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"Single cell analysis of colicin Ib biology in *Salmonella enterica* serovar Typhimurium" and "Protection of colicin M producing bacteria against self-killing"

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Ich, Stefanie J. Spriewald, erkläre hiermit an Eides statt, dass ich die vorliegende Dissertation mit dem Thema:

"Single cell analysis of colicin Ib biology in *Salmonella enterica* serovar Typhimurium"

and

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List of Abbreviations

Acc.no.	Accession number
Amp	Ampicillin
ANOVA	Analysis of variance
APS	Ammonium persulfate
AsnC	Asparagine synthetase A regulator
ATP	Adenosine triphosphate
BtuB	Vitamin B12 transporter
cba	Colicin B activity gene
cbi	Colicin B immunity gene
cfu	Colony forming units
cib	Colicin Ib activity gene
cib imm	Colicin Ib immunity gene
CirA	Colicin I receptor
Cm	Chloramphenicol
cma	Colicin M activity gene
CMI	ColM immunity protein
cmi	Colicin M immunity gene
Col	Colicin
CPM	Cytoplasma membrane
CsrA	Carbon storage regulator protein
CTAB	Cetyltrimethylammoniumbromid
CTCF	Corrected total cell fluorescence
DAPI	4',6-diamidino-2-phenylindole
ddH_2O	Double-distilled water
DMSO	Dimethyl S-oxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
ds	Double-stranded
DTPA	Diethylenetriamine pentaacetic acid
DTT	Dithiothreitol
E. coli (Ec)	*Escherichia coli*
e.g.	*exempli gratia*
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
FACS	Fluorescence-activated cell sorting
FepA	Ferrienterobactin receptor
FhuA	Ferrichrome-iron receptor

FkpA	Peptidyl prolyl cis-trans isomerase/chaperone
FLP	Flippase
FRT	Flippase recognition target (FRT) sites
FSC	Forward scatter
FtsH	ATP-dependent metalloprotease
Fur	Ferric uptake regulator
GFP	Green fluorescent protein
HA	Influenza hemagglutinin
HGT	Horizontal gene transfer
HRP	Horseradish peroxidase
IBD	Inflammatory bowel disease
IgG	Immunoglobulin G
IM	Inner membrane
IPTG	Isopropyl β –D-thiogalactopyranoside
IscR	Iron-sulphur (Fe-S) cluster-containing transcription factor
Kan	Kanamycin
LB	Luria-Bertani
LBA	Luria-Bertani including ampicillin
Lcn-2	Lipocalin-2
LexA	Locus for X-ray sensitivity A
LPS	Lipopolysaccharide
LysST	Lysis proteins of the ST64B prophage
MFI	Mean fluorescence intensity
mic	Microcin
MitC	Mitomycin C
NalAc	Nalidixic acid
ns	Not significant
o.n.	Overnight
OD_{600}	Optical density of 600 nm
OM	Outer membrane
Omp	Outer membrane protein
OmpLA	Outer membrane phospholipase A
p2	pColIB9
Pal	Peptidoglycan-associated lipoprotein
PBS	Phosphate buffered saline
P_{cib}	Promoter of colicin Ib
PCR	Polymerase chain reaction
pfu	Plaque forming units
PGL	Peptidoglycan layer
PMF	Proton motive force
PMN	Polymorphonuclear neutrophil

PMSF	Phenylmethylsulfonyl fluoride
P_{sicA}	Promoter of *sicA*
qPCR	Quantitative polymerase chain reaction
Rc	Receptor
RecA	Recombinase A
Rif	Rifampicin
RNA	Ribonucleic acid
RNase	Ribonuclease
RNS	Reactive nitrogen species
ROP	Repressor of primer
ROS	Reactive oxygen species
RT	Room temperature
S. Tm	*Salmonella enterica* serovar Typhimurium
SAR	Signal-anchor-release
SDS	Sodium dodecyl sulfate
sfGFP	Superfolder green fluorescent protein
sicA	*Salmonella* invasion chaperone A gene
Sm	Streptomycin
SPI	*Salmonella* pathogenicity island
ss	Single-stranded
SSC	Sideward scatter
SseI	*Salmonella* secreted effector protein
StD	Standard deviation
TEMED	Tetramethylethylenediamine
TI	Translocator
Tn*10*	Transposon 10
Tsx	Nucleoside specific channel-forming protein
ttss-1	Type III secretion systems encoded on *S.* Tm pathogenicity island 1 (T3SS-1)

List of Publications

Work published in the course of this thesis:

Nedialkova, L. P., Sidstedt M., Koeppel, M. B., **Spriewald S.**, Ring, D., Gerlach, R. G., Bossi L. & Stecher, B., 2015. Temperate phages promote colicin-dependent fitness of *Salmonella enterica* serovar Typhimurium. *Environmental Microbiology*

Spriewald S., Glaser J., Beutler M., Koeppel M. B. & Stecher B., 2015. Reporters for Single-Cell Analysis of Colicin Ib Expression in *Salmonella enterica* Serovar Typhimurium. *PLoS ONE* 10, e0144647

Other publications:

Bebeacua C., Fajardo L., Carlos J., Blangy S., Spinelli S., **Bollmann S.**, Neve H., Cambillau C. & Heller K., 2013. X-ray structure of a superinfection exclusion lipoprotein from phage TP-J34 and identification of the tape measure protein as its target. *Molecular microbiology,* 89, 152-165

Summary

Colicins are toxins produced under stress conditions by representatives of the Enterobacteriaceae family. Released in the environment they target closely related colicin-sensitive competitors. Thus, colicin-production is a wide-spread example for interference competition in complex bacterial ecosystems. Production of colicins is highly costly for the producer as the individual bacterium dies upon colicin-release by lysis. Therefore, colicins are only synthesized by a fraction of the (genetically identical) population while the remaining part of the population survives and can gain a fitness benefit over colicin-sensitive competitors. This strategy is termed 'division of labor'. It was previously shown that the enteric pathogen *Salmonella enterica* serovar Typhimurium SL1344 (*S*. Tm) produces colicin Ib (ColIb) which confers a competitive advantage to *S*. Tm over commensal, colicin-sensitive *E. coli* strains upon infection.

In the first part of this thesis, I investigated colicin Ib (*cib*) regulation of *S*. Tm at the single cell level. To this end a single- and a multi-copy *gfp*-reporter for the promoter of colicin Ib were generated. Comparative analysis revealed that the multi-copy *gfp*-reporter ($p^{P\,cib\,gfp}$) exhibits an optimal GFP signal intensity for a variety of applications. Further characterization showed that GFP intensity levels of $p^{P\,cib\,gfp}$ correlated well with intrinsic ColIb levels in individual bacteria. *Cib* expression is negatively regulated by the repressors LexA and Fur. Only a small fraction of the *S*. Tm population expressed *cib* under non-inducing conditions. In the course of this thesis the activity of the *cib* promoter (P*cib*) was further characterized in response to SOS-response induction and iron-limitation. When applied individually, both environmental conditions led to an unimodal increase of the proportion of GFP^+ *S*. Tm, however with an overall low GFP signal intensity. In contrast, the simultaneous exposure to both conditions resulted in a *S.* Tm population exhibiting unimodal and high GFP signal intensity. In conclusion, this first part of my thesis provided a thorough characterization of $p^{P\,cib\,gfp}$ which is a prerequisite to investigate the regulation of *cib* expression and release at the single cell level.

Colicin Ib belongs to colicin subgroup B, which do not encode a cognate lysis gene. Until recently, the release mechanism has been unclear. Our group showed that in *S.* Tm ColIb can be released in the course of prophage-mediated cell lysis. To simultaneously investigate prophage activity and ColIb release at the single cell level, appropriate *gfp*-reporter tools were generated. I could confirm that individual Col^+ bacteria only lyse in the presence of intact prophages. Furthermore, single cell analysis revealed that *cib* expression and induction of prophage-lysis genes are co-regulated within individual bacteria. Interestingly, only a fraction of $ColIb^+$ *S.* Tm induces prophage-mediated cell lysis and undergoes lysis.

Therefore I could show that *S.* Tm uses the strategy of 'division of labor' for the process of colicin release and not as it is the case for other colicins, for colicin production. In conclusion, this data reveal the mechanism how *S.* Tm gains a fitness benefit over competing *E. coli* in the inflamed intestine by 'division of labor'.

Generally, colicin-producing bacteria also synthesize a cognate immunity protein, which protects the producer population against self-killing. Here, we characterized an *E. coli* strain (Ec^{252R}) that produces ColM (*cma*) and ColB (*cba*). Interestingly, the strain was immune to ColB but highly susceptible to ColM, despite producing high amounts of this colicin. ColM is a peptidoglycan lipid-II-degrading enzyme that interferes with cell wall biogenesis. *Cba* and *cma* are encoded 'in tandem' on the same locus on a large conjugative plasmid. Expression of both genes is under control of the same promoter, which is located upstream of *cba*. In addition, the cognate immunity genes (*cbi* and *cmi*) are also encoded on the same locus. In this part of the thesis I aimed to unravel the underlying reason for ColM sensitivity of Ec^{252R}. First, I demonstrated that the immunity protein of Ec^{252R} is fully functional and therefore not the underlying reason for lack of ColM immunity of Ec^{252R}. However, *cmi* overexpression only partially protected ColM producers against 'self-killing'.

Next, a collection of 36 *E. coli* strains carrying the *cma* locus was characterized. Two strains could be identified which also exhibited the 'self-killing' phenotype. Interestingly, the majority of the strains harbor a short version of the *cba* gene (*cba*-remnant) and this genotype was associated with lack of ColM production. Based on our findings, we hypothesized that immunity to ColM is insufficient to protect ColM producers against self-killing. Thus, resistance to ColM, as seen in the vast majority of ColM producers must have been gained by other mechanisms. In particular, *E. coli* strains which have just received a ColBM plasmid are only partially protected against ColM by the ColM immunity protein and have to 'further evolve' by accumulating mutations rendering them resistant to ColM or by interfering with *cma* expression. According to this idea, Ec^{252R} might represent an isolate which recently acquired a functional ColBM plasmid but has not yet evolved towards ColM tolerance or downregulation of ColM production. Finally, a series of *in vitro* evolution experiments was performed to experimentally verify this idea. In conclusion, our data reveal novel insights into the biology of ColM, which represents an exceptional case among other colicins, as it shows high toxicity despite intact immunity.

Zusammenfassung

Colicine sind Toxine, die unter Stressbedingungen von Mitgliedern der Familie der Enterobacteriaceae gebildet werden. Vom Produzenten entlassen richtet sich ihre Toxizität gegen nah verwandte, kompetierende und Colicin sensitive Bakterien. Demzufolge ist die Produktion von Colicinen eine weitverbreitete Wettbewerbsstrategie zwischen Bakteriengruppen in komplexen Ökosystemen. Die Produktion von Colicinen ist für den Produzenten mit hohen Kosten verbunden, da die Freisetzung der Colicine die Zell-Lyse und somit den Tod des Produzenten zur Folge hat. Auf Grund dieser Tatsache wird das Colicingen nur in einem Teil der (genetisch identischen) Population exprimiert. Der verbliebende Teil der Population überlebt und erhält einen Fitness Vorteil gegenüber anderen kompetierende sensitiven Stämmen. Diese Strategie der „Arbeitsteilung“ dient dem Wohle der gesamten Population.

Vor kurzen wurde gezeigt, dass während einer Darmentzündung der darmpathogene Erreger *Salmonella enterica* serovar Typhimurium SL1344 (*S.* Tm) durch die Synthese von Colicin Ib (ColIb) einen kompetitiven Wachstumsvorteil gegenüber dem Kommensalen und Colicin sensitiven *E. coli* Stamm erlangt. Im ersten Teil dieser Arbeit, wurde die Regulation von ColIb (*cib*) in *S.* Tm auf Einzelzellebene untersucht. Für diesen Zweck wurden zwei unterschiedliche *gfp*-Reporter („single-copy“ und „multi-copy“ Reporter) hergestellt, mit denen sich die ColIb Promoter Aktivität bestimmen lässt. Eine vergleichende Analyse ergab, dass der „multi-copy“ *gfp*-Reporter ($p^{P_{cib\ gfp}}$) optimale GFP Intensitäts-Levels erzielt und somit für eine Vielzahl von Anwendungen geeignet ist. Des Weiteren zeigten die Untersuchen, dass die Bildung von GFP durch den Reporter mit intrinsischen ColIb Proteinmengen in der *S.* Tm Population hervorragend korreliert.

Frühere Arbeiten unserer Arbeitsgruppe konnten zeigen, dass die Expression von *cib* durch die Repressoren LexA und Fur negativ reguliert wird (Nedialkova *et al.*, 2014). Dieser Sachverhalt konnte nun im zweiten Teil dieser Arbeit auf Einzelzellebene bestätigt werden. Zunächst konnte gezeigt werden, dass unter nicht induzierten Bedingungen ColIb nur durch eine kleine Fraktion der *S.* Tm Population gebildet wird. Im weiteren Verlauf dieser Arbeit wurde mit Hilfe des *gfp*-Reporters die Aktivität des Colicin Ib Promoters (P*cib*) im Bezug auf die Induktion der SOS-Antwort, sowie Eisenmangel weiter charakterisiert. Jede Bedingung für sich führte zu einer unimodalen Zunahme von GFP^+ *S.* Tm Bakterien, allerdings mit einer durchschnittlich geringen GFP Signal Intensität. Im Gegensatz dazu führte die Induktion der SOS-Antwort und ein gleichzeitig vorliegender Eisenmangel zu einer unimodalen aber insgesamt starken GFP Signal Intensität. Zusammenfassend konnte festgestellt werden,

dass im ersten Teil dieser Dissertation eine sorgfältige Charakterisierung des *gfp*-Reporters $p^{P_{cib\ gfp}}$ gelungen ist. Daraufhin konnte der *gfp*-Reporter verwendet werden um die Regulation der *cib* Expression, sowie die Freisetzung von ColIb auf Einzelzellebene zu untersuchen.

Colicin Ib gehört zu der Klasse von Colicinen der Gruppe B und kodiert daher kein entsprechendes Gen, welches für die Lyse der Zelle und somit zur Freisetzung von intrazellularem Colicin verantwortlich ist. Bislang war daher unklar auf welche Weise ColIb aus der Zelle freigesetzt wird. Unsere Gruppe konnte vor kurzem zeigen, dass ColIb von *S.* Tm durch die Zelllyse freigesetzt wird (Nedialkova *et al.*, 2015). Diese Zelllyse wird durch Prophagen kodierte Lyse-Proteine verursacht und ColIb im Zuge der Aktivierung von Prophagen freigesetzt. Um die Expression von *cib*, sowie die Induktion von Prophagen gleichzeitig untersuchen zu können, wurden entsprechende *gfp*-Reporter hergestellt. Im Laufe meiner Dissertation konnte ich bestätigen, dass einzelne Col^+ Bakterien nur in Gegenwart von intakten Prophagen lysieren. Des Weiteren konnte ich zeigen, dass innerhalb einer Bakterienzelle die Expression von *cib* und die Induktion der Prophagen kodierten Lysegene ko-reguliert sind. Interessanterweise wurde des Weiteren festgestellt, dass nur ein kleiner Teil von $ColIb^+$ *S.* Tm Bakterien die Expression von Prophagen kodierten Lysegene induziert und im Anschluss lysiert. Basierend auf den Ergebnissen meiner Dissertation konnte nun erstmals gezeigt werden, dass *S.* Tm die Strategie der „Arbeitsteilung" für die Freisetzung von ColIb verfolgt, jedoch aber nicht, wie bei anderen Colicinen, für die Produktion des Colicins. Dies deutet erstmals auf den Mechanismus hin, wie *S.* Tm im entzündeten Darm durch „Arbeitsteilung" einen Fitnessvorteil gegenüber kompetierende *E. coli* Stämme erlangt.

