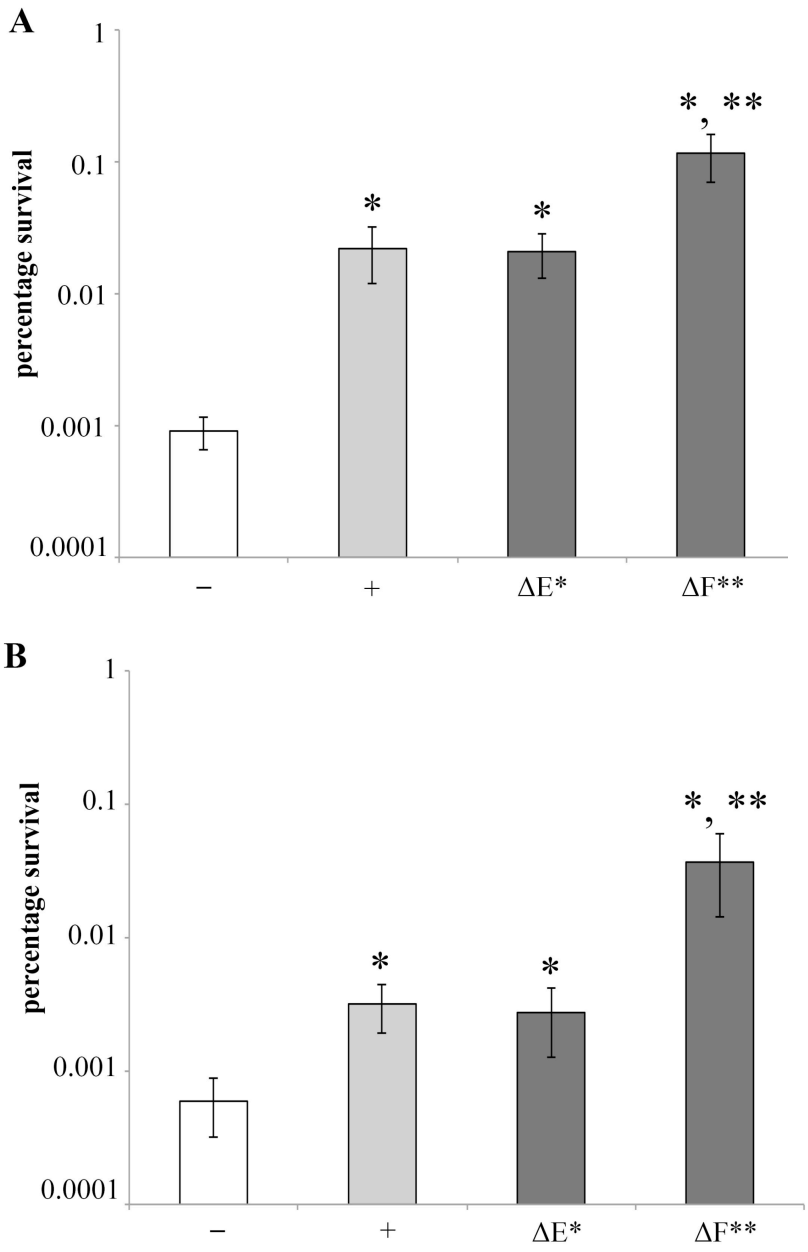
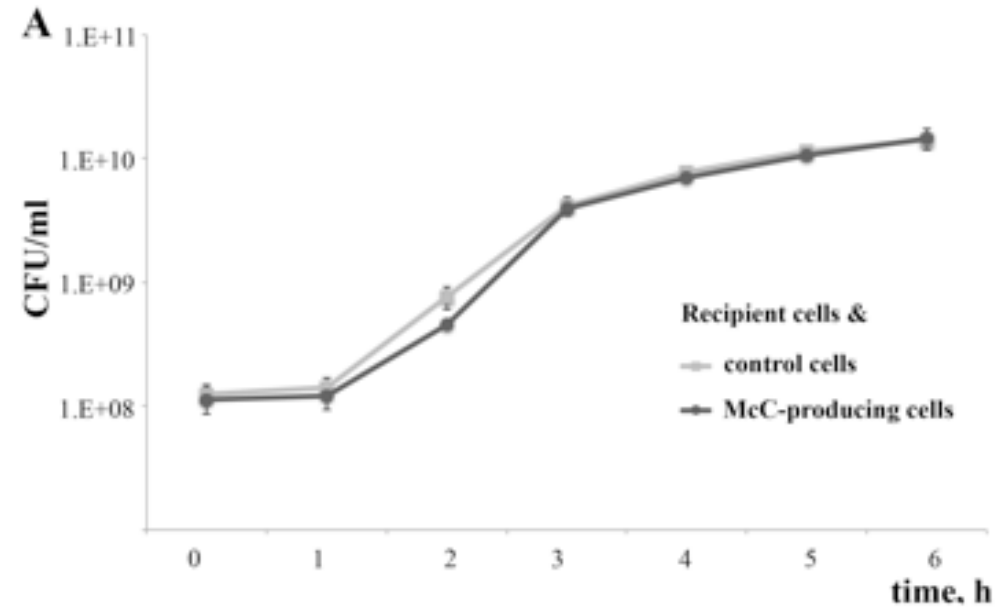


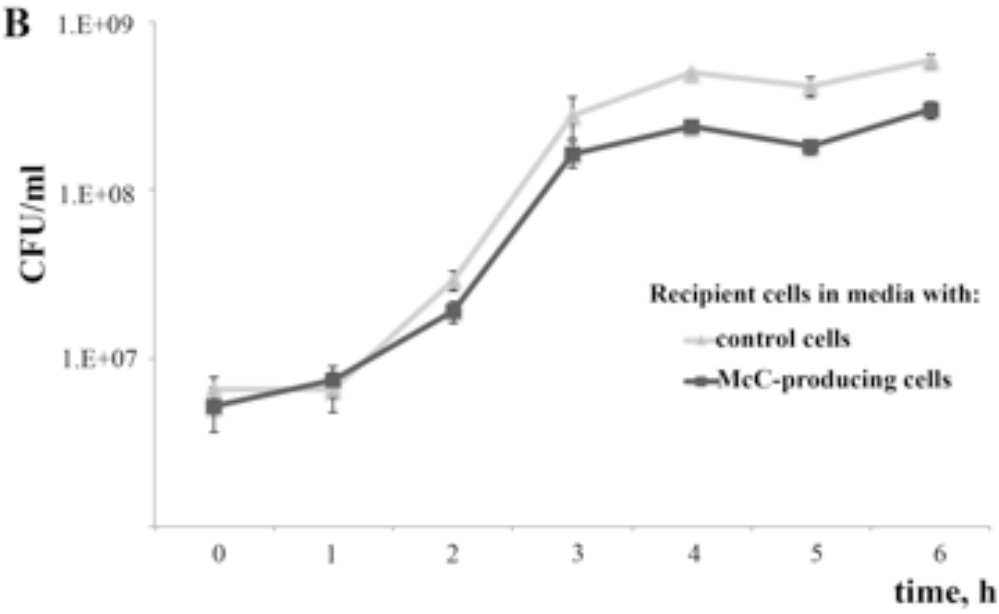
Fig. 24. Persister levels in wild-type and McC^{Eco}-resistant *yejA* mutant cells producing McC^{Eco} from complete plasmid-borne *mcc* operon or *mcc* operons with disrupted autoimmunity genes. Exponentially growing cells of MG1655 (A) or the isogenic deletion strains Δ*yejA* (B) harboring either the pp70 or pp70ΔmccF (same as pp70 but lacking functional *mccF*^{Eco} due to S118A mutation) or pp70ΔmccE (same as pp70 but carries *mccE*^{Eco} with inactivated acetyltransferase due to S553A and E572A mutations) were treated by 1 μg/ml of ciprofloxacin. The percentage of surviving cells after 4 h of antibiotic treatment was compared with that of the control strains carrying the empty vector plasmid (white bars) (log scale). Error bars indicate the standard deviations of the averages of at least 3 independent experiments, where * indicates p-value < 0.05 (two-

tailed Student's t-test) compared with control strains, and ** indicates p-value < 0.05 (two-tailed Student's t-test) compared with McC^{Eco}-producing strains.