Gewöhnlich bildet ein Colicin produzierendes Bakterium ein entsprechendes Immunitätsprotein, um sich vor der Toxizität des Colicins, und somit vor einem „Selbstmord" zu schützen. Im letzten Teil der Arbeit habe ich einen Colicin B (ColB) sowie Colicin M (ColM) produzierenden *E. coli* Stamm (Ec^{252R}) charakterisiert. Dieser Stamm ist zwar immun gegenüber ColB, aber zeigt eine sehr hohe Sensitivität gegenüber ColM. ColM ist nicht nur eines der kleinsten Colicine, es hat auch eine einzigartige Wirkungsweise. Im Gegensatz zu den derzeit bekannten Colicinen inhibiert ColM die Peptidoglycan Biosynthese. Dieses erfolgt im Periplasma durch die Hydrolyse des Phosphatesters zwischen dem Lipidcarrier Undecaprenol und der Mureinvorstufe N-Actelymuramyl-(Pentapeptid)-N-Acetylglucosamin. ColM (*cma*) sowie ColB (*cba*) sind hintereinander auf einem großen, konjugativen Plasmid kodiert (pColBM). Die Expression beider Gene wird von ein und demselben Promoter reguliert, welcher sich stromaufwärts von *cba* befindet. Entsprechende Immunitätsgene (*cbi* und *cmi*) sind ebenfalls auf demselben Lokus kodiert, werden aber in die entgegengesetzte Richtung transkribiert. Ziel war es in diesem Teil der Dissertation die Ursachen für die

Sensitivität von Ec^{252R} gegenüber dem selbstgebildeten ColM aufzuklären. Zunächst habe ich belegt, dass das von Ec^{252R} produzierte ColM Immunitätsprotein funktionell ist und daher nicht die Ursache für den durch ColM verursachten „Selbstmord“ von Ec^{252R} ist. Darüber hinaus konnten die Ergebnisse dieser Arbeit zeigen, dass die Produktion des ColM Immunitätsproteins im Allgemeinen den Produzenten nur teilweise gegenüber der Toxizität von ColM schützt. Im Anschluss wurde eine Sammlung bestehend aus 36 verschiedenen *E. coli* Stämmen, welche den *cma* Lokus tragen, charakterisiert. Zwei dieser Stämme zeigten ebenfalls einen ähnlichen „Selbstmord“ Phänotyp. Auffällig war, dass die Mehrheit dieser Stämme ein verkürztes *cba* Gen aufweist („*cba*-remnant“). Eine genauere Untersuchung ergab, dass die Verkürzung des *cba* Gens eine Inhibition der ColM Produktion zur Folge hat. Basierend auf diesen Ergebnissen stellte ich die Hypothese auf, dass die Immunität gegenüber ColM unzureichend ist und daher ColM Produzenten nicht ausreichend gegenüber einem „Selbstmord“ geschützt sind. Aus diesem Grund muss eine ColM-Resistenz, welche in der großen Mehrheit der ColM Produzenten beobachtet wurde, durch andere Mechanismen entstanden sein. Vor allem *E. coli* Stämme, welche gerade erst ein ColBM Plasmid aufgenommen haben, sind durch das ColM Immunitätsprotein gegenüber ColM nur partiell geschützt. Diese „neuen“ ColBM Produzenten müssten infolgedessen Mutationen anhäufen, welche eine ColM-Resistenz vermitteln oder mit der *cma* Expression interfrieren. In Anlehnung an diese Idee habe ich vermutet, dass Ec^{252R} eventuell ein Isolat repräsentiert, welches vor kurzem ein funktionales ColBM Plasmid aufgenommen hat. Dieser Stamm hat sich bisher jedoch noch nicht genügend angepasst, so dass sich eine ColM-Resistenz entwickeln konnte oder die Reduzierung der ColM Produktion bewirkt wurde. Um diese Idee experimentell zu verifizieren wurde schließlich eine Serie an *in vitro* Evolutionsexperimenten durchgeführt. Zusammenfassend lässt sich sagen, dass die Daten dieser Dissertation neue Erkenntnisse und Einblicke in die Biologie von ColM liefern. Laut meiner Ergebnisse repräsentiert ColM nicht nur ein Ausnahmefall im Bezug auf seine einzigartige Wirkungsweise innerhalb der Colicine, sondern auch ein Ausnahmefall im Bezug auf seine ungewöhnlich hohe Toxizität gegenüber seinem Produzenten, trotz eines intakten und funktionellen Immunitätsproteins.

1 Introduction

1.1 Colicin biology

Bacteria are found in all kind of environments such as e.g. soil, water or the human gut, were they form complex communities and interact with each other. Competition between microorganisms is one example of bacterial interaction (Hibbing *et al.*, 2010). Competition between microorganisms (interspecies competition) has an important role in microbial interactions as it drives community dynamics and thereby shapes biodiversity (Little *et al.*, 2008, Foster and Bell, 2012). It is also important for pathogens for the establishment of infections (Stecher *et al.*, 2007). Interspecies competition can occur in two different scenarios, by exploitative and interference competition (Little *et al.*, 2008). In case of exploitative competition, bacteria compete with other bacteria by consuming their resource (space and nutrients) and thus banish competitors from the captured ecological niche (Little *et al.*, 2008). In contrast to this, using interference competition bacteria compete directly with other bacteria and harm competitors by production of toxic compounds like antimicrobials (Cornforth and Foster, 2013). These produced compounds also include bacteriocins such as colicins (Majeed *et al.*, 2011).

1.1.1 Main characteristics of colicins

Colicins were first discovered in the name-giving species *Escherichia coli* (*E. coli*) by André Gratia in 1925 (Gratia, 1925) and since then have been also found in other species of Enterobacteriaceae like *Salmonella* spp., *Shigella* spp. and *Citrobacter* spp. (Cascales *et al.*, 2007). Colicins are small proteinaceous toxins and are classified as bacteriocins (Jacob *et al.*, 1953). In fact, the colicin is released by the producing bacterium, and kills susceptible closely related target bacteria (usually of the same species) (Riley and Wertz, 2002a). They are generally found with a high molecular mass ranging in size from around 42 to 75 kDa, with the exceptions of colicin M (~30 kDa) and colicin Js (11 kDa) (Šmarda and Šmajs, 1998, Šmajs and Weinstock, 2001). Colicins display a high killing efficiency, as only one molecule of the colicin, if successfully translocated, is sufficient to kill a sensitive target bacterium. These toxins are usually harmless for the producer if the cognate immunity protein is simultaneously synthesized. Colicins are mostly abundant in the mammalian gut and play an important role as competition factors for intestinal colonization by new strains including enteric pathogens (Cascales *et al.*, 2007).

According to the translocation machinery used to enter the target bacterium, colicins are classified in two groups (A and B). Group A colicins (e.g. colicin A, E1-E9) are translocated in a Tol-dependent manner, whereas group B colicins (e.g. colicin B, Ia, Ib, M) are imported into the target bacterium by the TonB-translocation machinery (Davies and Reeves, 1975a, Davies and Reeves, 1975b). More than 20 different colicins have been discovered so far. They do not only differ in size and in the dependency on the translocation machinery, but also display a wide range of killing mechanisms (**Table 1.1**). Colicins with nuclease activity (DNase or RNase), colicin M which comprises the ability to disrupt the peptidoglycan biosynthesis, and pore-forming ionophore colicins have been described (Cascales *et al.*, 2007). The latter are able to depolarize the bacterial cell membrane followed by an efflux of intracellular ions and death of the bacterium (Weaver *et al.*, 1981a).

Table 1.1: Characteristics of the most studied colicins: import and mode of action.

Colicin	1st receptor	2nd receptor (translocator)	Translocation system	Mode of action
Group A				
A	BtuB	OmpF	TolA, TolB, TolQ, TolR	Pore formation
E1	BtuB	TolC	TolA, TolR	Pore formation
E2	BtuB	OmpF	TolA, TolB, TolQ, TolR	DNase
E3	BtuB	OmpF	TolA, TolB, TolQ, TolR	16S-RNase
E4	BtuB	OmpF	TolA, TolB, TolQ, TolR	16S-RNase
E5	BtuB	OmpF	TolA, TolB, TolQ, TolR	t-RNase
E6	BtuB	OmpF	TolA, TolB, TolQ, TolR	16S-RNase
E7	BtuB	OmpF	TolA, TolB, TolQ, TolR	DNase
E8	BtuB	OmpF	TolA, TolB, TolQ, TolR	DNase
E9	BtuB	OmpF	TolA, TolB, TolQ, TolR	DNase
K	Tsx	OmpF	TolA, TolB, TolQ, TolR	Pore formation
N	LPS	OmpF	TolA, TolQ, TolR	Pore formation
S4	OmpW	OmpF	TolA, TolB, TolQ, TolR	Pore formation
U	OmpA	OmpF, LPS	TolA, TolB, TolQ, TolR	Pore formation
Group B				
B	FepA	unknown	TonB, ExbB, ExbD	Pore formation
D	FepA	unknown	TonB, ExbB, ExbD	t-RNase
Ia	CirA	CirA	TonB, ExbB, ExbD	Pore formation
Ib	CirA	CirA	TonB, ExbB, ExbD	Pore formation
M	FhuA	FhuA	TonB, ExbB, ExbD	Inhibition of peptidoglycan biosynthesis
5	Tsx	TolC	TonB, ExbB, ExbD	Pore formation
10	Tsx	TolC	TonB, ExbB, ExbD	Pore formation

BtuB: vitamin B_{12} transporter; CirA: colicin I receptor; FepA: ferrienterobactin receptor; FhuA: ferrichrome-iron receptor; Omp: outer membrane protein; Tsx: nucleoside specific channel-forming protein. Table modified from (Kim *et al.*, 2014).

All colicins have in common a tripartite domain structure consisting of the amino-terminal translocation domain (T), the receptor domain (R) and the carboxy-terminal cytotoxic domain (C) (**Figure 1.1A**). The initial step of colicin import is also similar to all colicins. First, the central R-domain binds with high affinity to its specific outer-membrane receptor, thus explaining the narrow-spectrum of colicin activity. Receptors for colicins can be either trimeric outer membrane porins (OmpF, OmpW, OmpA), or outer membrane transporters usually used to transport iron-chelate complexes (FhuA, FepA, Tsx, CirA), or vitamin B_{12} (BtuB), or lipopolysaccharides (LPS) in case of colicin N (Kleanthous, 2010, Johnson *et al.*, 2014). After binding, group A colicins recognize a translocator protein with their T-domain (e.g. OmpF), subsequently recruit the Tol- translocation system to the translocon and the T-domain passes through the outer membrane (OM). The Tol-translocation system consists of the inner membrane proteins TolA, TolQ, TolR, the periplasmic protein TolB and the peptidoglycan-associated lipoprotein Pal. The proteins TolQ and TolR are responsible for the proton-motive-force (PMF)-dependent activation of TolA (**Figure 1.1B**). However, group B colicins bind with the T-domain a second copy of the R-domain-bound receptor (e.g. ColIa uses two copies of CirA) followed by the recruitment of proteins of the TonB-dependent system (Jakes and Finkelstein, 2010). The TonB-machinery consists of the inner membrane proteins TonB, ExbB, ExbD, whereas ExbB and ExbD provide the required energy from the inner membrane PMF to energize TonB for the uptake of group B colicins (**Figure 1.1C**). After receptor-binding, the C-domain of the colicin crosses the OM by a yet unknown mechanism. Depending on the mode of action, the C-domain is integrated into the inner membrane (IM) (pore formers) while in case of colicin M it stays in the periplasm or is further translocated into the cytoplasm (nucleases). In case of nuclease colicins, the C-domain is cleaved off in a FtsH-dependent manner (ATP-dependent metalloprotease) in the IM and is further passaged through the IM into the cytoplasm (Kim *et al.*, 2014).

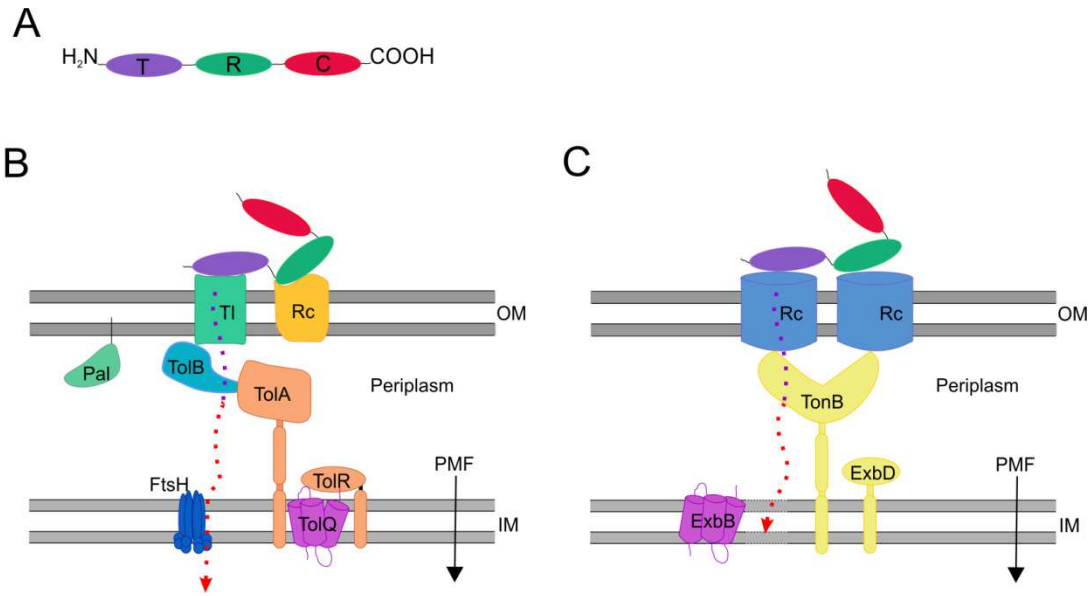

Figure 1.1.: Schematic view of Tol- and TonB-dependent import pathways for colicins. (A) The tripartite domain structure of colicins, consisting of an amino-terminal translocation domain [T], a central receptor binding domain [R] and a carboxy-terminal cytotoxic domain [C]. **(B)** Example of the import of a group A nuclease colicin (e.g. colicin K) using the Tol-dependent import machinery (TolA, TolB, TolQ, TolR and the peptidoglycan-associated lipoprotein Pal). At first the colicin binds with its central R-domain to its specific receptor [Rc]. In the next step the T-domain recognizes the translocator protein [TI], followed by the recruitment of the Tol-import machinery and its transfer through the outer membrane [OM] in the periplasm (indicated as purple dots). After this, the C-domain is also imported in the periplasm by a yet unknown mechanism, cleaved from the other domains in an FtsH-dependent manner and further translocated (indicated as red dots). **(C)** Example of the import of a group B pore-forming colicin by the TonB-translocation system (TonB, ExbB, ExbD). The colicin binds with its R-domain its specific receptor and with the T-domain a second copy, of this receptor, thereby recruiting the TonB-machinery. After this the T-domain is translocated through the OM in the periplasm (indicated as purple dots). As a consequence the C-domain is imported in the periplasm by a yet unknown mechanism and integrated in the IM to form a voltage gated ion channel (indicated as red dots). Both import machineries are energy-dependent and receive this energy from the proton motive force [PMF] by either TolR/TolQ (Tol-dependent translocation) or ExbB/ExbD (TonB-dependent translocation). Adapted by (Kim *et al.*, 2014).

1.1.2 Genetic organization and regulation of colicins

Colicin operons are encoded on colicinogenic plasmids and can be subdivided into two classes: type I and type II. Type I plasmids mostly encode operons of group A colicins and are small (6-10 kb), multi-copy (~20 copies/cell) and mobilizable. Type II plasmids generally encode operons of the group B colicins and are usually large (>40 kb), mono-copy and conjugative (Hardy *et al.*, 1973). Type II plasmids can also encode two different colicins, as for example pColBM, which encodes genes for colicin B and M (Köck *et al.*, 1987, Ölschläger *et al.*, 1984).

The genetic organization of nuclease colicins differ from that of pore-forming colicins. The colicin gene (= activity gene) of nuclease colicins is followed by the gene encoding for a cognate immunity protein that protects the colicin-producer against self-killing. The immunity gene is regulated by the promoter of the activity gene and, additionally, by its own

constitutive promoter, located within the activity gene. This allows constitutive expression of the immunity gene. For pore-forming colicins the immunity gene is not encoded on the same operon but on the opposite DNA strand downstream of the colicin activity gene, and is regulated by its own constitutive promoter. The operon of group A colicins also encodes a third gene downstream of the activity gene (pore-formers) or immunity gene (nucleases), which encodes for a lysis gene and ensures the release of colicins by the producing cell (**Figure 1.2**). In contrast, group B colicins usually do not encode for a lysis gene, except colicin 5, 10 and D. In general, all colicins are negatively regulated by a strong SOS-inducible promoter in a LexA (locus for X-ray sensitivity A)-dependent manner (Singh and Ghosh, 2012) and SOS-induction can yield a 1000-fold increase in colicin gene expression (Zgur-Bertok, 2012).

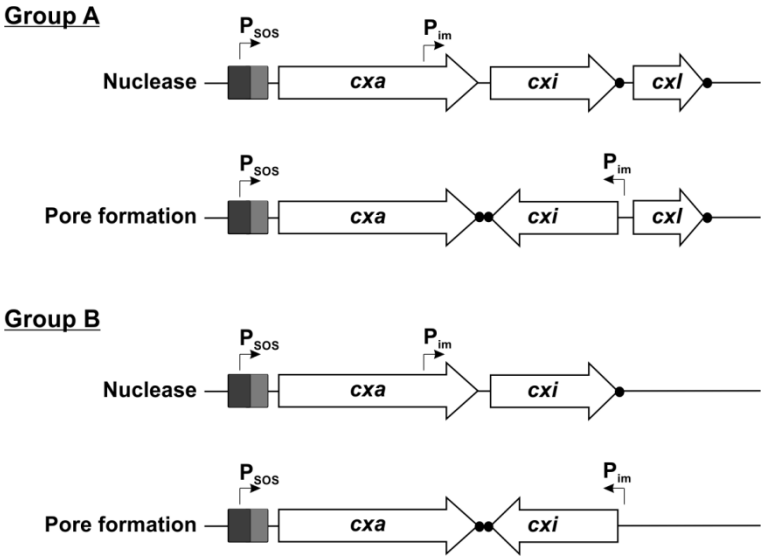

Figure 1.2: Gene structure of colicin operons. Colicin operons are generally regulated by the SOS-inducible promoter P_{SOS}. The operon of group A nuclease-type colicins encodes the colicin X activity gene (*cxa*), its cognate immunity gene (*cxi*) and the lysis gene (*cxl*). In contrast, group B, colicin operons (except colicin D, 5, 10) lacking the *cxl* gene. The operons of pore-forming colicins do not encode for the immunity gene *cxi*. They either encode the genes *cxa* and *cxl* (group A) or only the *cxa* gene (group B). In general, the immunity gene of pore-forming colicins is located on the opposite DNA strand and regulated by its own constitutive promoter (P_{im}). The immunity genes of nuclease-type colicins are additionally regulated by the P_{SOS} promoter. Transcription terminators are indicated as ●.

The SOS-response comprises a pathway for controlling DNA repair and is responsible for the regulation of more than 50 genes (Zgur-Bertok, 2013). For the regulation of the SOS-response two key regulator proteins are needed: the repressor protein LexA and the inducer protein RecA (recombinase A) (Butala *et al.*, 2009). Repression of SOS-inducible genes takes place by a LexA dimer binding to a short (20 bp) palindromic consensus sequence which is termed SOS-box and is located downstream of the Pribnow box. The SOS-response can be induced by exogenous factors (e.g. UV-radiation, chemical or oxidative compounds or antibiotics (like mitomycin C and ciprofloxacin) or by endogenous factors as stalled replication forks or metabolic by-products as reactive oxygen species (ROS) (Zgur-Bertok, 2013).

Concerning the main SOS-response pathways in general, upon SOS-induction (e.g. by UV irradiation) double stranded DNA breaks occur and are recognized by the RecBCD complex. This complex generates with its helicase and nuclease activity single stranded DNA (ssDNA) the substrate for RecA. Thereafter, ssDNA accumulates in the cell and is recognized and bound by RecA, which is thereby converted in its active form as a nucleoprotein filament. This induces auto-cleavage of free LexA dimers and lead to the dissociation of LexA from the SOS-box. As a consequence, this leads to de-repression of SOS-inducible promotors (Baharoglu and Mazel, 2014).

In case of colicin regulation two overlapping SOS-boxes are generally involved except for colicin Ia and Ib, which are regulated only by a single SOS-box differing from SOS-box consensus sequences of other colicins of *E. coli* (Gillor *et al.*, 2008). Recently it has been shown that colicin synthesis is additionally regulated by nutrient-responsive regulators such as ferric uptake regulator Fur (colicin Ib) (Nedialkova *et al.*, 2014), the transcription regulator IscR (iron–sulphur cluster regulator) (colicin E1, K, N) (Butala *et al.*, 2012) or transcription regulator AsnC (ColE2, ColE6, ColE8) (Kamenšek *et al.*, 2015) or the RNA-binding protein CsrA (carbon storage regulator protein) which post-transcriptionally regulates synthesis of colicin E7 and E2 lysis genes (Yang *et al.*, 2010, Hol *et al.*, 2014). Due to these additional regulators induction of colicin gene expression exhibits a short lag phase between SOS-induction and the induction of colicin gene expression (Herschman and Helinski, 1967, Salles *et al.*, 1987). Therefore, colicin synthesis occurs only upon severe and persistent SOS-response induction and additional regulator proteins ensure a tight repression of colicin and colicin lysis genes (Zgur-Bertok, 2012).

1.1.3 Colicin release mechanisms

The release mechanism of group A colicins and some colicins of group B (colicin D, 5 and 10) depends on production of the lysis protein. Colicin lysis proteins are small lipoproteins (27-35 amino acids), which are synthesized as precursor in the cytoplasm and further processed by lipid-modification events, resulting in a mature lysis protein anchored to the periplasmic leaflet of the IM or OM. This leads to membrane perturbation, so that the outer membrane phospholipase A (OmpLA) is activated (OmpLA*). OmpLA* permeabilizes the OM by hydrolyzing the OM-phospholipids and generates thereby fatty acids and lysophopholipids, promoting the release of colicins (Snijder and Dijkstra, 2000, Pugsley and Schwartz, 1984, Cavard, 1997). For colicin E7 release it was further shown that the mature lysis protein, anchored in the IM, activates OmpLA cooperatively with its cleaved stable signal peptide (Chen *et al.*, 2011).

The release mechanism of group B colicins, which do not encode a cognate lysis gene had been unclear for a long time (Cascales *et al.*, 2007). Recently another colicin lysis gene independent release mechanism has been described. Group B colicin Ib, which does not encode a colicin lysis gene, is released from the producing bacteria by prophage-mediated bacterial cell lysis (Nedialkova *et al.*, 2015) (**section 1.5.7**).

1.1.4 Immunity to colicins

Protection against self-killing due to colicin synthesis is usually circumvented by the simultaneous production of a cognate immunity protein. To neutralize the lethal toxicity of the colicin its immunity protein has to bind to the C-domain of the colicin (Cascales *et al.*, 2007). Immunity proteins of nuclease-type colicins (DNase- and RNase-type) have a molecular weight of about ~10 kDa and bind directly to their cognate colicin after their synthesis. This occurs either to the nuclease activity site directly, in case of immunity proteins of tRNase-type colicins (e.g. Im5 and ImD), or by binding the adjacent region of the active site, in case of DNase and rRNase colicins (e.g. colicins E2, E7, E9, E3, E4, E6) (Kleanthous *et al.*, 1999, Kleanthous and Walker, 2001, Luna-Chávez *et al.*, 2006). Both blocking mechanisms inhibit colicin binding to its substrate. Hence, the nuclease-type colicin, once synthesized, is bound directly to its cognate immunity protein, to prevent its lethal toxicity already inside the producer, and is released by the producer as an inactive complex. The immunity protein is released from this complex after the colicin has bound its receptor, but the mechanism is still unclear (Kim *et al.*, 2014). The immunity proteins of pore-forming colicins, having a molecular weight of about 13-21 kDa (Harkness and Ölschläger, 1991), are integral proteins located in the IM and are classified as A-type (for colicins A, B, N, and U) or E1-type (for colicins E1, 5, K, 10, Ia, and Ib) immunity proteins. The immunity proteins of both groups diffuse laterally in

the IM to bind their cognate colicin cytotoxic domain right before the colicin-formed channel opens. Therefore it does not prevent formation of the channel but the depolarization of the membrane. The integral embedded immunity proteins either bind the voltage-gated region of the colicin channel (E1-type e.g. colicin Ib) or to the adjacent region (A-type e.g. colicin B) (Espesset *et al.*, 1996, Nardi *et al.*, 2001, Zhang *et al.*, 2010).

The immunity protein of the enzymatically active colicin M has a calculated molecular weight of about 14 kDa (Harkness and Ölschläger, 1991, Usón *et al.*, 2012), but showed a molecular weight of 16 kDa determined by polyacrylamide gels (Olschläger and Braun, 1987). It is anchored in the IM similarly to the immunity proteins of pore-forming colicins and binds colicin M in the periplasm (Usón *et al.*, 2012), which is in contrast to the immunity proteins of the enzymatic nuclease-type colicins. However, colicin M is only lethal to a bacterium if translocated from the outside into the periplasm. Therefore colicin M does not need to be bound to its immunity protein the entire time (Harkness and Braun, 1990).

1.1.5 Colicin Ib in *Salmonella enterica* serovar Typhimurium

Salmonella enterica serovar Typhimurium (*S.* Tm) is a Gram-negative and facultative anaerobic bacterium which belongs to the family of Enterobacteriaceae. As intracellular pathogen of humans and warm-blooded animals, it causes self-limiting diarrhea (in humans) or a systemic typhoid-like disease (in mice) (Coburn *et al.*, 2007). Usually in the healthy intestine Enterobacteriaceae as *S.* Tm and *E. coli* are only present in low abundance (~0.1%) and are taken over by the residing obligate anaerobic microbiota (homeostasis) (Eckburg *et al.*, 2005). However, in response to inflammatory conditions, for instance when induced by enteropathogenic *S.* Tm (Stecher *et al.*, 2007), the ecology of the microbiota is altered, which in turn leads to a high increase (“bloom”) of facultative anaerobic Enterobacteriaceae as *S.* Tm and *E. coli* (Stecher *et al.*, 2013). For instance, induction of inflammation causes neutrophil transmigration, which produce lipocalin-2 that sequesters bacterial siderophores (e.g. enterochelin) (Raffatellu *et al.*, 2009), and stress-inducing reactive oxygen and nitrogen species (ROS and RNS) (Fialkow *et al.*, 2007, Winter *et al.*, 2013a). Furthermore, this leads to generation of anaerobic electron acceptors by the inflamed mucosa (e.g. NO_3^-, tetrathionate) which are used for anaerobic respiration by facultative anaerobic bacteria (Winter *et al.*, 2010a, Winter *et al.*, 2013b). Intriguingly, under inflammatory conditions *S.* Tm (*S.* Tm^{SL1344}) is not only able to outcompete the resident anaerobic microbiota but also commensal *E. coli*. This ability of *S.* Tm is conferred by the large (86.9 kb) low-copy, conjugative colicinogenic plasmid pCoIIB9 (further named as p2) which encodes, among other genes, for colicin Ib and its cognate immunity protein (Kröger *et al.*, 2012, Stecher *et al.*, 2012a, Clewell and Helinski, 1970). Colicin Ib production is induced by SOS-response

and Fe^{2+}-limitation (see **section 1.1.2**). Killing occurs by the binding of colicin Ib to its receptor CirA, followed by the TonB-dependent translocation (as for group B colicins) across the OM and the formation of pores in the IM, a process that leads to ion-leakage and cell death of a sensitive bacterium (Weaver *et al.*, 1981b). Additionally, it has been shown in *in vivo* studies that *S.* Tm^{SL1344} up-regulates colicin Ib gene expression in the inflamed mouse gut. This confers *S.* Tm^{SL1344} a strong growth advantage over competing, commensal and colicin Ib-sensitive *E. coli* in inflammation-induced Enterobacterial blooms (**Figure 1.3**) (Stecher *et al.*, 2013, Nedialkova *et al.*, 2014).

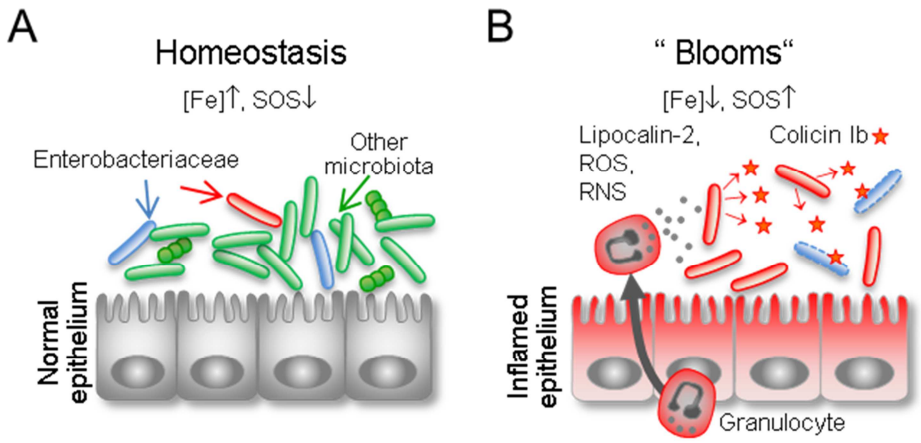

Figure 1.3: Inflammation induced colicin-dependent competition in Enterobacterial blooms. (A) In the healthy intestine, and thus upon homeostatic conditions, Enterobacteriaceae (red and blue) are low in numbers and suppressed by high numbers of the obligate anaerobic microbiota (green). **(B)** As a consequence of intestinal inflammation, induced for instance by enteropathogenic *S.* Tm (e.g. *S.* Tm^{SL1344}) the immune system of the host responds with e.g. neutrophil transmigration into the gut lumen. Iron-depleting agents as lipocalin-2 and reactive oxygen and nitrogen species (ROS, RNS) are produced and secreted which result in iron-limited conditions and activation of the SOS-response. Such environmental cues induce colicin Ib (red star) production and are thereby a trigger for colicin-dependent competition in Enterobacterial blooms. Adapted from (Nedialkova *et al.*, 2014).

1.1.6 Colicin M in *E. coli*

As a member of the Enterobacteriaceae family, *E. coli* is a Gram-negative and facultative anaerobic bacterium and closely related to *S.* Tm. It is primarily found in the gastrointestinal tract as commensal of vertebrates like mammals, birds and reptiles. In addition a variety of *E. coli* pathovars exist, causing intestinal (diarrhea) or extraintestinal (bacteraemia, meningitis or urinary tract infections) diseases (Donnenberg, 2002, Dozois and Curtiss, 1999, Hartl and Dykhuizen, 1984). Strains of the species *E. coli* are the main reservoir of colicinogenic plasmids, which serve competition for ecological niches (Cascales *et al.*, 2007). Colicin M is unique among the colicins produced by *E. coli*. It has a molecular weight of only ~30 kDa and is therefore one of the smallest colicins known so far. Usually, it is encoded together with colicin B (~55 kDa) and their cognate immunity proteins on large (~80-180 kb) conjugative colicin BM plasmids further named as pColBM (Johnson *et al.*, 2006, Christenson and Gordon, 2009, Ölschläger *et al.*, 1984). Among all known colicins, colicin M displays a unique cytotoxicity, as it interferes with the peptidoglycan synthesis (El Ghachi *et al.*, 2006). Notably, colicin M is only toxic for the cell if imported from the surrounding environment (Harkness and Braun, 1990). To this end, colicin M binds to the OM receptor FhuA, probably partially unfolds and, as a group B colicin, it is further translocated into the periplasm, using the TonB-dependent machinery (Zeth *et al.*, 2008). Once it is in the periplasm it is bound by FkpA (a peptidyl prolyl cis-trans isomerase/chaperone) which refolds the colicin M protein (Helbig *et al.*, 2011). Active colicin M cleaves lipid II, the undecaprenyl-phosphate-linked precursor, of the growing peptidoglycan layer resulting in undecaprenol and 1-pyrophospho-(N-Acetylmuramic-acid)-(pentapeptide). These products cannot be used further for the murein biosynthesis, resulting in cell wall damage and bacterial lysis (El Ghachi *et al.*, 2006).

1.2 Ecology of colicinogeny

Colicinogeny can shape microbial community diversity and dynamics, as shown by mathematical modeling, in *in vitro* and *in vivo* studies (Riley and Wertz, 2002b, Czárán *et al.*, 2002, Nedialkova *et al.*, 2014, Kirkup and Riley, 2004). About 10-50% of natural *E. coli* strains are colicin-producers, whereas an average of about 70% are resistant to at least one colicin and an average of 30% are resistant to all investigated colicins, with only a small fraction of colicin-sensitive bacteria (Riley and Gordon, 1999).

Interestingly, although colicin-producers comprise a high-killing potential, the three different species (sensitive, producer, resistant) can coexist, but only in a spatially structured environment (e.g. growth on agar plates or in biofilms) according to a “rock-paper-scissor”

game (Kerr *et al.*, 2002). This system is also designated as “non-transitive-interaction network” (**Figure 1.4**). The producer strain beats the sensitive strain, but in turn the producer bears the cost for colicin production and thus is beaten by the resistant strain. The resistant strain in turn is beaten by the sensitive strain as the sensitive strain does not bear the costs for colicin resistance. For instance, colicin resistance can be achieved by mutation of the receptor which can result, in case of mutation of the receptor CirA (ColIb receptor) in suboptimal iron-acquisition. In a well-mixed environment (e.g. liquid culture), the resistant strain would outcompete both the sensitive strain and the producer strain, therefore preventing coexistence of all phenotypes (Kerr *et al.*, 2002, Hibbing *et al.*, 2010).

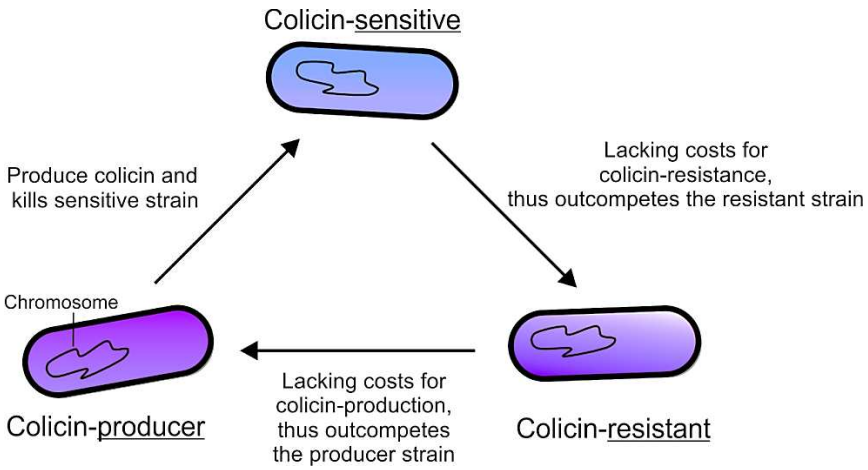

Figure 1.4: Rock-paper-scissors interplay in a non-transitive competition network. A colicin producing strain kills the colicin-sensitive strain due to colicin-mediated killing. The colicin-sensitive strain does not pay the costs for resistance and gain a competitive growth advantage over the colicin-resistant strain. The colicin-resistant strain in turn outcompetes the producer strain, as it does not pay the cost for colicin production. Adapted from (Hibbing *et al.*, 2010).