McC^{Eco} producing cells induce persistence during co-culturing

Since microcinogenic cells show increased persistence levels caused by the combined effect of internal and external McC^{Eco}, the ability of McC^{Eco} produced in the culture to induce persistence in non-microcinogenic cells were tested. To this end, cells transformed with pp70 (or control vector) with McC^{Eco}-sensitive "recipient" cells marked with a chromosomal tetracycline resistance gene were co-cultured. The co-culturing experiment was initiated by introducing equal amounts of both kinds of cells into a fresh medium containing ampicillin to maintain the McC^{Eco} producing plasmid or control vector. The overall CFU numbers in co-cultures with pp70 or control vector cells were identical during the course of the experiment (Figure 25A). When the amounts of tetracycline-resistant non-microcinogenic cells were monitored, no difference between co-cultures with or without the pp70 plasmid was observed during logarithmic stage of growth. However, when the cultures reached the stationary phase, the amount of tetracycline-resistant cells in co-cultures with pp70-transformed cells was found to be ~5-fold lower (Figure 25B). Since inhibitory concentrations of McC^{Eco} were present when the co-culture reached the stationary phase, McC^{Eco} exported into the medium by the producing cells must have caused the decrease in tetracycline-resistant cells. To get support for this notion, the level of persistence in McC^{Eco}-sensitive recipient cells was determined. As can be seen (Figure 25C), recipient cells co-cultured with McC^{Eco} producers exhibited 100-fold higher persistence levels than the same cells co-cultured with control non-producing cells or when grown in monoculture. Therefore, McC^{Eco} producers not only inhibit the growth of sensitive cells but also induce their persistence state.


A
1.E+11
1.E+10
1.E+09
1.E+08
CFU/ml
Recipient cells &
control cells
McC-producing cells
0
1
2
3
4
5
6
time, h


B
1.E+09
1.E+08
1.E+07
CFU/ml
Recipient cells in media with:
control cells
McC-producing cells
0
1
2
3
4
5
6
time, h

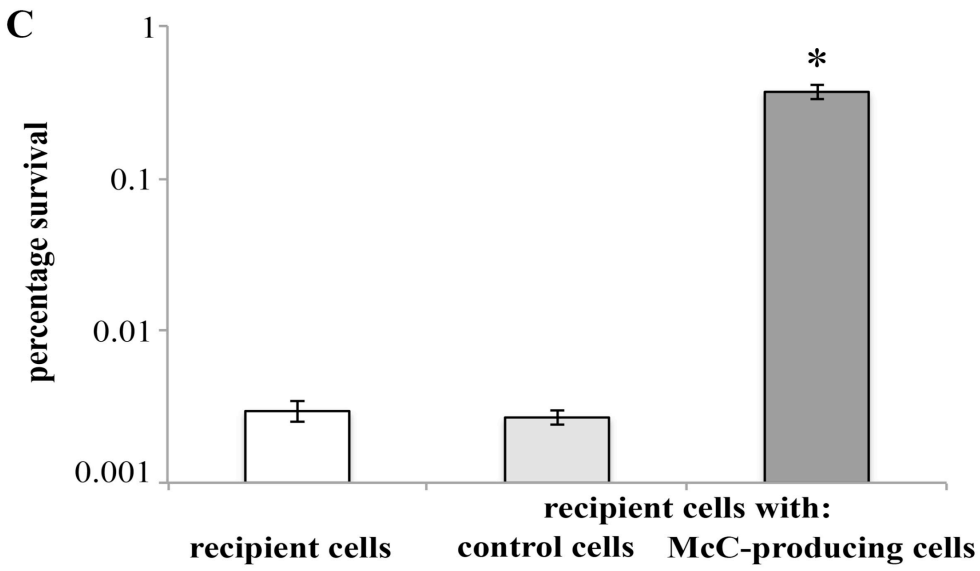

C
1
0.1
0.01
0.001
percentage survival
*
recipient cells
recipient cells with:
control cells
McC-producing cells

Fig. 25. McC^{Eco}-producing cells induce persistence in McC^{Eco}-non-producing cells during co-cultivation. (A and B). Growth curves of co-cultures of recipient tetracycline-resistant cells with McC^{Eco}-producing cells or non-producing control cells are shown. Total (recipient and McC^{Eco}-producing/non-producing (control)) cell counts are shown in (A). Amounts of recipient cells grown with McC^{Eco} producing or non-producing control cultures are shown in (B). (C) Persister levels in recipient cells grown alone (white bar) or co-cultivated with McC^{Eco}-producing (dark grey bar) or non-producing control cells (grey bar). Error bars indicate the standard deviations of averages of at least 3 independent experiments, where * indicates p-value < 0,005 (two-tailed Student's t-test) compared with the control strain.

Summary 2

1. Microcin C from *E. coli* is a potent inducer of persisters in *E. coli* populations.
2. McC^{Eco} increases persistence in a concentration-dependent way.
3. McC^{Eco}-dependent persistence is dependent on ppGpp, Lon protease, inorganic polyphosphate and genomic Type II toxin-antitoxin modules.
4. McC^{Eco}-producing bacteria have increased levels of persistence than it is potentiated by the MccF auto-immunity protein.
5. McC^{Eco}-producing cells induce persistence in sensitive cells during co-cultivation.

Overall, our data demonstrate that McC^{Eco} action is similar to that of HipA, which targets a different aminoacyl-tRNA synthetase. Persistence induced by McC^{Eco} is mediated by (p)ppGpp and requires chromosomally encoded toxin-antitoxin modules. McC^{Eco}-producing cells have increased persistence levels due to the combined effect of McC^{Eco} imported from the cultured medium and intracellularly synthesized antibiotic. However, unlike HipA, a cell-autonomous persistence inducing agent, McC^{Eco} acts on sensitive cells from without and is therefore a non-autonomous persistence-inducing agent. Presumably, any agent targeting an aminoacyl-tRNA synthetase will also act in the same way.

However, our results indicate that the interaction between McC producers and non-microcinogenic sensitive cells may be more complex since McC production also increases persistence in the recipient cells population. The observed increase is stronger than in the producing cells as recipient cells lack the McC export pump and dedicated detoxifying enzymes. Thus, taking persistence into account, there is an underscoring complex interaction in bacterial communities where an antagonistic compound produced by one community member can be beneficial for other members by increasing their ability to withstand antibiotics.