1.3 Evolution of colicin diversity

Evolutionary processes (e.g. mediated by mutations) are also common among colicins and contribute to the diversity of colicinogenic-bacterial population. It is assumed that colicins evolve by two different possibilities depending on the environmental abundancy. In an environment of less abundant colicins, as it would be in general the case for nuclease-type colicins (less abundant than pore-forming colicins), mutations are highly likely to appear in the colicin immunity region. This would result in a strain broadened in its immunity capacity against its own and relative colicins. This might be followed by an additional mutation in the colicin region close to the immunity gene. This generated novel colicin is able to kill the ancestral colicin producing strain and thus, outcompetes the yet more abundant ancestral colicin-producer. Upon conditions of high colicin-abundance, thus very likely for pore-forming

colicins, “domain-swapping” of protein-sequence with high similarities occurs by recombination. Interestingly, this domain-swapping was also shown for nuclease-type colicins (e.g. E2), demonstrating that colicin diversity, as a result of the occurrence of mutations, is dependent on colicin-abundance and sequence similarity rather than the mode of action (Riley and Wertz, 2002a). Another mechanism which contributes to the diversity of bacterial populations is the acquisition of additional colicin genes (e.g. pColBM). This confers a competitive advantage against single-colicin producer and a broadened killing capacity against sensitive cells, as these strains can produce colicins which bind to a variety of receptors (Gordon and O'Brien, 2006, Gordon *et al.*, 2007, Ölschläger *et al.*, 1984).

1.4 Heterogeneous gene expression in bacteria

Based on experimental bulk assays, it was assumed that a population of bacteria, derived from a common ancestor bacterium, consists of isogenic bacterial cells, which should all exhibit the same phenotype. Due to microscopy based single cell analysis techniques, it was discovered that in a population of isogenic bacteria, individuals can exhibit different phenotypic traits (e.g. gene expression, growth rates, cell-size), even if grown under the same environmental conditions. This phenomenon is designated as “phenotypic heterogeneity” (Raj and van Oudenaarden, 2008). Bistable (or bimodal) gene expression can lead to phenotypic heterogeneity in an isogenic population, e.g. due to feedback loops. For instance, changing nutrient concentrations in the surrounding environment can be sensed by the bacterial signaling network. In case the corresponding network comprises a bistable gene expression, the population is split into two phenotypically distinct subpopulations. It is also possible that the network comprises a multistable gene expression pattern, which results in more than two phenotypically distinct subpopulations (Smits *et al.*, 2006). For an isogenic population phenotypic heterogeneity can lead to an increase in the overall fitness of a population by two different scenarios, called “bet-hedging” and “division of labor” (**Figure 1.5**). Both can increase fitness of the genotype (Ackermann, 2015). The bet-hedging strategy can promote the fitness of a population in fluctuating environments. For instance, an isogenic population of bacteria can be composed of a fast growing subpopulation and a subpopulation with a phenotype reduced or inhibited in growth. The latter exhibits a detrimental phenotype compared to the other subpopulation, but it might be better adapted to other environments. This has been shown for *E. coli* persister cells which can tolerate antibiotic-exposure (Balaban *et al.*, 2004). Another example of bet-hedging is explained by variation in gene expression of metabolic pathways generated by a positive feedback loop, as shown for the lac-operon in *E. coli* (Elowitz *et al.*, 2002, Ozbudak *et al.*, 2004). Interestingly, the lysis-lysogeny life cycle switch of bacteriophage lambda (generated by

double-negative feedback loop; **section 1.4**) depends as well on environmental conditions (e.g. nutrient or phage particle concentrations) and thus was shown to follow the bet-hedging strategy (Toman *et al.*, 1985, Arkin *et al.*, 1998).

The fitness of a population can also be increased by division of labor. In contrast to bet-hedging, this second strategy depends on the interaction between subpopulations. Here, subpopulations can fulfill simultaneously different functions which are otherwise costly or impossible to be fulfilled by a single bacterium. Therefore, in case of division of labor, a subpopulation sacrifices its fitness as “common good” to increase the fitness of the overall population (Ackermann, 2015). Formation of biofilms by *Bacillus subtilis* is one example of division of labor, as this process depends on the production of the major matrix protein EPS (extracellular polymeric substance), which is costly and reduces the bacterial fitness. EPS is only produced by a subpopulation of *Bacillus subtilis* and, therefore, EPS-production by the subpopulation serves as common good for the fitness of the remaining population (van Gestel *et al.*, 2014). Another example is the expression of virulence genes for the Type III secretion system encoded on *Salmonella* pathogenicity island I (*ttss-1*) of *S.* Tm. Upon infection of the gut *S.* Tm bifurcates into two subpopulations consisting of bacteria either expressing *ttss1* or not. *Ttss-1* expressing bacteria invade the intestinal mucosa, thus initiating an immune response of the host, and are killed by immune defenses. The other subpopulation which does not express *ttss1* stays in the gut lumen and benefits from gut inflammation, as this enables *S.* Tm to outcompete the intrinsic gut microbiota (Stecher *et al.*, 2007, Ackermann *et al.*, 2008). Colicin production is also a form of division of labor. As known, colicins are only produced by a fraction of the population (Mrak *et al.*, 2007, Kamensek *et al.*, 2010) and this is costly because the producers need to lyse in order to release colicins (Cascales *et al.*, 2007). In fact, this suicidal behavior is disastrous for the individual colicin-producing bacteria but beneficial for the remaining population. For example, in inflammation-induced Enterobacterial blooms in the gut, *S.* Tm outcompetes colicin Ib sensitive commensal *E. coli* by colicin Ib-dependent killing (Nedialkova *et al.*, 2014) (**section1.1.5**).

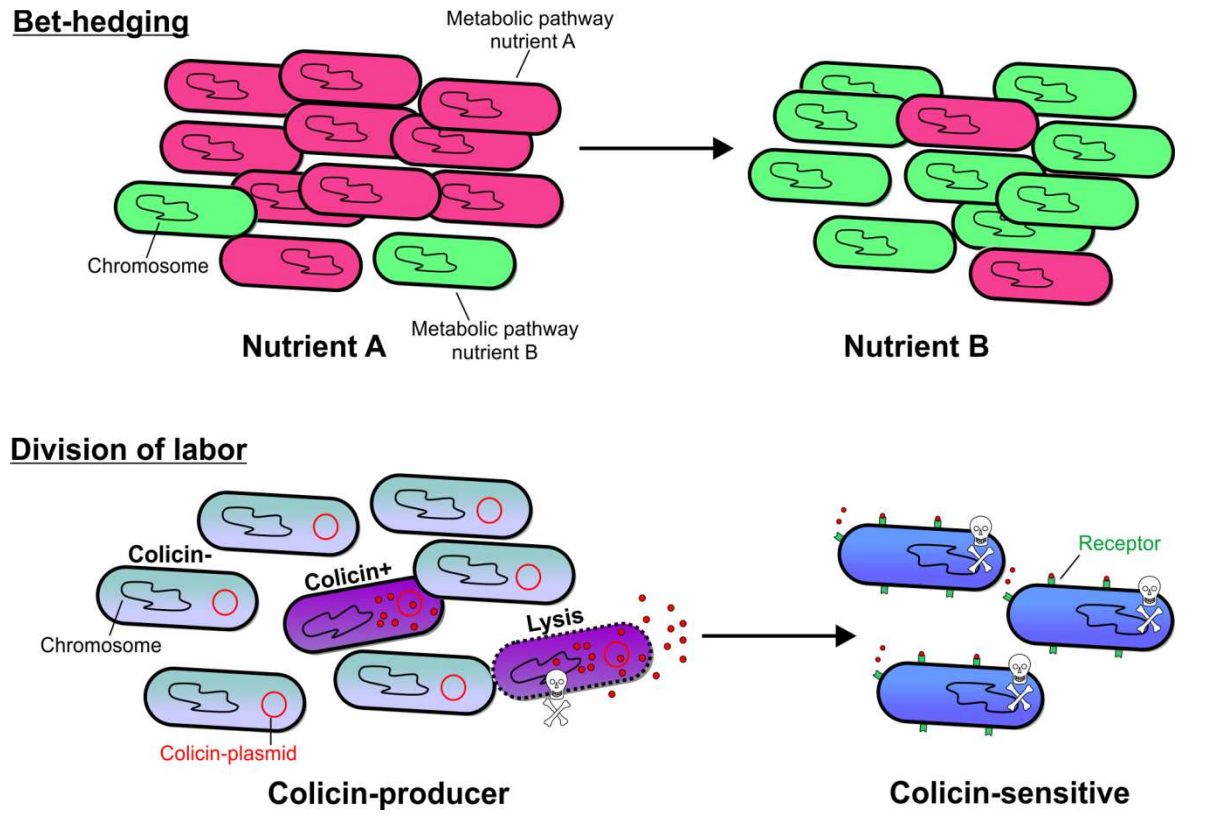

Figure 1.5: Phenotypic heterogeneity in bacterial populations. Phenotypic heterogeneity can increase the fitness of a bacterial population by bet-hedging (upper panel) or division of labor (lower panel). The bet-hedging strategy is of advantage in fluctuating environments. For instance, phenotypic heterogeneity achieved by bet-hedging can result in at least two phenotypically distinct subpopulations, consisting of nutrient A-adapted bacteria (pink) and bacteria with a phenotype suited for growth in nutrient B (green), and thus showing a reduced fitness in nutrient A conditions. If environmental conditions changes to nutrient B the survival of the overall population is rescued by the green subpopulation. Phenotypic heterogeneity by division of labor depends on bacterial-cooperation. This strategy can increase the fitness of a population for example by colicin-production. Therefore, only a fraction of producers actually synthesizes colicin (colicin+; purple), release the toxin by lysis and thus kill sensitive bacteria. Due to the suicidal behavior of individual bacteria, the remaining population (light blue) can gain a fitness benefit over the colicin sensitive population (blue).

1.5 Bacteriophages

1.5.1 Main characteristics

Bacteriophages (or phages) were first discovered in the early 20th century and named by D'Herelle according to the observations that phages infect bacteria and lyse or devour (greek: *phagein*) the bacteria (Twort, 1915, d'Herelle, 1917). They are defined as obligate intracellular parasites (= virus), because they need to infect bacterial (or archeae) host cells to propagate (Madigan *et al.*, 2009). Phages are highly abundant in the environment (>10^{31} phage particles are present in the biosphere) and are found ubiquitously just like their bacterial host cells (Comeau *et al.*, 2008).

1.5.2 Phage life cycle

Phages infect and replicate inside the host either by the lytic or the lysogenic life cycle (**Figure 1.6**). In both cases, phage-infection of the bacterial host is generally initiated by the highly specific attachment of phages to a specific surface structure of the host cell. These receptors are outer membrane structures presented at the bacterial surface such as lipopolysaccharides, teichoic acids, pili and flagella (Heller, 1992). Subsequently, the phage genome is injected into the host cell and circularizes as protection mechanism against degradation by the host exonucleases (Campbell, 2003). Depending on the phage type, different mechanisms are employed for replication. Virulent phages (e.g. phage T4), which undergo a lytic life cycle, redirect directly the host cell metabolism, resulting in the inhibition of all cellular processes not needed for phage synthesis. Subsequently, phage particles are assembled and mature phages are released by cell lysis. Temperate phages, which reproduce via a lysogenic life cycle, can either directly follow the lytic life cycle or undergo lysogeny. Lysogeny is a result of the integration of the phage-genome as prophage in the host chromosome (e.g. phage λ). Alternatively, the phage-genome stays in the cytoplasm as plasmid-like structure (e.g. phage P1). As a result, temperate phages replicate together with the host genome and its replication machinery (Salmond and Fineran, 2015). At any time prophages can be induced to excise from the host chromosome and switch to the lytic life cycle. Phage particles are synthesized and finally the mature phage particles are released by cell lysis. For instance, induction of the host SOS-response (e.g. by UV-radiation, oxidative stress, antibiotics, and starvation) can result in prophage induction (Oppenheim *et al.*, 2005, Zgur-Bertok, 2013). Filamentous phages (e.g. M13) are the exception as instead of lysing the host cell to release mature phage particle, they secrete mature phage particles. Thus filamentous phages do not kill but slow down the growth of the host cell (Russel, 1995).

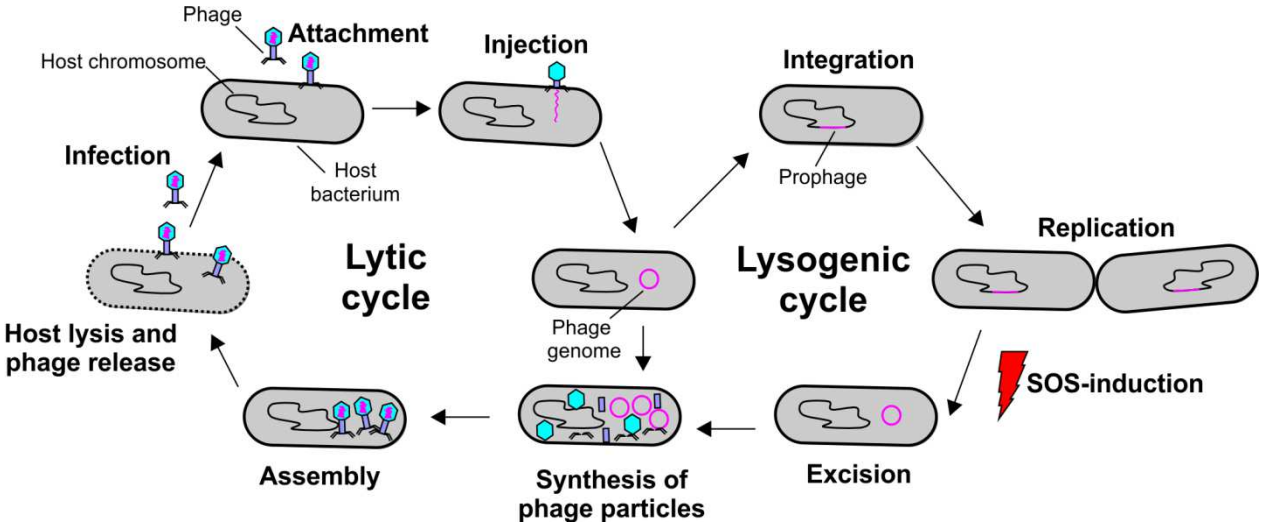

Figure 1.6: Exemplary life cycle of a temperate phage. The phage life cycle starts by the attachment of the phage to a specific receptor, presented on the host outer membrane. Subsequently, the phage genome is injected into the host cell, were it directly circularizes. At this step the phage decides weather it will follow the lytic or the lysogenic cycle. In case it decides for the lysogenic cycle it will integrate its genome in the host chromosome as prophage. Thus, it is replicated together with the host cell. Upon stressful bacterial growth conditions (e.g. SOS-response induction) the phage genome exits the host chromosome and starts the lytic cycle. The phage genome is replicated and phage particles are synthesized and assembled. This is followed by the host lysis and release of mature and infectious phage particles, which are able to infect new host cells. Adapted from (Campbell, 2003).

1.5.3 Induction of temperate phages

In case of the temperate phage λ, the genetic switch between the lysogenic and the lytic state depends on the phage-encoded strong repressor protein cI. CI represses the promoters P_R and P_L of the lytic genes and regulates its own expression (P_M). Thus the phage remains in the dormant state (Wegrzyn *et al.*, 2012). As mentioned in **section 1.1.2**, upon induction of the SOS-response, e.g. by UV irradiation, RecA is activated (Rec*), inducing, among SOS-regulated genes like LexA, also the auto-cleavage of cI dimers (Sauer *et al.*, 1982). However, in contrast to LexA, the cleavage of cI is less efficient, suggesting that prophages are only induced upon a severe and permanent DNA damage (Slilaty *et al.*, 1986). Additionally, Liu and co-workers could show a delay in the expression of the prophage late genes, after the genetic switch to the lytic cycle (Liu *et al.*, 2013). After the cleavage of cI, the prophage genes of the lytic cycle are no longer repressed. Subsequently, the repressor protein cro is produced and represses cI-expression via the promotor P_M and also other genes needed for maintenance of lysogeny (Court *et al.*, 2007, Wegrzyn *et al.*, 2012).

1.5.4 Evolutionary aspects of temperate phages

It is well known that phages contribute to shape the bacterial community dynamics. For instance, temperate phages integrate into the host genome as prophages and thus increase the fitness of the host cell (lysogenic conversion). Lysogenic conversion implies that the host cell acquires prophage-encoded genes, which are usually worthless for the phage itself, but e.g. encode for antibiotic resistance genes (Quirós *et al.*, 2014), virulence factors or superinfection exclusion systems (Brüssow *et al.*, 2004). The latter protect the host against phage superinfection by blocking the DNA-injection of other phages (e.g. lytic phages) (McGrath *et al.*, 2002, Bebeacua *et al.*, 2013). Examples for prophage encoded virulence factors are the shiga toxin of *E. coli* O157:H7 (O'Brien *et al.*, 1989, O'Brien *et al.*, 1984), the cholera toxin of *Vibrio cholerae* (Waldor and Mekalanos, 1996) and the effector protein SopE of *S.* Tm (Friebel *et al.*, 2001). Bacterial fitness can also be negatively influenced by integration of prophages in virulence genes of the host cell, thereby causing insertion mutations (Coleman *et al.*, 1989, Lee and Iandolo, 1985). Furthermore, bacterial community dynamics can be shaped by phage-dependent horizontal gene transfer (HGT), by transduction (Zinder and Lederberg, 1952, Modi *et al.*, 2013) or by phage-mediated cell lysis causing the release of bacterial genetic material which can be acquired by surrounding competing bacteria (e.g. transformation) (Kloos *et al.*, 1994). Therefore, bacteriophages are able to promote the spread of antibiotic resistances by several mechanisms (Modi *et al.*, 2013, Huddleston, 2014, Quirós *et al.*, 2014).

1.5.5 Phage release by bacterial host lysis

Phage-dependent cell lysis needs to be very tightly regulated, as cell lysis should occur at the right time to ensure completion of synthesis and maturation of the phage particles. Icosahedral phages with ssDNA and ssRNA encode for a single lysis protein, which is thought to inhibit the biosynthesis of the peptidoglycan layer (PGL). Disruption at the developing septum of the cell is suggested to cause the lysis (Bernhardt *et al.*, 2001a, Bernhardt *et al.*, 2001b, Bernhardt *et al.*, 2002). In contrast, host cell lysis mediated by dsDNA phages (e.g. phage λ or phage 21) depends on several phage lysis genes (*S*, *R*, *Rz* and *Rz1*), which are usually encoded in a cassette regulated by the late gene promoter *pR'* (**Figure 1.7A**) (Young, 2013). The cell lysis of Gram negative bacteria mediated by dsDNA phages involves 3 steps: (*i*) permeabilization of the cytoplasmic membrane (CPM), (*ii*) degradation of the peptidoglycan layer and (*iii*) fusion of the inner (IM) and outer membrane (OM) (Rajaure *et al.*, 2015, Young, 2013). Two fundamentally different phage-dependent lysis pathways exist: (*i*) the canonical holin-endolysin model and (*ii*) the pinholin-SAR (signal-anchor-release) endolysin model (Young, 2013). Phages using the canonical holin-

endolysin lysis model (e.g. phage λ; **Figure 1.7B**) produce the small membrane protein holin, encoded by the *S* gene, which accumulates in the CPM as an inactive complex with its cognate antiholin protein. In the meantime the active muralytic enzyme endolysin, encoded by the *R* gene, is produced and accumulates in the cytoplasm. At a certain time the holins are triggered to form aggregates, called “rafts” (1-3 per cell) consisting of hundreds of holin molecules. It is thought that the formation of these holin-“rafts” depolarizes the CPM, which can activate the holin-antiholin complex. This in turn causes the arrangement of holes (0.34-1 µm) which allow the passage of endolysins and the endolysin-dependent degradation of the PGL (Young, 2013). In contrast, the pinholin-SAR endolysin model (e.g. by phage 21) produces the protein pinholin together with its cognate antipinholin protein and the muralytic SAR (signal-anchor-release) endolysin (Young, 2013). SAR endolysins are translocated by the Sec-transport machinery of the host and anchored in the periplasmic leaflet of the IM. In the meantime inactive pinholin-antipinholin complexes are produced and accumulate in the CPM. After triggering, pinholins are activated, a process followed by the arrangement of rafts, causing the formation of ~10^3 “pinholes” with a diameter of about 2 nm. This in turn results in the depolarization of the CPM. The muralytic SAR endolysins are released from the CPM and change to their enzymatically active form, which leads to the degradation of the PGL (Young, 2013, Young, 2014). In the last step of the host cell lysis, two additional proteins are needed to form a complex that spans the entire periplasm (= spanin complex). One of these is the type II integral inner membrane protein Rz (= i-spanin) and the other one is the outer membrane lipoprotein Rz1 (= o-spanin). After the PGL is degraded the spanin complex disrupts the OM by mediating the fusion of the inner and outer membrane (Rajaure *et al.*, 2015, Berry *et al.*, 2008).

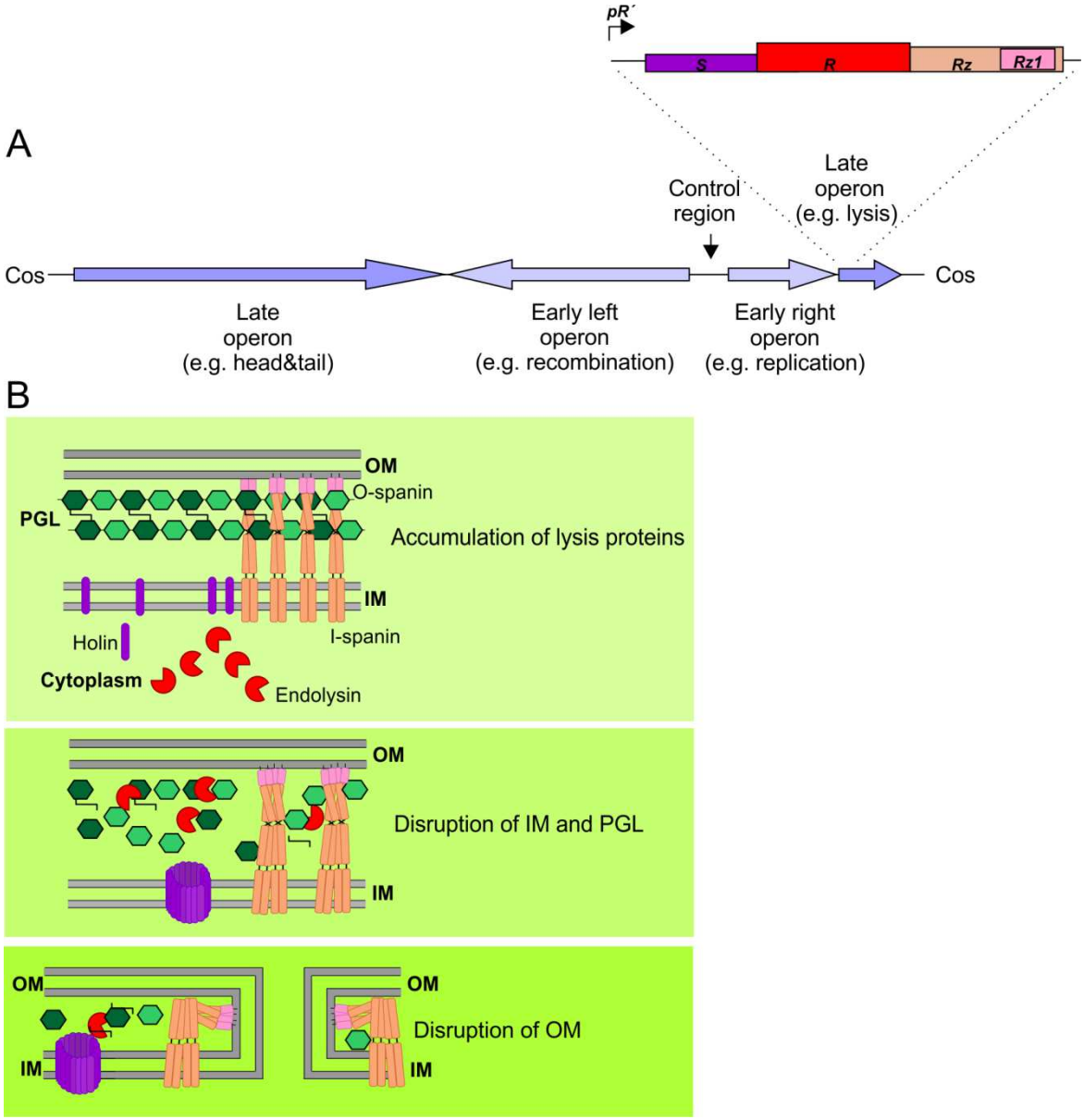

Figure 1.7: The canonical holin-endolysin lysis model of phage λ. (**A**) Linearized genome of phage λ, flanked by the cohesive (cos) sites used for circularization inside of the host. Control region encodes among others the transcription regulators cI and cro. Adapted from (Rajagopala *et al.*, 2011). Gene cassette organization of the lambdoid prophage lysis genes (*S, R, Rz and Rz1*) is shown as inset. The expression of *S* (holin), *R* (encodes endolysin), *Rz* (i-spanin) and *Rz1* (o-spanin) is regulated by the phage late gene reporter *pR´*. (**B**) Schematic process of the host cell lysis by a lambdoid phage. Initially, phage lysis starts with the accumulation of the phage lysis proteins. Holins accumulate in the inner membrane (IM), endolysins in the cytoplasm and spanins in the IM (i-spanins) and outer membrane (OM; o-spanins) and form periplasmic spanning complexes. Upon "triggering", holins form "rafts" which mediates hole-formation. Using these holes the muralytic endolysins can transit into the periplasm and degrade the peptidoglycanlayer (PG). This results in the oligomerization of the PG-liberated spanins. Subsequently conformational changes of the spanins lead to the disruption of the OM by the IM-OM fusion. Adapted from (Rajaure *et al.*, 2015, Young, 2014).

1.5.6 Prophages encoded by *S.* Tm^{SL1344}

As already mentioned in **section 1.5.4**, phages shape the community dynamics of their host strains by several mechanisms. This could also been shown for *S.* Tm strains, which differ in the number and type of their prophages (Porwollik and McClelland, 2003, Figueroa-Bossi and Bossi, 2004, Bossi *et al.*, 2003). Phage typing is thereby a very useful tool to support global *Salmonella* surveillance (Baggesen *et al.*, 2010). *S.* Tm^{SL1344} harbors four different prophages, which are inducible upon SOS-response activated by the antibiotic mitomycin C: the P2-like prophage SopEϕ and the lambdoid prophages Gifsy-1, Gifsy-2 and ST64B (Mirold *et al.*, 1999, Figueroa-Bossi and Bossi, 2004, Figueroa-Bossi *et al.*, 1997, Frye *et al.*, 2005) (**Figure 1.8**).

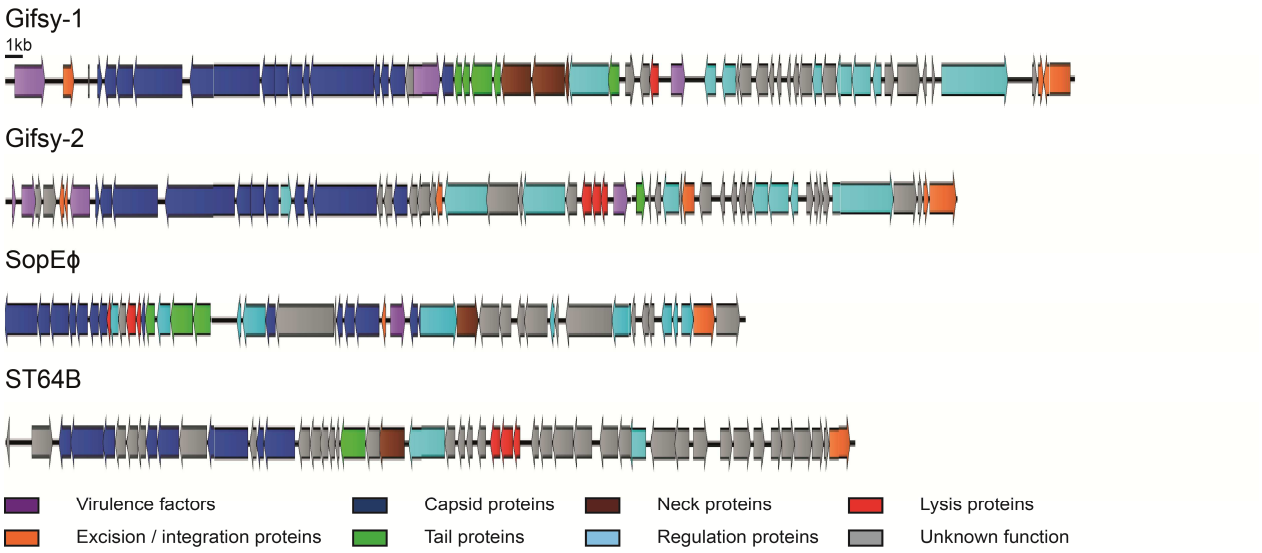

Figure 1.8: Genetic organizations of the prophage genomes in *S.* Tm^{SL1344}. Depicted are the open reading frames (ORF) of the Gifsy-1, Gifsy-2, SopEϕ and ST64B phage-genome, respectively. The ORFs encodes for excision/integration proteins (orange), regulation proteins (light blue), virulence factors (purple), phage structure proteins (capsid= blue; neck= brown; tail= green) proteins, lysis proteins and for proteins with unknown functions (grey). The direction of the gene-transcription is shown by arrows. From (Nedialkova *et al.*, 2015).

The P2-like prophage SopEϕ encodes the effector protein SopE an important virulence factor of *S.* Tm. The effector protein SopE is exported by the Type III secretion system encoded on *Salmonella* pathogenicity island I (T3SS-1) and promotes invasion of epithelial cells and thus enhances *S.* Tm-induced intestinal inflammation by triggering the release of pro-inflammatory cytokines. Furthermore it supports nitrate respiration upon inflammatory conditions (Lopez *et al.*, 2012, Müller *et al.*, 2009, Mirold *et al.*, 1999). Lambdoid prophages Gifsy-1 and Gifsy-2 also encode for different virulence genes which contribute to the pathogenicity and fitness of the strain required for efficient infection. Gifsy-1 encodes for instance for effector proteins SseI and GipA which are needed for intracellular survival of *Salmonella* and exported by the

Type III secretion system encoded on *Salmonella* pathogenicity island II (T3SS-2) (Miao and Miller, 2000, McLaughlin *et al.*, 2009, Fàbrega and Vila, 2013), (Stanley *et al.*, 2000). However, prophage Gifsy-2 encodes for [Cu, Zn] superoxide dismutase SodCI. By this, *S.* Tm is able to convert superoxide radicals to hydrogen peroxide and is thus protected against reactive oxygen species (ROS) (Figueroa-Bossi *et al.*, 2001). Prophage ST64B was first isolated from *S.* Tm^{DT64} strain, and it was shown to produce tailless non-infectious phages due to a reversible frameshift mutation in the tail genes (Mmolawa *et al.*, 2003, Figueroa-Bossi and Bossi, 2004). Although it was demonstrated that *S.* Tm strains harboring the prophage ST64B have a slight benefit for surviving in the blood, its contribution to virulence is not clear yet (Herrero-Fresno *et al.*, 2014).

1.5.7 Contribution of temperate phages to colicin Ib release from *S.* Tm^{SL1344}

It has been recently discovered that temperate phages are the key mediators of ColIb release in *S.* Tm^{SL1344} (Nedialkova *et al.*, 2015). Therefore *S.* Tm^{SL1344} gains a fitness benefit over colicin-sensitive bacteria in the inflamed gut (Nedialkova *et al.*, 2014). Lysis proteins encoded by prophage ST64B seem to be the main contributors for ColIb release by *S.* Tm^{SL1344}, but interestingly prophages Gifsy-1 and Gifsy-2 do also contribute to the release of ColIb (Nedialkova *et al.*, 2015). Furthermore, in the same study it has been shown that prophage-mediated ColIb release is not only restricted to *Salmonella* but is also true for *E. coli* strains carrying functional prophages. For instance an originally sensitive *E. coli* strain (e.g. Ec^{MG1655}), harboring ColIb plasmid p2, expresses *cib* in a similar way as *S.* Tm^{SL1344}, but is not able to release ColIb. In contrast to this, p2-harboring strain Ec^{MG1655} lysogenic for phage 933W (Ec^{Stx} p2) is able to express *cib* and to release ColIb due to prophage-mediated cell lysis. Furthermore, it has been shown that upon SOS-inducing conditions (using mitomycin C), the expression of ST64B lysis genes is delayed (~60 min) compared to the *cib* expression. This enables *S.* Tm to produce and accumulate sufficient amounts of colicin Ib before the cells lyse and release the toxin (Nedialkova *et al.*, 2015).

2 Aims of the Thesis

Colicins are protein toxins commonly produced by Enterobacteriaceae family-members. Once released into the environment, colicins are able to kill sensitive closely-related target bacteria, whereas the producing bacteria are protected by a simultaneously synthesized immunity protein. Colicin production is highly costly as the producer needs to lyse and, thus, to die to release colicins. Hence, colicins are only produced by a fraction of the bacterial population (division of labor).