Although McC^{Eco} has high antimicrobial activity levels, it strongly induces persistent cell formation in sensitive bacteria, which will limit its practical use, especially when combined with bactericidal antibiotics.

Conclusion

The world population currently faces a global antibiotic crisis, as the most effective early antibiotics now are losing their ability to act powerfully against bacterial pathogens. The main reason for this is increased bacterial resistance to drugs. Growing resistance of pathogenic strains makes the discovery of new antimicrobial agents very important, and the modification of natural antimicrobial peptides, including microcins, is a highly promising direction for such research.

In this work was shown that microcin C from *E. coli* induces persistence in *E. coli*. Analogously to HipA, which inhibits glutamyl-tRNA synthetase, McC^{Eco}, an inhibitor of aspartyl-tRNA synthetase, causes production of (p)ppGpp, which activates the (p)ppGpp-dependent persistence formation pathway that is responsible for about 90% of the observed increase in persisters. Formation of the remaining, (p)ppGpp-independent persisters depends on the toxin-antitoxin systems encoded in the *E. coli* genome.

While the levels of persistence caused by McC^{Eco} are dependent on its concentration, the persistence increases obtained in the presence of concentrations that inhibit cell growth are the highest ever reported. Differences in persistence levels caused by agents inhibiting different aminoacyl-tRNA synthetases could be due to the involvement of specific mechanisms activated by the inhibition of a particular aminoacyl-tRNA synthetase or may have to do with the frequency of use of a particular aminoacyl-tRNA or the extent of (p)ppGpp synthesis activation by specific uncharged tRNAs.

The increase in persistence is observed at sub-inhibitory concentrations of McC^{Eco}. This finding led us to hypothesize that growing McC^{Eco}-producing cultures may also have increased persistence levels. The producing cells should maintain a steady-state level of internal McC^{Eco} determined by the balance between the synthesis rate and the export rate by the $MccC^{Eco}$ pump and detoxification by $MccE^{Eco}$ and $MccF^{Eco}$ enzymes. When the concentration of McC^{Eco} in the medium is high, additional McC may be imported into the producing cells by the YejABEF transporter, further increasing the concentration of intracellular McC^{Eco}. McC^{Eco} from the intracellular pool could be processed by proteases leading to accumulation of toxic processed McC^{Eco} and stimulating persistence. Indeed, persister cells in McC^{Eco} producing cultures were directly observed and it was demonstrated that import of McC^{Eco} produced earlier accounts for about 50% of the overall increase in persistence levels seen in producing cultures. The remaining increase

must be due to processing of internally generated McC^{Eco} molecules that have not been exported. So the autoimmunity enzymes $MccE^{Eco}$ and $MccF^{Eco}$ are not able to fully counter accumulation of processed McC^{Eco} in producing cells. Analysis of cells harboring *mcc* mutant plasmids shows that $MccF^{Eco}$ is the primary contributor to detoxification of intracellular McC^{Eco} in the producing cells, since inactivation of this enzyme, but not of $MccE^{Eco}$, leads to additional increase in persistence levels.

The additional proteolytic processing step undertaken during the biosynthesis of the active compound encoded by the *Y. pseudotuberculosis mcc* operon was studied. The importance of TldD/E protease for this step was shown. The additional step immediately after processing and right before the compound export may help solve the problem of self-intoxication of producing cells that inevitably arises due to processing by aminopeptidases of peptide-nucleotides accumulating inside the producing cells. Relying on a much longer, possibly structured MccA peptide should increase the intracellular stability of post-translationally modified $MccA^{Yps}$ and prevent the accumulation of toxic aspartamidyl-cytidylate that will inhibit protein synthesis in the producer. Though nothing is known about the regulation of TldD/E activity, one can speculate that its activation under specific conditions may allow *Y. pseudotuberculosis* carrying the *mcc* operon to produce massive amounts of McC^{Yps} from a depot of previously synthesized inactive pro-McC^{Yps}. That role of TldD/E protease is a one of the possible explanation of the widespread presence of *tld* genes. It was shown that they fulfill an important function in bacterial physiology like a role in protein quality control and in the activation and degradation of different natural products (for example, microcin B17) or toxin-antitoxin modules (for example, CcdA) [170, 171, 174].

The levels of McC produced by cells carrying the *mcc* cluster are sufficient to inhibit growth of neighboring sensitive cells, which is thought to be beneficial for microcinogenic cells in conditions when nutrient availability is limited [6, 11]. Our results show, however, that the interaction between McC^{Eco} producers and non-microcinogenic sensitive cells may be more complex, since McC^{Eco} production also increases persistence in the population of recipient cells. The observed increase is stronger than in the producing cells since recipient cells lack the McC^{Eco} export pump and dedicated detoxifying enzymes.

All of knowledge about McC^{Eco} as persistence inductor agent we may generalize on all McC^{Eco}-like peptide-nucleotide compounds that target an aminoacyl-tRNA synthetase, for example, McC^{Yps} on the grounds of all the evidence about importance of an aminoacyl-tRNA synthetase inhibition for bacterial persistence induction.

Thus, at least from the point of view of persistence, McC-like compounds producing cells seem to contribute to the "public good" of the community, ensuring their own survival and that of others. This property may contribute to the wide distribution of microcinogenicity in phylogenetically diverse bacteria.

Bibliography

1. Madison L.L. 1997. The leader peptide is essential for the post-translational modification of the DNA-gyrase inhibitor microcin B17. Mol Microbiol.Vol. 23. - P. 161-168;

2. Oman T.J. 2010. Follow the leader: the use of leader peptides to guide natural product biosynthesis. Nat Chem Biol. Vol. 6. - P. 9-18;

3. Rebuffat S. 2012. Microcins in action: amazing defense strategies of Enterobacteria. Biochem Soc Trans Vol. 40. - P. 1456-1462;

4. S. Duquesne, D. Destoumieux-Garzrón, J. Peduzzi And S. Rebuffat. 2007 Microcins, gene-encoded antibacterial peptides from enterobacteria. Nat. Prod. Rep., 24, 708–734;

5. Fomenko D.E., Metlitskaya A.Z., Peduzzi J., Goulard C., Katrukha G.S., Gening L.V., Rebuffat S., Khmel I.A., 2003. Microcin C51 plasmid genes: possible source of horizontal gene transfer. Antimicrob Agents Chemother 47:2868–2874;

6. Baquero F. 1978. Microcin plasmids: a group of extrachromosomal elements coding for low-molecular-weight antibiotics in Escherichia coli. J Bacteriol. Vol. 135. - P. 342-347;

7. Kurepina N.E. 1990. Microcin R51 and a plasmid determining its synthesis. Mol Gen Mikrobiol Virusol.P. 12-17;

8. Novoa M.A. 1986. Cloning and mapping of the genetic determinants for microcin C7 production and immunity. J Bacteriol. Vol. 168. - P. 1384-1391;

9. Gonzalez-Pastor J.E. 1994. The smallest known gene. Nature. Vol. 369. - P. 281;

10. Gonzalez-Pastor J.E. 1995. Structure and organization of plasmid genes required to produce the translation inhibitor microcin C7. J Bacteriol.Vol. 177. - P. 7131-7140;