Aim 1. It was previously shown that colicin Ib (ColIb)-production confers a fitness benefit to *Salmonella* Typhimurium strain SL1344 (*S.* Tm) over sensitive *E. coli* upon inflammation-induced Enterobacterial "blooms" in the gut. Based on this data it was hypothesized that heterogeneous colicin Ib (*cib*) gene expression increases the fitness of *S.* Tm against commensal and colicin-sensitive *E. coli*. In the first part of this thesis, reporter tools shall be generated and characterized to analyze *cib* expression *in vitro* under different environmental conditions at the single cell level. This will shed light on the question if heterogeneous *cib* expression increases the fitness of the pathogen against a sensitive-competitor.

Aim 2. Group A colicins are usually released by the synthesis of a cognate lysis protein. In contrast, in case of group B colicins cognate lysis genes are absent and release can be mediated by prophage-mediated cell lysis (e.g. ColIb; (Nedialkova *et al.*, 2015)). The second part of this thesis aims at analyzing regulation of *cib* expression and prophage activation at the single cell level in *S.* Tm. To this end, reporter tools shall be generated to analyze prophage lysis gene expression in individual cells. Together with the *cib* reporter established in the first part of this thesis, these reporters shall be employed to determine the fraction of bacteria which produce ColIb and are prone to lysis due to prophage-mediated cell lysis.

Aim 3. In the course of the current work an *E. coli* strain (Ec^{252R}) was found to produce and release a bacteriocin against which it is insufficiently protected ('self-killing' phenotype). A transposon library screen revealed that ColM production is responsible for this phenotype. This was highly surprising, as colicin-producers are thought to be sufficiently protected against self-killing by cognate immunity. Based on this result, it was hypothesized that (*i*) Ec^{252R} may not harbor or produce a functional immunity protein, or (*ii*) after acquisition of a colicin plasmid, the production of a cognate immunity protein alone is insufficient to protect the producer against self-killing. The third aim of the thesis was to validate these hypotheses and shed light on the 'self-killing' phenotype of ColM producers.

3 Materials and Methods

3.1 Materials

3.1.1 Strains and plasmids

Table 3.1: Strains

Strains	*Strain ID*	*Relevant characteristics*	*Antibiotic resistance*	*Reference*
***S.* Tm strains**				
S. Tm^{wt}	SB300	*S.* Tm wild type strain SL1344	Sm^{50}	(Hoiseth and Stocker, 1981)
S. Tm p2$^{\Delta cib}$	M990-2	SB300, *cib imm::aphT*	Sm^{50}, Kan^{30}	(Stecher *et al.*, 2012a)
S. Tm p2$^{cib\text{-}HA}$	M1400	SB300, *cib::*HA *aphT*	Sm^{50}, Kan^{30}	Spriewald *et al.*,2015
S. Tm^{WT}	MA6118	*S.* Tm wild type strain SL1344	Sm^{50}	(Figueroa-Bossi *et al.*, 2001)
S. Tm$^{\Delta Ph\ \Delta pRSF1010}$	MA7891	MA6118, ΔGifsy-1 ΔGifsy-2 ST64B*::aphT*, SopEΦ*::cat*, ΔpRSF1010-SL1344	Kan^{30}, Cm^{30}	(Alonso *et al.*, 2005)
S. Tm$^{\Delta Ph}$	LPN27-1	MA7891, pRSF1010-SL1344	Sm^{50}, Kan^{30}, Cm^{30}	(Nedialkova *et al.*, 2015)
S. Tm p2$^{cib::sfgfp}$	SJB15-2	SB300, *cib imm::sfgfp aphT,* pWKS30	Sm^{50}, Kan^{30}, Amp^{100}	Spriewald *et al.*,2015
S. Tm$^{lysST::T7\ pol}$	SJB34	SB300, ST64B *(SL1344_1955-SL1344_1957)::T7 gene1 aphT*	Sm^{50}, Kan^{30}	This study
S. Tm$^{lysST::sfgfp}$ p2$^{cib\text{-}HA}$	SJB36-1	SB300, *cib::*HA *aphT;* ST64B *(SL1344_1955-SL1344_1957)::sfgfp*	Sm^{50}, Kan^{30}	This study
***E. coli* (Ec) strains**				
Ec$^{DH5\alpha}$	DH5α	*F$^-$, Φ80lacZΔM15, Δ(lacZYA-argF), U169, recA1, endA1, hsdR17(r_k^-, m_k^+), phoA, supE44, thi-1, gyrA96, relA1, λ$^-$*	-	Invitrogen
Ec^{MG1655}	MG1655	*E. coli* K-12 wild type strain MG1655, F$^-$, *λ$^-$*, *ilvG$^-$*, *rfb-50*, *rph-1*	Sm^{50}	(Moller *et al.*, 2003)
Ec$^{BL21(DE3)}$	BL21 (DE3)	BL21(DE3), *B F$^-$ dcm ompT hsdS (rB$^-$ mB$^-$) gal l (DE3),*	-	Stratagene
Ec ^{SM10}	SM10 (λ- pir)	*thi-1, leu, tonA, lacY, supE, recA*::RP4-2-Tc::Mu, *λ-pir*	Kan^{30}	(Simon *et al.*, 1983)
Ec^{252R}	252R	*E. coli* mouse isolate, pColBM	Rif^{45}, Sm^{50}	This study
Ec$^{252R\ Tn10}$	MAD3	252R, pColBM::Tn*10*	Rif^{45}, Sm^{50}, Kan^{30}	This study
Ec^{P13C5}	P13C5	252R, *virE::*Tn*10*	Rif^{45}, Sm^{50}, Kan^{30}	This study

Strains	*Strain ID*	*Relevant characteristics*	*Antibiotic resistance*	*Reference*
Ec^{P10A10}	P10A10	252R, *cma::*Tn*10*	Rif^{45}, Sm^{50}, Kan^{30}	This study
Ec^{252R} pColBM$^{cba::cat}$	MAD4-1	252R, *cba::cat*;	Rif^{45}, Sm^{50}, Cm^{10}	This study
Ec^{252R} pColBM$^{cma::cat}$	MAD5-1	252R, *cma::cat*	Rif^{45}, Sm^{50}, Cm^{10}	This study
Ec^{252R} pColBM^{cmaHA}	SJB3	252R, *cma::*HA *cat*	Rif^{45}, Sm^{50}, Cm^{10}	This study
Ec^{252R} pColBM^{cmiHA}	SJB4	252R, *cmi::*HA *cat*	Rif^{45}, Sm^{50}, Cm^{10}	This study
Ec^{Cl139}	Cl139	pColBM-Cl139 *leu*, *lac*, *thi*, λ^r	unknown	Prof. Klaus Hantke, Tübingen
Ec^{252R} pColBM$^{\Delta cba}$	SJB5	252R, *Δcba*	Rif^{45}, Sm^{50}	This study
Ec^{252R} pColBM$^{\Delta cma}$	SJB6	252R, *Δcma*	Rif^{45}, Sm^{50}	This study
$Ec^{BL21(DE3)\ \Delta fhuA}$	SJB7	BL21(DE3), *B F⁻ dcm ompT hsdS (rB⁻ mB⁻) gal l (DE3), fhuA::cat*	Cm^{30}	This study
$Ec^{DH5\alpha\ \Delta fhuA}$	SJB8	DH5α, *fhuA::cat*	Cm^{30}	This study
$Ec^{MG1655\ \Delta fhuA}$	SJB20	MG1655, *fhuA::cat*	Sm^{50}, Cm^{30}	This study
Ec^{Stx}	MBK13	MG1655 lysogenized with 933W phage, *stx2A::luc aphT*	$NalAc^{60}$, Sm^{50}, Kan^{30}	(Nedialkova *et al.*, 2015)
$Ec^{Stx\Delta SR}$	MBK14	MG1655 lysogenized with 933W phage, *stx2A R S::luc aphT*	$NalAc^{60}$, Sm^{50}, Kan^{30}	(Nedialkova *et al.*, 2015)
Ec^{MG1655} pColBM^{B1144}	SJB21-2	MG1655, pColBM^{B1144}	$NalAc^{60}$, Sm^{50}, Amp^{100}	This study
Ec^{Stx} pColBM^{B1144}	SJB22-2	MBK13, pColBM^{B1144}	$NalAc^{60}$, Sm^{50}, Amp^{100}, Kan^{30}	This study
$Ec^{Stx\Delta SR}$ pColBM^{B1144}	SJB23-2	MBK14, pColBM^{B1144}	$NalAc^{60}$, Sm^{50}, Amp^{100}, Kan^{30}	This study
Ec^{MG1655} pColBM^{B1144} S6	SJB21-2-Set6 clone	derivative of SJB21-2	$NalAc^{60}$, Sm^{50}, Amp^{100}	This study
Ec^{Stx} pColBM^{B1144} S6	SJB22-2-Set6 clone	derivative of SJB22-2	$NalAc^{60}$, Sm^{50}, Amp^{100}, Kan^{30}	This study
$Ec^{Stx\Delta SR}$ pColBM^{B1144} S6	SJB23-2-Set6 clone	derivative of SJB23-2	$NalAc^{60}$, Sm^{50}, Amp^{100}, Kan^{30}	This study
Ec^{MG1655} pColBM^{B1144} G5	SJB21-2-G5	derivative of SJB21-2	$NalAc^{60}$, Sm^{50}, Amp^{100}	This study
Ec^{Stx} pColBM^{B1144} G5	SJB22-2-G5	derivative of SJB22-2	$NalAc^{60}$, Sm^{50}, Amp^{100},	This study

Strains	*Strain ID*	*Relevant characteristics*	*Antibiotic resistance*	*Reference*
			Kan^{30}	
$Ec^{Stx\Delta SR}$ pColBM^{B1144} G5	SJB23-2-G5	derivative of SJB23-2	$NalAc^{60}$, Sm^{50}, Amp^{100}, Kan^{30}	This study
***E. coli* clinical isolates**				
Ec^{B2025}	B2025	ColM	unknown	D. Šmajs and J. Bosák, Brno
Ec^{B411}	B411	ColM	unknown	D. Šmajs and J. Bosák, Brno
Ec^{B516}	B516	ColM	unknown	D. Šmajs and J. Bosák, Brno
Ec^{B522}	B522	ColM	unknown	D. Šmajs and J. Bosák, Brno
Ec^{B526}	B526	ColM	unknown	D. Šmajs and J. Bosák, Brno
Ec^{B537}	B537	ColM	unknown	D. Šmajs and J. Bosák, Brno
Ec^{B593}	B593	ColM	unknown	D. Šmajs and J. Bosák, Brno
Ec^{B1216}	B1216	ColM	unknown	D. Šmajs and J. Bosák, Brno
Ec^{B1218}	B1218	ColM	unknown	D. Šmajs and J. Bosák, Brno
Ec^{B1311}	B1311	ColM	unknown	D. Šmajs and J. Bosák, Brno
Ec^{B459}	B459	ColM	unknown	D. Šmajs and J. Bosák, Brno
Ec^{B1314}	B1314	ColM	unknown	D. Šmajs and J. Bosák, Brno
Ec^{B1327}	B1327	ColM	unknown	D. Šmajs and J. Bosák, Brno
Ec^{B1688}	B1688	ColM	unknown	D. Šmajs and J. Bosák, Brno
Ec^{B3253}	B3253	ColM	unknown	D. Šmajs and J. Bosák, Brno
Ec^{B7}	B7	ColB, ColM, micV	unknown	D. Šmajs and J. Bosák, Brno
Ec^{B54}	B54	ColE1, ColM	unknown	D. Šmajs and J. Bosák, Brno
Ec^{B72}	B72	ColE1, ColM	unknown	D. Šmajs and J. Bosák, Brno
Ec^{B147}	B147	I2*, ColM, micV	unknown	D. Šmajs and J. Bosák, Brno
Ec^{B195}	B195	ColB, ColM	unknown	D. Šmajs and J. Bosák, Brno
Ec^{B1011}	B1011	ColB, ColM	$NalAc^{30}$, Kan^{30}, Amp^{100}	D. Šmajs and J. Bosák, Brno
Ec^{B1069}	B1069	ColB, ColM	unknown	D. Šmajs and J. Bosák, Brno
Ec^{B1144}	B1144	ColB, ColM	$NalAc^{60}$, Kan^{30},	D. Šmajs and J. Bosák, Brno

Strains	*Strain ID*	*Relevant characteristics*	*Antibiotic resistance*	*Reference*
			Amp^{100}	
Ec^{B1156}	B1156	ColB, ColM, micM	unknown	D. Šmajs and J. Bosák, Brno
Ec^{B1161}	B1161	ColB, ColM, micH47	unknown	D. Šmajs and J. Bosák, Brno
Ec^{B202}	B202	ColE1, ColM	unknown	D. Šmajs and J. Bosák, Brno
Ec^{B203}	B203	ColB, ColM	unknown	D. Šmajs and J. Bosák, Brno
Ec^{B236}	B236	ColM	unknown	D. Šmajs and J. Bosák, Brno
Ec^{B255}	B255	ColM, ColN	unknown	D. Šmajs and J. Bosák, Brno
Ec^{B338}	B338	ColM	unknown	D. Šmajs and J. Bosák, Brno
Ec^{B358}	B358	ColM	unknown	D. Šmajs and J. Bosák, Brno
Ec^{B383}	B383	ColE1, ColB, ColM, micV	unknown	D. Šmajs and J. Bosák, Brno
Ec^{B805}	B805	ColM	unknown	D. Šmajs and J. Bosák, Brno
Ec^{B848}	B848	ColE1, ColM	unknown	D. Šmajs and J. Bosák, Brno
Ec^{B852}	B852	ColE1, ColM	unknown	D. Šmajs and J. Bosák, Brno

Amp: ampicillin; Cm: chloramphenicol; Kan: kanamycin; NalAc: nalidixic acid; Rif: Rifampicin; Strep: streptomycin; mic: microcin; *: bacteriocin-like

Table 3.2: Plasmids

Plasmids	*Plasmid ID*	*Copy number*	*Relevant characteristics*	*Antibiotic resistance*	*Reference*
p2795	p2795	high	pSK, *ori* F1 and *ori* pUC	Amp^{100}, Kan^{30}	(Husseiny and Hensel, 2005)
pWKS30	pWKS30		pSC101 origin of replication;	Amp^{100}	(Wang and Kushner, 1991)
pJA1	pJA1	low	R6K γ-origin, Tn*10*, *lacIq*, P_{tac}-ATS transposase	Amp^{100}, Kan^{30}	(Alexeyev and Shokolenko, 1995), (Badarinaraya na *et al.*, 2001)
pKD46	pKD46	low	λ Red-expression under control of pBAD, temperature-sensitive	Amp^{100}	(Datsenko and Wanner, 2000)
pWRG7	pWRG7	medium	Promoterless *sfgfp-aphT* lambda red template vector	Kan^{30}	(Spriewald *et al.*, 2015)
pSU315	pSU315	low	R6K *ori*V, FRT-flanked *HA-aphT,* lambda red template vector	Amp^{100}, Cm^{30}	(Uzzau *et al.*, 2001)

Plasmids	*Plasmid ID*	*Copy number*	*Relevant characteristics*	*Antibiotic resistance*	*Reference*
pCP20	pCP20	low	*FLP*$^{+}$, λ cI857^{+}, λ p_R Rep^{ts}	Amp^{100}, Cm^{30}	(Cherepanov and Wackernagel, 1995)
pSJB4	pSJB4	high	*cma-6x-his*	Kan^{30}	This study
p^{control}	pSJB17	high	*ori pBR322*, *ori F1*, control plasmid	Amp^{100}	(Spriewald *et al.*, 2015)
p$^{P_{cib}}$	pSJB16	high	*ori pBR322*, *ori F1*, *cib* promoter (P_{cib})	Amp^{100}	(Spriewald *et al.*, 2015)
p^{gfp}	pM968	high	*ori pBR322*, *ori F1*, carrying *gfpmut2* coding region without promoter	Amp^{100}	(Stecher *et al.*, 2004)
p$^{P_{cib}\ gfp}$	pM1437	high	*ori pBR322*, *ori F1*, P_{cib}::*gfpmut2*	Amp^{100}	(Nedialkova *et al.*, 2014)
p$^{P_{rpsM}\ gfp}$	pM979	high	*ori pBR322*, *ori F1*, constitutive *gfpmut2*-reporter plasmid; P_{rpsM} (ribosomal *rpsM* promoter)	Amp^{100}	(Stecher *et al.*, 2004)
p$^{P_{T7}\ sfgfp}$	pJLG1	low	pM955, P_{T7}::*sfgfp*	Amp^{100}	This study
p$^{T7\ Pol}$	pJLG2	low	p2795, *T7 gene 1 aphT*	Amp^{100}, Kan^{30}	This study
pM946	pM946	low	*ori pSC101*, pWKS30 cured of P_{lac}, P_{T7} *gfpmut3b*	Amp^{100}	(Stecher *et al.*, 2004)
p^{empty}	pM955	low	*ori pSC101*, pWKS30 cured of P_{lac}	Amp^{100}	This study
p$^{P_{sicA}\ gfp}$	pM974	high	*ori pBR322*, *ori F1*, P_{sicA}::*gfpmut2*	Amp^{100}	(Stecher *et al.*, 2004), (Stecher *et al.*, 2007)
p$^{cmi\ 252R}$	pMAD7	low	pM955, 252R P_{cmi} *cmi*	Amp^{100}	This study
p$^{cmi\ CI139}$	pMAD8	low	pM955, CI139 P_{cmi} *cmi*	Amp^{100}	This study

Amp: ampicillin; Cm: chloramphenicol; Kan: kanamycin

3.1.2 Oligonucleotides

All oligonucleotides used in this study were synthesized by Metabion (Martinsried).