11. Roush R.F. 2008. Maturation of an Escherichia coli ribosomal peptide antibiotic by ATP-consuming N-P bond formation in microcin C7. J Am Chem SocVol. 130. - P. 3603-3609;

12. Metlitskaya A. 2009. Maturation of the translation inhibitor microcin C. J Bacteriol. Vol. 191. - P. 2380-2387;

13. Novikova M., Kazakov T., Vondenhoff G.H., Semenova E., Rozenski

J., Metlytskaya A., Zukher I., Tikhonov A., Van Aerschot A., Severinov K., 2010. MccE provides resistance to protein synthesis inhibitor microcin C by acetylating the processed form of the antibiotic. J Biol Chem 285:12662-9;

14. Zeng Y. 2009. Characterization of two seryl-tRNA synthetases in albomycin-producing Streptomyces sp. strain ATCC 700974 // Antimicrob Agents Chemother. Vol. 53. - P. 4619-4627;

15. Kulikovsky A. 2014. The molecular mechanism of aminopropylation of the peptidenucleotide antibiotic microcin C. J Am Chem Soc.Vol. 136. - P. 11168-11175;

16. Kazakov T. 2008. Escherichia coli peptidase A, B, or N can process translation inhibitor microcin C. J Bacteriol. Vol. 190. - P. 2607-2610;

17. Law C.J. 2008. Ins and outs of major facilitator superfamily antiporters Annu Rev Microbiol. Vol. 62. - P. 289-305;

18. Tikhonov A., Kazakov T., Semenova E., Serebryakova M., Vondenhoff G., Van Aerschot A., Reader J.S., Govorun V.M., Severinov K., 2010. The mechanism of microcin C resistance provided by the MccF peptidase. J Biol Chem 285:37944-52;

19. Fomenko D. 2009. Regulation of microcin C51 operon expression: the role of global regulators of transcription. Res Microbiol. Vol. 152. - P. 469-479;

20. Moreno F. 2002. The regulation of microcin B, C and J operons // Biochimie. Vol. 84. - P. 521-529;

21. Diaz-Guerra L. 1989. appR gene product activates transcription of microcin C7 plasmid genes. J Bacteriol. Vol. 171. - P. 2906-2908;

22. Zukher I. 2014. Ribosome-controlled transcription termination is essential for the production of antibiotic microcin C. Nucleic Acids Res. Vol. 42. - P. 11891-11902;

23. Khmel I.A. 1993. Isolation and characterization of Escherichia coli strains producing microcins of B and C types. FEMS Microbiol Lett. Vol. 111. - P. 269- 274;

24. Novikova M. 2007. The Escherichia coli Yej transporter is required for the uptake of translation inhibitor microcin C. J Bacteriol Vol. 189. - P. 8361-8365;

25. Garcia-Bustos J.F. 1985. Structure and mode of action of microcin 7, an antibacterial peptide produced by Escherichia coli. Antimicrob Agents Chemother Vol. 27. - P. 791-797;

26. Metlitskaya A. 2006. Aspartyl-tRNA synthetase is the target of peptide

nucleotide antibiotic Microcin C. J Biol Chem. Vol. 281. - P. 18033-18042;

27. Garcia-Bustos J.F. 1984. Microcin 7: purification and properties // Biochem Biophys Res Commun. Vol. 119. - P. 779-785;

28. Quinones M. Sequence and gene expression analyses of plasmid pHPM8 from Helicobacter pylori reveal the presence of two operons with putative roles in plasmid replication and antibiotic activity. Plasmid. - 2001. - Vol. 46. - P. 223-228;

29. Hofreuter D. Characterization of two cryptic Helicobacter pylori plasmids: a putative source for horizontal gene transfer and gene shuffling // J Bacteriol. - 2002. - Vol. 184. - P. 2755-2766;

30. Severinov, K.; Semenova, E.; Kazakov, A.; Kazakov, T.; Gelfand, M. S. Low-molecular-weight post-translationally modified microcins. Mol. Microbiol. 2007, 65, 1380-1394;

31. Bantysh, O.; Serebryakova, M.; Makarova, K. S.; Dubiley, S.; Datsenko, K. A.; Severinov, K. Enzymatic synthesis of bioinformatically predicted microcin C-like compounds encoded by diverse bacteria. MBio. 2014, 5, e01059-14;

32. Serebryakova, M.; Tsibulskaya, D.; Mokina, O.; Kulikovsky, A.; Nautiyal, M.; Van Aerschot, A.; Severinov, K.; Dubiley, S. A Trojan-Horse Peptide-Carboxymethyl-Cytidine Antibiotic from *Bacillus amyloliquefaciens J. Am. Chem. Soc*. **2016**, *138*, 15690-15698;

33. Dodds D.R., 2017. Antibiotic Resistance, a Current Epilogue. Biochemical Pharmacology 134: 139-146;

34. Michael C.A., Dominey-Howes D., Labbate M., 2014. The antibiotic resistance crisis: causes, consequences, and management, Front. Public Health 2:145;

35. Martens E., Demain A.L., 2017. The antibiotic resistance crisis, with a focus on the United States. The Journal of Antibiotics 70:520–526

36. Ventola C.L., 2015. The antibiotic resistance crisis. Part 1: causes and threats, P.T. 40: 277–283;

37. Boucher H.W., Talbot G.H., Bradley J.S., Edwards J.E., Gilbert V. Rice L.B. 2009. Bad bugs, no drugs: no ESKAPE! An update from the Infectious Diseases. Society of America, Clin. Infect. Dis. 48: 1–12;

38. Rice L.B., 2010, Progress and challenges in implementing the research on ESKAPE pathogens, Infect. Control Hosp. Epidemiol. 31:S7–S10;

39. Wright G.D., 2010. Q&A: Antibiotic resistance: where does it come from and what can we do about it? BMC Biol. 8: 123;

40. Yılmaz C., Özcengiz G., 2017. Antibiotics: Pharmacokinetics, toxicity, resistance and multidrug efflux pumps. Biochemical Pharmacology 133:43–62;

41. Blair J.M., Webber M.A., Baylay A.J., Ogbolu D.O., Piddock L.J., 2015. Molecular mechanisms of antibiotic resistance. Nat Rev Microbiol 13(1):42-5;

42. Karam G., Chastre J, Wilcox M.H. , Vincent J.L., 2016. Antibiotic strategies in the era of multidrug resistance. Crit. Care 20:136;

43. J. Davies, 2007. Microbes have the last word, EMBO Rep. 8:616–621;

44. Chang S, Sievert DM, Hageman JC, et al. Infection with Vancomycin-resistant Staphylococcus aureus Containing the vanA Resistance Gene. N Engl J Med. 2003; 348(14):1342–47;

45. Fair R.J., Tor Y., 2014. Antibiotics and bacterial resistance in the 21st century, Perspect. Medicin. Chem. 6:25–64;

46. Wozniak R.A.F., Waldor M.K., 2010. Integrative and conjugative elements: mosaic mobile genetic elements enabling dynamic lateral gene flow. Nat. Rev. Microbiol. 8:552–563;

47. Marti E., Variatza E., Balcázar J.L., 2014. Bacteriophages as a reservoir of extended spectrumvb-lactamase and fluoroquinolone resistance genes in the environment. Clin. Microbiol. Infect. 20:O456–O459;

48. Brown-Jaque M., Calero-Cáceres W., Muniesa M., 2015. Transfer of antibiotic resistance genes via phage-related mobile elements. Plasmid 79:1–7;

49. Costerton JW, Cheng KJ, Geesey GG, Ladd TI, Nickel JC, Dasgupta M, Marrie TJ. 1987. Bacterial biofilms in nature and disease. Annu Rev Microbiol. 41:435-64;

50. Sultana ST, Call DR & Beyenal H. 2016. Eradication of Pseudomonas aeruginosa biofilms and persister cells using an electrochemical scaffold and enhanced antibiotic susceptibility. npj Biofilms and Microbiomes 2, 2 (2016):doi:10.1038/s41522-016-0003-0;

51. Verstraeten N., Knapen W., Fauvart M., Michiels J., 2016. A historical perspective on bacterial persistence, in: Methods in Molecular Biology. Springer Science, New York, pp. 3–13;

52. Dhar N., McKinney J.D., 2010. Mycobacterium tuberculosis persistence mutants identified by screening in isoniazid-treated mice. Proc. Natl. Acad. Sci. 107, 12275–12280;

53. Balaban N.Q., Merrin J., Chait R., Kowalik L., Leibler S., 2004. 709 Bacterial persistence as a phenotypic switch. Science 305, 1622–1625;

54. Keren I., Kaldalu N., Spoering A.L., Wang Y., Lewis K., 2004. Persister cells and tolerance to antimicrobials. FEMS Microbiol. Lett. 230, 13–18;

55. Bigger J.W., 1944. Treatment Of Staphylococcal Infections With Penicillin. Lancet Ii: 497-500;

56. Harms A., Maisonneuve E., Gerdes K., 2016. Mechanisms of Bacterial Persistence During Stress and Antibiotic Exposure. Science 354;

57. Keren, I., Minami, S., Rubin, E., Lewis, K., 2011. Characterization and transcriptome analysis of Mycobacterium tuberculosis persisters. MBio 2, e00100-11;

58. Keren, I., Shah, D., Spoering, A.L., Kaldalu, N., Lewis, K., 901 2004. Specialized persister cells and the mechanism of multidrug tolerance in Escherichia coli. J. Bacteriol. 186, 8172–8180;

59. De Groote, V.N., Verstraeten, N., Fauvart, M., Kint, C.I., Verbeeck, A.M., Beullens, S., Cornelis, P., Michiels, J., 2009. Novel persistence genes in Pseudomonas aeruginosa identified by high throughput screening. FEMS Microbiol. Lett. 297, 73–79;

60. Lewis K., 2010. Persister Cells. Annu Rev Microbiol 64:357-72;

61. Kint, C.I., Verstraeten, N., Fauvart, M., Michiels, J., 2012. New-found fundamentals of bacterial persistence. Trends Microbiol. 20, 1–9;

62. Maisonneuve, E., Castro-Camargo, M., Gerdes, K., 2013. (p)ppGpp controls bacterial persistence by stochastic induction of toxin-antitoxin activity. Cell 154, 1140–1150;

63. Shan, Y., Lazinski, D., Rowe, S., Camilli, A., Lewis, K., 2015. Genetic basis of persister tolerance to aminoglycosides in Escherichia coli. MBio 6, e00078-15;

64. Korch, S.B., Hill, T.M., 2006. Ectopic overexpression of wild-type and mutant hipA genes in Escherichia coli: effects on macromolecular synthesis and persister formation. J. Bacteriol. 188, 3826–3836;

65. Pedersen, K., Christensen, S.K., Gerdes, K., 2002. Rapid induction and reversal of a bacteriostatic condition by controlled expression of toxins and antitoxins. Mol. Microbiol. 45, 501–510;

66. Vázquez-Laslop, N., Lee, H., Neyfakh, A.A., 2006. Increased persistence in Escherichia coli caused by controlled expression of toxins or other unrelated proteins. J. Bacteriol. 188, 3494–3497;

67. Kwan, B.W., Valenta, J.A., Benedik, M.J., Wood, T.K., 2013. Arrested protein synthesis increases persister-like cell formation. Antimicrob. Agents Chemother. 57, 1468–1473;

68. Pearl, S., Gabay, C., Kishony, R., Oppenheim, A., Balaban, N.Q., 2008. Nongenetic individuality in the host-phage interaction. PLoS Biol. 6, e120;

69. Wiuff, C., Andersson, D.I., 2007. Antibiotic treatment in vitro of phenotypically tolerant bacterial populations. J. Antimicrob. Chemother. 59, 254–263;

70. Wiuff, C., Zappala, R., Regoes, R.R., Garner, K., Baquero, F., Levin, B.R., 2005. Phenotypic tolerance: antibiotic enrichment of non-inherited resistance in bacterial populations. Antimicrob. Agents Chemother. 49, 1438–1494;

71. Allison, K.R., Brynildsen, M.P., Collins, J.J., 2011. Heterogeneous bacterial persisters and engineering approaches to eliminate them. Curr. Opin. Microbiol. 14, 593–598;

72. Amato, S.M., Brynildsen, M.P., 2015. Persister heterogeneity arising from a single metabolic stress Curr. Biol. 25, 2090–2098;

73. Levin, B.R., Concepción-Acevedo, J., Udekwu, K.I., 2014. Persistence: a copacetic and parsimonious hypothesis for the existence of non-inherited resistance to antibiotics. Curr. Opin. Microbiol. 21,18–21;

74. Adams, K., Takaki, K., Connolly, L., 2011. Drug tolerance in replicating mycobacteria mediated by a macrophage-induced efflux mechanism. Cell 145, 39–53;

75. Nguyen, D., Joshi-Datar, A., Lepine, F., Bauerle, E., Olakanmi, O., Beer, K., McKay, G., Siehnel, R., Schafhauser, J., Wang, Y., Britigan, B.E., Singh, P.K., 2011. Active starvation responses mediate antibiotic tolerance in biofilms and nutrient-limited bacteria. Science 334, 982–986;

76. Maisonneuve, E., Gerdes, K., 2014. Molecular mechanisms underlying bacterial persisters. Cell 157, 539–548;

77. Wakamoto, Y., Dhar, N., Chait, R., Schneider, K., Signorino-Gelo, F., Leibler, S., McKinney, J.D., 2013. Dynamic persistence of antibiotic-stressed mycobacteria. Science 339, 91–95;

78. Pu, Y., Zhao, Z., Li, Y., Zou, J., Ma, Q., Zhao, Y., Ke, Y., Zhu, Y., Chen, H., Baker, M.A.B., Ge, H., Sun, Y., Xie, X.S., Bai, F., 2016. Enhanced efflux activity facilitates drug tolerance in dormant bacterial cells. Mol. Cell 62, 284–294;

79. Kussell E., Kishony R., Balaban N.Q., Leibler S., 2005. Bacterial Persistence: A Model Of Survival In Changing Environments. Genetics 169:1807–1814;

80. Stepanyan, K., Wenseleers, T., Duéñez-1094 Guzmán, E.A., Muratori, F., Van den Bergh, B., Verstraeten, N., De Meester, L., Verstrepen, K.J., Fauvart, M., Michiels, J., 2015. Fitness trade-offs explain low levels of persister cells in the opportunistic pathogen Pseudomonas aeruginosa. Mol. Ecol. 24, 1572–1583;