Table 3.3: Oligonucleotides

Designation	Sequence (5' → 3')
Ampli4_Fwd	TGAGATAGGTGCCTCACTG
Ampli4_Rev	ACAGGGCGCGTAAATCAATC
CBA CBI Primer For	ATGACCAGCAATAAAGATAAG
CBA complete Rev	ATCTTGGAGATAAATACAAGG
CBA remnant Pri. Rev	GATGAGTTGAATCACAAGATC
cba-check-down	GCGTAATTTTTGATCTGTAC
cba-check-up	CATGACATATGCGATTTTAC
cba-fwd-ko	TGGTTATGTGTACAGTATTTAATTTTAATTGATTGTTTTTAAAG TCAAAGAGGTTTTCTATATGAATATCCTCCTTAGTT
cba-rev-ko	ATGCCATTACTTGTCATTAAATATTTTAAAGGGAGGCAGTAAC ACTGCCTTCCTTTTAATGTGTAGGCTGGAGCTGCTTC
Check up_ SFGFP /RFP _rev	CATGCTGGAGTTCTTCGCC
Check up_ SFGFP_fwd	GTGGCTCAGTTCTGTAACT
check up_pSJB16	AGGTTTTCACCGTCATCA
check_ST64_for	CTGTTTGGCGGCCTTTTC
check_ST64_rev	GGA TAT AAA AAC GCC CCG
cma-check-down	TCTCTCATGGGGTTTGTTCG
cma-check-up	TTGTGAGGAGGGATATGTTG
cma-fwd-ko	GAATGACTAATACACTGTTATAAAGGCTGCATAAAAAGGCCG GAACCCCGGCCCTTATAATATGAATATCCTCCTTAGTT
cma-HA-fwd	CTGGTAAAGAGTACCAGATACTGCTTCCTGGTGAAATTCACA TTAAAGAAAGTGGTAAGCGATATCCGTATGATGTTCCT
cma-HA-rev	GCGAATGACTAATACACTGTTATAAAGGCTGCATAAAAAGGC CGGAACCCCGGCCCTTATAATATGAATATCCTCCTTAG
cma-rev-ko	GGCGGAGGCGTTAGCCAGCGGCGAACTCTGTTATCTTGTTA ACTTATAAGGAGTTATGTTGTGTAGGCTGGAGCTGCTTC
cmi check down rev	ACTGCTTCCTGGTGAAATTC
cmi check up forward	CCGATGCTGTCACAATGCTG
cmi-HA-fwd	GTAAGAGAATGTATTATGGCATGTACCATTGTGAGGAGGGAT ATGTTGAATATGCGAATGACTATCCGTATGATGTTCCT
cmi-HA-rev	GGTAAGCGATAATATAAGGGCCGGGGTTCCGGCCTTTTTATG CAGCCTTTATAACAGTGTAATATGAATATCCTCCTTAG
cmi-histag_fwd	ATAGCTAGCATGGAAACCTT
cmi-histag_rev2	CGCAAGCTTTCGCTTACCACT
cmi-pro-fwd-XbaI	CCCGGATCCACTGCTTCCTGGTGAAATTC
cmi-pro-rev-BamHI	CCCTCTAGACCGATGCGGTCACAATGCTG
Colicin-HA-fwd	GTTAATGATAAGTTTATTGAGCAGGTCAATAAACTTATTGGTA TCTATCCGTATGATGTTCCTGA
Colicin-HA-rev	ACTTAATTTTTATATTCTGCAAAGCCCTTTCAGGAAAATGAATA TGAATATCCTCCTTAG
Col-lux_check_for	GACATGCCATTTTCTCCT
Col-lux_check_rev	GATGTAAACGTGACACAG
Col-ÜE-XbaI	CCCTCTAGACCTCAGAGGATGAAGGAGATAC
Col-ÜE-XhoI	CCCCTCGAGGATGTAAACGTGACACAGCT
FhuA_sequ-fwd1	GGATGGCGAGTTGCCATC
FhuA_sequ-fwd2	GACAGCCTGTTCCAGAC

Designation	Sequence (5' → 3')
FhuA_sequ-rev	CGTGTATTCCTGCATAAC
FhuA-KO_fwd	CTTTATAATAATCATTCTCGTTTACGTTATCATTCACTTTACAT CAGAGATATACCATGTGTAGGCTGGAGCTGCTTC
FhuA-KO_rev	CCTGCATAACAGCCAACTTGTGAAATGGGCACGGAAATCCGT GCCCCAAAAGAGAAAATATGAATATCCTCCTTAGTT
hp-F*	GAATTACGATGAAAATAACG
hp-R2*	TCATTCTGGCGATTTCAATA
InsideT7	TAGCATCTTGACGACGCGGTG
pJLG2 seq Fw	GCTCTGAAGTTCCTATACT
pJLG2 seq Rev	GCTATGACCATGATTACG
pJLG1proof fwd	GGGGATGTGCTGCAAGG
pJLG1proof rev	GAGCTGACTGGGTTGAAG
pJLG1 SFGFP SD BamHI Fw	GATCCTAAGGAGGGTAAGCATGCGCAAAGGCGAAG
pJLG1 SFGFP EcoRI Rev	GTAGAATTCTTATTATTTATACAGTTCATCCATGCCA
PsicA_2_fwd	GGCAAAGACGTTATTCAGCC
PsicA_2_rev	GCCCAATGAATACATCGCTAC
R/S outside Rev	GAGATGCGCAGAAATGACAA
SFGFP_cib_fwd	TATACGTAAGCAGTTAATTCATTTGTTTTCCTCAGAGGATGAA GGAGATACCGAATGCGCAAAGGCGAAGAACTGTTTAC
SFGFP_cib_rev	TGTGATTATGTTATCACGCACCGGTACACGATAACAATAAAG GAGAAAACAGCGTGTAGGCTGGAGCTGCTTC
SFGFP-fwd	GATAAGCTTATGCGCAAAGG
SFGFP-rev	CTTTCTAGAGAATAGGAACTTC
ST64B-sfgfp-fwd	TCCACTCACCCGATACCCGGGTAAACAGTCTCCCGGACAGG GGGAGGTCATGCGCAAAGGCGAAGAACTGTTTAC
ST64B-sfgfp-rev	ACGAGGCATTTTCATGAAAGTCACTTGTCAAATTTCTATGTGA TGGAAACGTGTAGGCTGGAGCTGCTTC
Stx2 Operon Fw	GATCGGTATGTTGAGCGTGA
Stx2 Operon Rev	TGCTCAGTCTGACAGGCAAC
Stx2A outside Fw	AGACGGTCAGGGAAGTTCAG
T7	TAATACGACTCACTATAGGG
T7 pol FW NotI	CATGCGGCCGCATGAACACGATTAACATCGCTAAG
T7 pol Rev XhoI	TCACTCGAGTTACGCGAACGCGAAGTC
T7 seq left 2	CTAAGGGTCTACTCGGTGGC
T7 seq right 2	TCACGCTCACAGATTCCCAA
T7_ST64B_fwd	TCCACTCACCCGATACCCGGGTAAACAGTCTCCCGGACAGG GGGAGGTCATGAACACGATTAACATCGCTAAGAA

* from (Christenson and Gordon, 2009)

3.1.3 Chemicals

Table 3.4:Sources of the used chemicals and enzymes.

Chemicals and enzymes	Source
Acrylamide mix 30%	Serva (Heidelberg)
Agar bacto™	Becton, Dickinson and Company (BD) (Heidelberg)
Ammonium persulfate (APS)	Roth (Karlsruhe)
Benzonase nuclease (Novagen)	Merck Chemicals (Schwalbach)
Bovine serum albumin fraction (BSA), PAA	GE Healthcare (Munich)
Bromphenolblue	Roth (Karlsruhe)
$CaCl_2$	Merck Chemicals (Schwalbach)
Chloroform	Roth (Karlsruhe)
Cetyltrimethylammoniumbromid (CTAB)	Roth (Karlsruhe)
4',6-diamidino-2-phenylindole (DAPI)	Roth (Karlsruhe)
D-glucose	Roth (Karlsruhe)
Diethylenetriamine Pentaacetic Acid (DTPA)	Sigma-Aldrich Chemie (Munich)
dNTP Set	Thermo Fisher Scientific Biosciences (St. Leon-Rot)
DreamTaq PCR Master Mix (2 x)	Thermo Fisher Scientific Biosciences (St. Leon-Rot)
Dithiothreitol (DTT)	Roth (Karlsruhe)
ECL detection system	GE Healthcare (Munich)
Immobilion Western Chemoluminescent HRP substrate	Merck Chemicals (Schwalbach)
Ethylenediaminetetraacetic acid (EDTA)	Roth (Karlsruhe)
Ethylene glycol tetraacetic acid (EGTA)	Roth (Karlsruhe)
Ethanol ROTIPURAN® ≥99,5 %, p.a	Roth (Karlsruhe
FastStart Taq DNA Polymerase Kit	Roche (Mannheim)
Glycerol	Roth (Karlsruhe)
Glycine	MP Biomedicals
High-fidelity PCR enzyme	Thermo Fisher Scientific Biosciences (St. Leon-Rot)
Immobilion Western Chemoluminescent HRP substrat	Merck Chemicals (Schwalbach)
K_2HPO_4	Roth (Karlsruhe)
KCl	Fluka, Sigma-Aldrich Chemie (Munich)
KH_2PO_4	Roth (Karlsruhe)
L-(+)-Arabinose	Sigma-Aldrich Chemie (Munich)
Lysozyme from chicken egg white	Sigma-Aldrich Chemie (Munich)
MacConkey agar	Oxoid, Thermo Fisher Scientific Biosciences (St. Leon-Rot)
MacConkey agar	Roth (Karlsruhe)
Methanol ROTIPURAN® ≥99,5 %, p.a	Roth (Karlsruhe)
Milk powder	Roth (Karlsruhe)
Mitomycin C (MitC)	Roth (Karlsruhe)
Na_2HPO_4 unhydrated	Roth (Karlsruhe)
Na_2HPO_4 x $2H_2O$	Roth (Karlsruhe)
NaAcetate	Roth (Karlsruhe)

Chemicals and enzymes	Source
NaCl	Roth (Karlsruhe)
NH_4Cl	Sigma-Aldrich Chemie (Munich)
Peptone	Oxoid, Thermo Fisher Scientific Biosciences (St. Leon-Rot)
Phenol/chloroform/isoamylalcohol	Roth (Karlsruhe)
Phenylmethylsulfonyl fluoride (PMSF),	Serva (Heidelberg)
Propidium iodide	Sigma-Aldrich Chemie (Munich)
ProteinaseK	Roth (Karlsruhe)
Restriction enzymes	Thermo Fisher Scientific Biosciences (St. Leon-Rot)
Sodium dodecyl sulfate (SDS)	Serva (Heidelberg)
Sodium Azide	Merck Chemicals (Schwalbach)
T4 DNA ligase	Thermo Fisher Scientific Biosciences (St. Leon-Rot)
Tergitol	Sigma-Aldrich Chemie (Munich)
Tetramethylethylenediamine (TEMED)	Biomol Feinchemikalien (Hamburg)
Tissue-Tek Optimal Cutting Temperature (O.C.T.) compound	Sakura Finetek, (Torrance)
Tris	MP Biomedicals (Eschwege)
Triton X-100	Roth (Karlsruhe)
Tryptone	Roth (Karlsruhe)
Tween 20	Roth (Karlsruhe)
VECTASHIELD(R) Mounting Medium	Biozol (Eching)
Yeast extract	MP Biomedicals (Eschwege)
Zebra Spin desalting columns (5 ml)	Thermo Fisher Scientific Biosciences (St. Leon-Rot)
β –D-thiogalactopyranoside (IPTG)	Roth (Karlsruhe)

Table 3.5: Antibiotics

Antibiotics	Final concentration	Source
Ampicillin sodium salt	100 µg/ml	Roth (Karlsruhe)
Chloramphenicol	30 µg/ml 10 µg/ml (*E. coli* 252R derivatives)	Roth (Karlsruhe)
Kanamycin sulphate	30 µg/ml	Roth (Karlsruhe)
Nalidixic acid	60 µg/ml	Roth (Karlsruhe)
Rifampicin	45 µg/ml, dissolved in $_{dd}H_2O$	Roth (Karlsruhe)
Streptomycin sulphate	50 µg/ml	Roth (Karlsruhe)

Table 3.6: DNA and protein standards

Item	Source
Page ruler prestained protein ladder	Thermo Fisher Scientific Biosciences (St. Leon-Rot)
GeneRuler 1 kb DNA ladder	Thermo Fisher Scientific Biosciences (St. Leon-Rot)

Table 3.7: Sources of purification kits

Kit	Source
NucleoSpin Gel and PCR clean-up kit	Macherey-Nagel (Düren)
NucleoSpin Plasmid kit	Macherey-Nagel (Düren)
NucleoSpin Tissue	Macherey-Nagel (Düren)
Plasmid Plus Midi Kit	QIAGEN (Hilden)

Table 3.8: Consumables

Consumables	Source
Microscope slides, SuperFrost®	Thermo Fisher Scientific Biosciences (St. Leon-Rot)
TC-Platten Orange 96-well (F-well)	Orange Scientific (Braine-l'Alleud, Belgium)
Filter Millex 0.22 µm	Merck Chemicals (Schwalbach)
Cryotubes	Thermo Fisher Scientific Biosciences (St. Leon-Rot)
Cultube sterile culture tubes, tube with cap, polystyrene; 17 mm x 95 mm H	Simport (Beloeil, Canada)
Nitrocellulose membrane	GE Healthcare (Munich)
HisTrap column (5 ml)	GE Healthcare (Munich)
HiTrap desalting column (5 ml)	GE Healthcare (Munich)
Electroporation Cuvette LE (1 mm)	PAQlab (VWR International GmbH Life Science Competence Center, Erlangen)

3.1.4 Buffers, media and supplements

If not otherwise stated, components of media, buffers and solutions are solved in $_{dd}H_20$.