81. Elowitz, M.B., Levine, A.J., Siggia, E.D., Swain, P.S., 2002. Stochastic gene expression in a single cell. Science 297, 1183–1186;

82. Ackermann, M., 2015. A functional perspective on phenotypic heterogeneity in microorganisms. Nat. Rev. Microbiol. 13, 497–508;

83. Rotem, E., Loinger, A., Ronin, I., Levin-Reisman, I., Gabay, C., Shoresh, N., Biham, O., Balaban, N.Q., 2010. Regulation of phenotypic variability by a threshold-based mechanism underlies bacterial persistence. Proc. Natl. Acad. Sci. 107, 12541–12546;

84. Germain E., Castro-Roa D., Zenkin N., Gerdes K., 2013. Molecular mechanism of bacterial persistence by HipA. Mol Cell 52:248-54;

85. Kaspy I., Rotem E., Weiss N., Ronin I., Balaban N.Q., Glaser G., 2013. HipA-mediated antibiotic persistence via phosphorylation of the glutamyl-tRNA-synthetase. Nat Commun 4 :3001;

86. Cheverton, A.M., Gollan, B., Przydacz, M., Wong, C.T., Mylona, A., Hare, S.A., Helaine, S., 2016. A Salmonella toxin promotes persister formation through acetylation of tRNA. Mol. Cell;

87. Cataudella, I., Sneppen, K., Gerdes, K., Mitarai, N., 2013. Conditional cooperativity of toxin - antitoxin regulation can mediate bistability between growth and dormancy. PLoS Comput. Biol. 9, e1003174;

88. Cataudella, I., Trusina, A., Sneppen, K., Gerdes, K., Mitarai, N., 2012. Conditional cooperativity in toxin-antitoxin regulation prevents random toxin activation and promotes fast translational recovery. Nucleic Acids Res. 40, 6424–6434;

89. Fung, D.K.C., Chan, E.W.C., Chin, M.L., Chan, R.C.Y., 2010. Delineation of a bacterial starvation stress response network which can mediate. Antimicrob. Agents Chemother. 54, 1082–1093;

90. Maisonneuve, E., Shakespeare, L.J., Girke, M., Gerdes, K., 2011. Bacterial persistence by RNA endonucleases. Proc. Natl. Acad. Sci. 108, 13206–13211;

91. Amato, S.M., Brynildsen, M.P., 2014. Nutrient transitions are a source of persisters in Escherichia coli biofilms. PLoS One 9, e93110;

92. Amato, S.M., Orman, M.A., Brynildsen, M.P., 2013. Metabolic control of persister formation in Escherichia coli. Mol. Cell 50, 475–487;

93. Mordukhova, E.A., Pan, J.-G., 2014. Stabilization of homoserine-O-cuccinyltransferase (MetA) creases the frequency of persisters in Escherichia coli under stressful conditions. PLoS One 9, e110504;

94. Murakami, K., Ono, T., Viducic, D., Kayama, S., Mori, M., Hirota, K., Nemoto, K., Miyake, Y., 2005. Role for rpoS gene of Pseudomonas aeruginosa in antibiotic tolerance. FEMS Microbiol. Lett. 242, 161–167;

95. Hong, S.H., Wang, X., O'Connor, H.F., Benedik, M.J., Wood, T.K., 2012. Bacterial persistence increases as environmental fitness decreases. Microb. Biotechnol. 5, 509–522;

96. Wu, Y., Vulic, M., Keren, I., Lewis, K., 2012. Role of oxidative stress in persister tolerance. Antimicrob. Agents Chemother. 56, 4922–4926;

97. Dörr, T., Lewis, K., Vulić, M., 2009. SOS response induces persistence to fluoroquinolones in Escherichia coli. PLoS Genet. 5, e1000760;

98. Johnson, P.J.T., Levin, B.R., 2013. Pharmacodynamics, population dynamics, and the evolution of persistence in Staphylococcus aureus. PLoS Genet. 9, e1003123;

99. Bhargava, N., Sharma, P., Capalash, N., 2014. Pyocyanin stimulates quorum sensing-mediated tolerance to oxidative stress and increases persister cells population in Acinetobacter baumannii. Infect. Immun. 8, 3417–3425;

100. Leung, V., Lévesque, C.M., 2012. A stress-inducible quorum-sensing

peptide mediates the formation of persister cells with noninherited multidrug tolerance. J. Bacteriol. 194, 2265–2274;

101. Möker, N., Dean, C.R., Tao, J., 2010. Pseudomonas aeruginosa increases formation of multidrug-tolerant persister cells in response to quorum-sensing signaling molecules. J. Bacteriol. 192,1946–1955;

102. Vega, N.M., Allison, K.R., Khalil, A.S., Collins, J.J., 2012. Signaling-mediated bacterial persister formation. Nat. Chem. Biol. 8, 431–433;

103. Arnoldini, M., Vizcarra, I.A., Peña-Miller, R., Stocker, N., Diard, M., Vogel, V., Beardmore, R.E., Hardt, W.-D., Ackermann, M., 2014. Bistable expression of virulence genes in Salmonella leads to the formation of an antibiotic-tolerant subpopulation. PLoS Biol. 12, e1001928;

104. Gardner, A., West, S.A., Griffin, A.S., 2007. Is bacterial persistence a social trait? PLoS One 2, e752;

105. Christensen, L.D., Van Gennip, M., Jakobsen, T.H., Alhede, M., Hougen, H.P., Høiby, N., Bjarnsholt, T., Givskov, M., 2012. Synergistic antibacterial efficacy of early combination treatment with tobramycin and quorum-sensing inhibitors against Pseudomonas aeruginosa in an intraperitoneal foreign-body infection mouse model. J. Antimicrob. Chemother. 67, 1198–1206;

106. Lebeaux D, Ghigo JM, Beloin C. 2014. Biofilm-related infections: bridging the gap between clinical management and fundamental aspects of recalcitrance toward antibiotics. Microbiol Mol Biol Rev. Sep; 78(3):510-43;

107. Stewart PS. 2015. Antimicrobial Tolerance in Biofilms. Microbiol Spectr. 2015 Jun; 3(3): 10.1128/microbiolspec. MB-0010-2014;

108. Spoering A.L., Lewis K., 2001. Biofilms and Planktonic Cells of Pseudomonas Aeruginosa Have Similar Resistance Killing by Antimicrobials. J Bacteriol. 183(23):6746-51;

109. Boehm A, Steiner S, Zaehringer F, Casanova A, Hamburger F, Ritz D, Keck W, Ackermann M, Schirmer T, Jenal U. 2009. Second messenger signalling governs Escherichia coli biofilm induction upon ribosomal stress. Mol Microbiol;72(6):1500-16;

110. Lebeaux D, Chauhan A, Létoffé S, Fischer F, de Reuse H, Beloin C, Ghigo JM. 2014. pH-mediated potentiation of aminoglycosides kills bacterial persisters and eradicates in vivo biofilms. J Infect Dis. 1;210(9):1357-66;

111. Jiafeng L, Fu X, Chang Z. 2015. Hypoionic shock treatment enables aminoglycosides antibiotics to eradicate bacterial persisters. Sci Rep; 5:14247;

112. Duan X, Li Y, Du Q, Huang Q, Guo S, Xu M, Lin Y, Liu Z, Xie J. 2016. Mycobacterium Lysine ε-aminotransferase is a novel alarmone metabolism related persister gene via dysregulating the intracellular amino acid level. Sci Rep; 6:19695;

113. Gefen, O., Gabay, C., Mumcuoglu, M., Engel, G., Balaban, N.Q., 2008. Single-cell protein induction dynamics reveals a period of vulnerability to antibiotics in persister bacteria. Proc. Natl. Acad. Sci. 105, 6145–6149;

114. Van den Bergh, B., Michiels, J.E., Fauvart, M., Michiels, J., 2016. Should we develop screens for multidrug tolerance? Expert Rev. Anti. Infect. Ther. 7210;

115. Wood TK. 2016. Combatting bacterial persister cells. Biotechnol Bioeng;113(3):476-83;

116. Moyed, H.S., Bertrand, K.P., 1983. hipA, a newly recognized gene of Escherichia coli K-12 that affects frequency of persistence after inhibition of murein synthesis. J Bacteriol. 155, 768–775;

117. Slattery, A., Victorsen, A.H., Brown, A., Hillman, K., Phillips, G.J., 2013. Isolation of highly persistent mutants of Salmonella enterica serovar Typhimurium reveals a new toxin-antitoxin module. J. Bacteriol. 195, 647–657;

118. Torrey, H.L., Keren, I., Via, L.E., Lee, J.S., Lewis, K., 2016. High persister mutants in Mycobacterium tuberculosis. PLoS One 11, e0155127;

119. Hansen, S., Lewis, K., Vulić, M., 2008. Role of global regulators and nucleotide metabolism in antibiotic tolerance in Escherichia coli. Antimicrob. Agents Chemother. 52, 2718–2726;

120. Ma, C., Sim, S., Shi, W., Du, L., Xing, D., Zhang, Y., 2010. Energy production genes sucB and ubiF are involved in persister survival and tolerance to multiple antibiotics and stresses in Escherichia coli. FEMS Microbiol. Lett. 303, 33–40;

121. Hu, Y., Coates, A.R.M., 2005. Transposon mutagenesis identifies genes which control antimicrobial drug tolerance in stationary-phase Escherichia coli. FEMS Microbiol. Lett. 243, 117–124;

122. Li, Y., Zhang, Y., 2007. PhoU is a persistence switch involved in persister formation and tolerance to multiple antibiotics and stresses in Escherichia coli. Antimicrob. Agents Chemother. 51, 2092–2099;

123. Girgis, H.S., Harris, K., Tavazoie, S., 2012. Large mutational target size for rapid emergence of bacterial persistence. Proc. Natl. Acad. Sci. 109, 12740–12745;

124. Spoering, A.L., Vulic, M., Lewis, K., Vulić, M., Lewis, K., 2006. GlpD and PlsB participate in persister cell formation in Escherichia coli. J. Bacteriol. 188, 5136–44;

125. Wu, N., He, L., Cui, P., Wang, W., Yuan, Y., Liu, S., Xu, T., Zhang, S., Wu, J., Zhang, W., Zhang, Y., 2015. Ranking of persister genes in the same Escherichia coli genetic background demonstrates varying importance of individual persister genes in tolerance to different antibiotics. Front. Microbiol. 6, 1003;

126. Orman, M.A., Brynildsen, M.P., 2015. Inhibition of stationary phase respiration impairs persister formation in E. coli. Nat. Commun. 6, 7983;

127. Verstraeten, N., Knapen, W., Fauvart, M., Michiels, J., 2015. Membrane depolarization-triggered responsive diversification leads to antibiotic tolerance. Microb. Cell 2, 299–301;

128. Doniselli N, Rodriguez-Aliaga P, Amidani D, Bardales JA, Bustamante C, Guerra DG, Rivetti C, 2015. New insights into the regulatory mechanisms of ppGpp and DksA on Escherichia coli RNA polymerase-promoter complex. Nucleic Acids Res 43:5249-62;

129. Hirsch, M., and T. Elliott, 2002. Role of ppGpp in rpoS stationary-phase regulation in Escherichia coli. J Bacteriol 184:5077-5087;

130. Jude, F., T. Kohler, P. Branny, K. Perron, M. P. Mayer, R. Comte, and C. van Delden, 2003. Posttranscriptional control of quorum sensing- dependent virulence genes by DksA in Pseudomonas aeruginosa. J Bacteriol 185:3558-3566;

131. Mogull, S. A., L. J. Runyen-Janecky, M. Hong, and S. M. Payne, 2001. dksA is required for intercellular spread of Shigella flexneri via an RpoS-independent mechanism. Infect Immun. 2001;69 (9) :5742-51;

132. Turner, A. K., M. A. Lovell, S. D. Hulme, L. Zhang-Barber, and P. A. Barrow, 1998. Identification of Salmonella typhimurium genes required for colonization of the chicken alimentary tract and for virulence in newly hatched chicks. Infect Immun 66:2099-2106;

133. Webb, C., M. Moreno, M. Wilmes-Riesenberg, R. Curtiss, 3rd, and J. W. Foster, 1999. Effects of DksA and ClpP protease on sigma S production and virulence in Salmonella typhimurium. Mol Microbiol 34:112-123;

134. Murphy, H., and M. Cashel, 2003. Isolation of RNA polymerase suppressors of a (p)ppGpp deficiency. Methods Enzymol 371:596-601;

135. Paul, B. J., M. M. Barker, W. Ross, D. A. Schneider, C. Webb, J. W. Foster, and R. L. Gourse, 2004. DksA: a critical component of the transcription initiation machinery that potentiates the regulation of rRNA promoters by ppGpp and the initiating NTP. Cell 118:311-322;

136. Paul, B. J., M. B. Berkmen, and R. L. Gourse, 2005. DksA potentiates direct activation of amino acid promoters by ppGpp. Proc Natl Acad Sci USA 102: 7823-7828;

137. Page, R., Peti, W., 2016. Toxin-antitoxin systems in bacterial growth arrest and persistence. Nat. Chem. Biol. 12, 208–214;

138. Van Melderen, L., 2010. Toxin-antitoxin systems: why so many, what for? Curr. Opin. Microbiol. 13,781–785;

139. Hall AM, Gollan B, Helaine S. Toxin-antitoxin systems: reversible toxicity. Curr Opin Microbiol. 2017 Mar 6;36: 102-110;

140. Dörr, T., Vulić, M., Lewis, K., 2010. Ciprofloxacin causes persister formation by inducing the TisB toxin in Escherichia coli. PLoS Biol. 8, e1000317;

141. Black, D.S., Kelly, A.J., Mardis, M.J., Moyed, H.S., 1991. Structure and organization of hip, an operon that affects lethality due to inhibition of peptidoglycan or DNA synthesis. J. Bacteriol. 173, 5732–5739;

142. Shah, D., Zhang, Z., Khodursky, A., Kaldalu, N., Kurg, K., 1070 Lewis, K., 2006. Persisters: a distinct physiological state of E. coli. BMC Microbiol. 6, 53;

143. S.P. Bernier, D. Lebeaux, A.S. DeFrancesco, A. Valomon, G. Soubigou, J.Y. Coppée, J.M. Ghigo, C. Beloin. 2013. Starvation, together with the SOS response, mediates high biofilm-specific tolerance to the fluoroquinolone ofloxacin. PLoS Genet;

144. Unoson, C., Wagner, E.G.H., 2008. A small SOS-induced toxin is targeted against the inner membrane in Escherichia coli. Mol. Microbiol. 70, 258–270;

145. Korch, S.B., Henderson, T.A., Hill, T.M., 2003. Characterization of the hipA7 allele of Escherichia coli and evidence that high persistence is governed by

(p)ppGpp synthesis. Mol. Microbiol. 50, 1199–1213;

146. Tian C, Roghanian M, Jørgensen MG, Sneppen K, Sørensen MA, Gerdes K, Mitarai N. 2016. Rapid curtailing of the stringent response by toxin-antitoxin module-encoded mRNases. J Bacteriol 198:1918–1926;

147. Gerdes, K., Rasmussen, P.B., Molin, S., 1986. Unique type of plasmid maintenance function: postsegregational killing of plasmid-free cells. Proc. Natl. Acad. Sci. 83, 3116–3120;

148. Persky, N.S., Ferullo, D.J., Cooper, D.L., Moore, H.R., Lovett, S.T., 2009. The ObgE/CgtA GTPase influences the stringent response to amino acid starvation in Escherichia coli. Mol. Microbiol.73, 253–266;

149. Gomez, J. E. & McKinney, J. D. M. tuberculosis persistence, latency, and drug tolerance. Tuberculosis 84, 29–44 (2004);

150. Gotuzzo, E. et al. Association between specific plasmids and relapse in typhoid fever. J. Clin. Microbiol. 25, 1779–1781 (1987);

151. Foxman, B. Recurring urinary tract infection: incidence and risk factors. Am. J. Public Health 80, 331–333 (1990);

152. Österlund, A., Popa, R., Nikkilä, T., Scheynius, A. & Engstrand, L. Intracellular reservoir of Streptococcus pyogenes in vivo: a possible explanation for recurrent pharyngotonsillitis. Laryngoscope 107, 640–647 (1997);

153. Okoro, C. K. et al. High−resolution single nucleotide polymorphism analysis distinguishes recrudescence and reinfection in recurrent invasive nontyphoidal Salmonella typhimurium disease. Clin. Infect. Dis. 54, 955–963 (2012);

154. Russo, T. A., Stapleton, A., Wenderoth, S., Hooton, T. M. & Stamm, W. E. Chromosomal restriction fragment length polymorphism analysis of Escherichia coli strains causing recurrent urinary tract infections in young women. J. Infect. Dis. 172, 440–445 (1995);

155. Bingen, E. et al. DNA restriction fragment length polymorphism differentiates recurrence from relapse in treatment failures of Streptococcus pyogenes pharyngitis. J. Med. Microbiol. 37, 162–164 (1992);

156. Michiels J.E., Van Den Bergh B., Verstraeten N., Michiels J., 2016. Molecular Mechanisms And Clinical Implications Of Bacterial Persistence. Drug Resist Updat. 29:76-89;

157. Mulcahy, L. R., Burns, J. L., Lory, S. & Lewis, K. Emergence of Pseudomonas aeruginosa strains producing high levels of persister cells in patients with cystic fibrosis. J. Bacteriol. 192, 6191–6199 (2010);

158. Schumacher, M. A. et al. HipBA–promoter structures reveal the basis of heritable multidrug tolerance. Nature 524, 59–64 (2015);

159. LaFleur, M. D., Qi, Q. & Lewis, K. Patients with long− term oral carriage harbor high−persister mutants of Candida albicans. Antimicrob. Agents Chemother. 54, 39–44 (2010);

160. Levin B.R., Rozen D.E., 2006. Non-Inherited Antibiotic Resistance. Nat Rev Microbiol 4:556-62;

161. Bjedov, I., Tenaillon, O., Gérard, B., Souza, V., Denamur, E., Radman, M., Taddei, F., Matic, I., 2003. Stress-induced mutagenesis in bacteria. Science 300, 1404–1409;

162. Loewe, L., Textor, V., Scherer, S., 2003. High deleterious genomic mutation rate in stationary phase of Escherichia coli. Science 302, 1558–1560;

163. Beaber, J.W., Hochhut, B., Waldor, M.K., 2004. SOS response promotes horizontal dissemination of antibiotic resistance genes. Nature 427, 72–74;

164. Ryder, V.J., Chopra, I., O'Neill, A.J., 2012. Increased mutability of staphylococci in biofilms as a consequence of oxidative stress. PLoS ONE 7, e47695;

165. Savage, V.J., Chopra, I., O'Neill, A.J., 2013. Staphylococcus aureus biofilms promote horizontal transfer of antibiotic resistance. Antimicrob. Agents Chemother. 57, 1968–1970;

166. Bryers, J. D. Medical biofilms. Biotechnol. Bioeng. 100, 1–18 (2008);

167. Lyczak, J. B., Cannon, C. L. & Pier, G. B. Lung infections associated with cystic fibrosis. Clin. Microbiol. Rev. 15, 194–222 (2002);

168. Jernberg, C., Löfmark, S., Edlund, C. & Jansson, J. K. Long−term impacts of antibiotic exposure on the human intestinal microbiota. Microbiology 156, 3216–3223 (2010);

169. Ghilarov, D.; Serebryakova, M.; Stevenson, C. E. M.; Hearnshaw, S. J.; Volkov, D.; Maxwell, A.; Lawson, D. M.; Severinov, K. The Origins of Specificity in the Microcin-Processing Protease TldD/E. *Structure.* **2017**, DOI: http://dx.doi.org/10.1016/j.str.2017.08.006;

170. Ghilarov D, Serebryakova M, Stevenson CEM, Hearnshaw SJ, Volkov D, Maxwell A, Lawson DM, Severinov K. The Origins of Specificity in the Microcin-Processing Protease TldD/E. Structure. 2017. pii: S0969-2126(17)30259-9;

171. Allali, N., et al.. 2002. The Highly Conserved TldD and TldE Proteins of Escherichia coli Are Involved in Microcin B17 Processing and in CcdA Degradation. J. Bacteriol., 184(12): p. 3224-3231;

172. Ramisetty B.C., Ghosh D., Chowdhury M.R., Santhosh R.S., 2016. What is the link between stringent response, endoribonuclease encoding Type II toxin-antitoxin systems and persistence? Front Microbiol. 7:1882;

173. Germain E., Roghanian M., Gerdes K., Maisonneuve E., 2015. Stochastic induction of persister cells by HipA through (p)ppGpp-mediated activation of mRNA endonucleases. Proc Natl Acad Sci USA 112:5171-6;

174. Murayama, N., Shimizu, H., Takiguchi, S., Baba, Y., Amino, H., Horiuchi, T., Sekimizu, K., and Miki, T. (1996). Evidence for involvement of Escherichia coli genes pmbA, csrA and a previously unrecognized gene tldD, in the control of DNA gyrase by letD (ccdB) of sex factor F. J. Mol. Biol. 256, 483–502;

175. Baba, T.; Ara, T.; Hasegawa, M.; Takai, Y.; Okumura, Y.; Baba, M.; Datsenko, K. A.; Tomita, M.; Wanner, B. L.; Mori, H. Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. *Mol. Syst. Biol.* 2006, *2*, 2006.0008.