Table 3.9: Media for bacterial growth and for cryostorage

Media	Component	Final concentration
Luria-Bertani (LB) medium	NaCl	85.6 mM
	Yeast extract	0.5% (w/v)
	Tryptone	1% (w/v)
LB-agar	NaCl	85.6 mM
	Yeast extract	0.5% (w/v)
	Tryptone	1% (w/v)
	Agar	1.5% (w/v)
LB-softagar	NaCl	85.6 mM
	Yeast extract	0.5% (w/v)
	Tryptone	1% (w/v)
	Agar	0.7% (w/v)
Peptone-glycerol (P/G) broth	Peptone	2% (w/v)
	Glycerol	5% (v/v)

Table 3.10: Buffers for multiple applications

Buffers	Component	Final concentration
Phosphate Buffered Saline (PBS) 10x	KCl	26.8 mM
	Na_2HPO_4 anhydrous	43 mM
	KH_2PO_4	17.6 mM

Table 3.11: Buffers for affinity purification of recombinant proteins

Buffers	Component	Final concentration
Loading buffer (pH 7.8)	Na_2HPO_4	40 mM
	NaCl	300 mM
	Imidazol	5 mM
Lysis buffer (pH 7.8)	Na_2HPO_4	40 mM
	NaCl	300 mM
	Imidazol	5 mM
	PMSF	2 mM
	Benzonase nuclease	0.0125 U/µl
Elution buffer (pH 7.8)	Na_2HPO_4	40 mM
	NaCl	300 mM
	Imidazol	500 mM
Exchange buffer 1 (pH 7.4)	Na_2HPO_4	20 mM
	NaCl	100 mM

Table 3.12: Buffers for SDS-polyacrylamide gel preparation

	Components	Final concentration
5% stacking gel	Acrylamide mix 30%	17% (v/v)
	Tris (pH 6.8)	125 mM
	SDS	1.73 mM
	APS	4.4 mM
	TEMED	0.1% (v/v)
12% resolving gel	Acrylamide mix 30%	40% (v/v)
	Tris (pH 8.8)	5700 mM
	SDS	3.5 mM
	APS	4.4 mM
	TEMED	0.04% (v/v)
15% resolving gel	Acrylamide mix 30%	50% (v/v)
	Tris (pH 8.8)	5700 mM
	SDS	3.5 mM
	APS	4.4 mM
	TEMED	0.04% (v/v)

Table 3.13: Buffers for SDS-PAGE and western blot

Buffer	Components	Final concentration
Protein loading buffer (5x)	Tris-Cl (pH 6.8)	250 mM
	DTT	500 mM
	SDS	346.8 mM
	Bromphenolblue	7.5 mM
	Glycerol	50% (v/v)
Running- buffer (10x)	Tris	249.3 mM
	Glycine	2500 mM
	SDS	34.7 mM
Transfer-buffer	Tris	25 mM
	Glycine	191.8 mM
	Methanol	5% (v/v)
Blocking solution in 1X PBS	Tween 20	0.1% (v/v)
	Milkpowder (Roth)	5% (w/v)

Table 3.14: Solution for coomassie staining of SDS-polyacrylamide gels

Solution	Components	Final concentration
Coomassie staining solution	Coomassie Brilliant Blue R250	0.3% (w/v)
	Methanol	45% (v/v)
	Acetic acid (100%)	10% (v/v)
Coomassie fixing solution	Methanol	50% (v/v)
	Acetic Acid (100%)	10% (v/v)

Table 3.15: Buffers for genomic DNA extraction

Buffer	Components	Final concentration
TE buffer (pH 8.0)	Tris/HCl	10 mM
	EDTA	1 mM
CTAB/NaCl	NaCl	0.7 M
	CTAB	10%

Table 3.16: Permeabilization buffers for intrabacterial staining

Buffer	Components	Final concentration
Permeabilization buffer A (pH 8.0)	Tris	20 mM
	EDTA	50 mM
	TritonX-100	0.1% (v/v)
	Glucose	99.9 mM
Permeabilization buffer B (pH 8.0)	Tris	25 mM
	EDTA	10 mM
	Glucose	99.9 mM

3.1.1. Antibodies

Table 3.17: Antibodies

Name of antibody	Recognized antigen	Origin	Final dilution	Source
Primary antibody				
ADI-SPA-880 (Hsp 70)	DnaK (E. coli)*	Mouse	1:200 (IF) 1:500 (WB)	Enzo Life Sciences (Lörrach)
HA probe (Y11)	Influenza hemagglutinin (HA)	Rabbit	1:200 (IF) 1:500 (WB)	Santa Cruz
Anti-ColM (rabbit polyclonal)	$His_{(6x)}$-tagged ColM (recombinant)	Rabbit	1:5000 (WB)	This study
Anti-GFP	Green Fluorescent Protein (GFP)	Rabbit	1:200 (IF)	antibodies-online.com
Secondary antibody (and label)				
Anti-mouse IgG (Rhodamine Red™-X)	Mouse IgG	Goat	1:200 (IF)	Invitrogen
Anti-rabbit IgG (HRP)	Rabbit IgG	Goat	1:10,000 (WB)	GE-Healthcare (Munich)
Anti-mouse IgG (HRP)	Mouse IgG	Goat	1:10,000 (WB)	Sigma-Aldrich Chemie (Munich)
Anti-rabbit IgG (DyLight™549)	Rabbit IgG	Goat	1:400 (IF)	Jackson
Anti-rabbit IgG (DyLight™649)	Rabbit IgG	Goat	1:400 (IF)	Jackson

*Also recognizes *S.* Tm DnaK; HRP: horseradish peroxidase; IF: immunofluorescence; WB: western blot

3.2 Methods

3.2.1 Bacterial growth

3.2.1.1 Bacterial growth conditions

If not otherwise stated bacteria (from –80 °C cryostocks) were streaked on LB agar (**Table 3.9**) supplemented with appropriate antibiotic(s) and incubated o.n. at 37 °C. Afterwards, a bacterial colony was used to inoculate liquid LB media (**Table 3.9**) supplemented with the selective antibiotic(s) and incubated over night (o.n.) in the rotor wheel at 37 °C.

3.2.1.2 Long-term storage of bacterial strains

Initially, bacteria were grown o.n. at 37 °C on LB agar. A single colony was used to inoculate 5 ml LB media (supplemented with or without selective antibiotics) and incubated o.n. at 37 °C and 180 rpm. Afterwards this o.n. culture was spun down at 4 °C and 4030 g for

20 min. The pellet was resuspended with 1 ml P/G broth (**Table 3.9**) and stored in 1 ml cryotubes (**Table 3.8**) at -80 °C.

3.2.1.3 Generation of samples for the intracellular immunofluorescence staining

Bacteria were streaked from -80 °C cryostocks on LB agar containing 100 µg/ml Ampicillin and incubated o.n. at 37 °C. The next day three individual colonies were picked and transferred in 100 µl 1x PBS (**Table 3.10**). The bacterial suspension were mixed and 20 µl was used to inoculate 2 ml liquid LB medium supplemented with 100 µg/ml Ampicillin, followed by incubation for 12 h at 37 °C in the rotor wheel in test tubes (Cultube sterile culture tubes, tube with cap, polystyrene; 17 mm x 95 mm H; **Table 3.8**). This o.n. culture was used to inoculate (1:20) 2 ml LB subcultures supplemented with 100 µg/ml Ampicillin, without any further supplements, or with mitomycin C (MitC; 0.25 µg/ml or 0.5 µg/ml), or with diethylenetriamine pentaacetic acid (DTPA; 100 µM), or supplemented with both. These subcultures were incubated for 4 h at 37 °C in the rotor wheel. Generation of samples for the intracellular immunofluorescence staining in **section 4.2.2** was done without supplementation of ampicillin.

3.2.1.4 Bacterial growth conditions for immunoblot and flow cytometry

A single bacterial colony was used to inoculate 3 ml LB medium (supplemented with appropriate antibiotics) and incubated for 12 h at 37 °C in the rotor wheel. This (o.n.) culture was diluted 1:20 in 2 ml LB (supplemented with the appropriate antibiotics) and subcultured for further 4 h at 37 °C in the rotor wheel. If required, subcultures were additionally supplemented with 0.25 µg/ml, or 0.5 µg/ml MitC, and/or 100 µM DTPA.

3.2.1.5 Growth in 96-well plates

Bacteria were streaked from -80 °C cryostocks on LB agar containing appropriate antibiotics and incubated o.n. at 37 °C. The following day a single bacterial colony was used to inoculate 3 ml LB medium and incubated for 12 h at 37 °C in the rotor wheel in test tubes (Cultube sterile culture tubes, tube with cap, polystyrene; 17 mm x 95 mm H; **Table 3.8**). This o.n. culture was diluted and normalized to an OD_{600} of 0.025 in 3 ml fresh LB medium. After an incubation time of 2 h at 37 °C in the rotor wheel, this subculture was diluted to an OD_{600} of 0.25 in fresh LB medium only or with fresh LB supplemented with 0.5 µg/ml MitC or 100 µM DTPA or both (final concentration). From each culture condition 200 µl/well were transferred in a sterile 96-well plate and incubated in total for 4 h at 37 °C while shaking.

3.2.1.6 Growth for live cell microscopy

Bacteria were streaked from -80 °C cryostocks on LB agar containing appropriate antibiotics and incubated o.n. at 37 °C. The following day a single bacterial colony was used to inoculate 3 ml LB medium and incubated for 12 h at 37 °C in the rotor wheel in test tubes

(Cultube sterile culture tubes, tube with cap, polystyrene; 17 mm x 95 mm H; **Table 3.8**). This o.n. culture was used to inoculate 2 ml LB medium in a ratio 1:20 and incubated for 2 h at 37 °C (in the rotor wheel). From this subculture a bacterial sample of 10^6 cells/ml in 1x PBS was prepared for live cell microscopy using the CellASIC® ONIX Microfluidic Platform (Millipore).

3.2.2 Molecular biological methods

3.2.2.1 Polymerase Chain Reaction (PCR)

In general a final volume of 50 µl was prepared. The reaction mixture contained 10-100 ng DNA template, 0.6 µM oligonucleotides and 1x Dream Taq Mastermix (Fermentas, St. Leon Roth). For amplicons >1.5 kb, the reaction mix contained 2 U FastStart^{Tm} Taq DNA Polymerase and 1x FastStart^{Tm} buffer supplemented with 20 mM $MgCl_2$ (Roche, Mannheim) as well as 0.2 mM dNTPs (Roche, Mannheim). Oligonucleotides used in this thesis are listed in **Table 3.3**.

In order to verify the specificity and yield of the reaction, 1/10 of the reaction mix was loaded on a 1% agarose gel and separated by gel electrophoresis according to their size. The following standard PCR program was used (**Table 3.18**).

Table 3.18: Standard PCR protocol

Standard PCR protocol:		
95 °C	5 min	
95 °C	30 sec	x 35
X °C	30 sec	
72 °C	~1 min/1 kb	
72 °C	10 min	
8 °C	∞	

3.2.2.2 Restriction hydrolysis

The restriction hydrolysis of DNA was done by restriction enzymes obtained from Thermo Fisher Scientific Biosciences (St. Leon-Rot) and the reaction was performed according to the manufacturer instructions.

3.2.2.3 Purification and gel extraction of DNA

The Purification of PCR-amplicons as well as the extraction of hydrolyzed DNA from 1% agarose gels was performed using the NucleoSpin® Gel and PCR Clean up kit (**Table 3.7**) according to the manufacturer's instructions.

3.2.2.4 DNA-Ligation

Ligation of DNA fragments, which had previously been purified from a 1% agarose gel, was performed with the T4 ligase (**Table 3.4**) and conducted according to the manufacturer instructions. The protocol was adjusted to a final reaction volume of 18 µl.

3.2.2.5 Sequencing

DNA was sent for sequencing to GATC-Biotech (Munich), according to the company requirements.

3.2.2.6 Generation of electro-competent bacterial cells

To generate electro-competent bacterial cells, 20 ml LB media (supplemented with appropriate antibiotic(s)) were inoculated using an o.n. culture in a 1:20 ratio and incubated at 37 °C and 180 rpm until OD_{600} of ~ 0.5-0.8 was reached. Subsequently, this subculture was washed and concentrated with ice cold $_{dd}H_20$ two times (1 volume and ½ volume) by centrifugation (4030 x g, 4° C, 15 min). Afterwards, bacterial cells were washed with 1/10 culture volume ice cold 10% glycerol by a further centrifugation step (4030 x g, 4° C, 15 min). After a final centrifugation step, bacterial cells were resuspended in 1/50 volume ice cold 10% glycerol and transferred in collection tubes in aliquots of 80 µl. These aliquots were shock frozen in liquid nitrogen and stored at -80 °C.

3.2.2.7 Transformation

Frozen electro-competent bacterial cells (80 µl) were thawed on ice and 1-6 µl of plasmid, linear DNA, or DNA ligation mix was added to the cells, mixed, transferred into an ice cold electroporation cuvette (1 mm) and pulsed at 1800 V/cm for 5 ms using the Gene Pulser Xcell (Bio-Rad). Subsequently, bacterial cells were suspended with 900 µl LB medium, transferred into a new collection tube and incubated for 1 h at 37° C and 950 rpm. Afterwards, bacterial cells were spun down at RT (6297 x g, 1 min). The cell pellet was resuspended with 100 µl LB medium and plated on LB agar containing the appropriate antibiotic(s) and incubated o.n. at 37 °C.

3.2.2.8 Conjugation

Donor and acceptor strains were grown individually at 37 °C o.n. in 3 ml liquid LB medium (supplemented with appropriate antibiotic(s)). The next day, 1 ml of each o.n culture was washed 3 times in LB medium by centrifugation at RT (1 min at 6297 x g). Then, the pellet of the donor strain was resuspended with 100 µl LB medium and this cell suspension was used

to resuspend the pellet of the acceptor strain. The mixture of acceptor and donor cells was transferred as a big spot (2 x 2 cm) on a LB agar without any antibiotics and incubated o.n. at 37 °C. The next day, the conjugation mixture was washed from the plate with 2 ml LB medium and transferred into a collection tube. This cell suspension was serially diluted 10-fold up to 10^{-4}. 50 µl of each dilution was plated on LB agar supplemented with the antibiotic(s) needed to select for successful conjugation events and the plates were incubated o.n. at 37 °C.

3.2.2.9 Transduction

Transduction (Schmieger, 1972) was used, to transfer a new generated allele containing an antibiotic resistance cassette, generated by lambda Red recombination, into a clean *S.* Tm background strain (Datsenko and Wanner, 2000); **section 3.2.2.14**). For this purpose, a P22 phage lysate of the donor strain was generated. In a second step, this phage lysate was used to transduce the allele of interest into a new bacterial background strain.

Generation of P22 phage lysate

At first, 3 ml LB medium supplemented with the appropriate antibiotic(s) and 5 mM $CaCl_2$ was inoculated with the donor strain and incubated o.n. at 37 °C in the rotor wheel. The next day, 10^3 pfu of a P22-phage lysate (stock solution: 10^6 pfu/ml) (Schmieger, 1972) were used to infect 1 ml o.n. bacterial culture, mixed and incubated for 15 min at 37 °C. Subsequently, the suspension was transferred to a baffled flask containing 10 ml LB medium and incubated o.n. at 37 °C and 180 rpm. After this, 100 µl chloroform were added to this phage containing culture and incubated at RT for 15 min while shaking. Afterwards, the culture was spun down (4 °C, 4030 x g and 15 min), 1 ml of the supernatant was filtered (0.45 µM) and transferred into a new 1.5 ml screwcap tube and stored at 4 °C. To confirm sterility of the phage lysate, 50 µl were plated on LB agar and incubated o.n. at 37 °C.

P22 transduction

3 ml LB medium supplemented with the appropriate antibiotic(s) and 5 mM $CaCl_2$ was inoculated with the acceptor strain and incubated o.n. at 37 °C in the rotor wheel. The next day, 10 µl of the generated donor phage lysate were added to 100 µl of the o.n. culture, mixed and incubated for 15 min at 37° C. Subsequently, 900 µl of LB supplemented with 10 mM EGTA were added. After incubation of 1 h at 37 °C and 850 rpm, cells were spun down (1 min, 10000 x g, RT). The pellet was resuspended in 100 µl LB and plated on LB agar supplemented with 10 mM EGTA and the appropriate antibiotics to select for transduction events. Plates were incubated o.n. at 37 °C. Colonies were streaked on fresh LB agar containing 10 mM EGTA and the appropriate antibiotics for in total three times.

3.2.2.10 Extraction of plasmid DNA from bacterial cells

The extraction of plasmid DNA from bacterial cells was done using the NucleoSpin® Plasmid Quick-Pure kit (**Table 3.7**) following the instruction of the manufacturer for low copy plasmids. If higher amounts of plasmid DNA were needed, plasmids were extracted using the QIAGEN Plasmid Plus Midi-Kit Table 3.7 according to the manufacturer instructions following the protocol for low copy plasmids.

3.2.2.11 Determination of DNA concentration

A NanoDrop Spectrophotometer ND-1000 (PEQLAB Biotechnology) was used to measure spectrophotometrically the DNA concentration at 260 nm absorbance (A260) as well as the A_{260}/A_{280} ratio. An A_{260}/A_{280} ratio of about 1.8-2.0 was considered as pure DNA.

3.2.2.12 Isolation of chromosomal DNA

For the isolation of bacterial chromosomal DNA 1 ml of an o.n. culture was harvested by centrifugation for 2 min at 19283 x g. Bacterial cell pellet was resuspended in 567 µl TE-buffer (**Table 3.15**), 30 µl 10% SDS and 3 µl Proteinase K (20 mg/ml). After the incubation for 1 h at 55 °C, 100 µl of 5 M NaCl and 80 µl CTAB/NaCl (**Table 3.15**) were added. The suspension was further incubated for 10 min at 65 °C. Subsequently, an equal volume of phenol/chloroform/isoamylalcohol (Roth, Karlsruhe) was added and the suspension was mixed by vortexing for 10 sec and spun down for 5 min at 19283 x g at RT. The supernatant containing chromosomal DNA was transferred into a new 1.5 ml collection tube and DNA was precipitated by adding 2.5 volume of 100% EtOH and 1/10 volume of 3 M NaAcetate, mixed by inverting and spun down for 30 min at 4 °C and 19283 x g. The supernatant was removed carefully and the DNA pellet was washed using 2.5 volume of 70% EtOH. After the last centrifugation step for 15 min at 4 °C and 19283 x g, the DNA pellet was air-dried at RT and finally resuspended with 20-50 µl of TE buffer or $_{dd}H_20$ and stored at 4 °C, or for long time storage at -20 °C. Chromosomal DNA isolation for the transposon mutagenesis assay (**section 3.2.2.16**) was done using the Kit NucleoSpin Tissue (**Table 3.7**), following the instructions of the protocol “support protocol for bacteria” of the manufacturer.

3.2.2.13 PCR on bacterial lysates

To verify generated bacterial mutants a single bacterial colony was transferred into a 1.5 ml collection tube containing 100 µl $_{dd}H_20$. This bacterial suspension was mixed an incubated at 95 °C for 10 min. Immediately after, the tube was cooled down on ice and 8 µl was added to the PCR reaction mix (final volume of 20 µl) containing: 1 µM oligonucleotides and 1x DreamTaq PCR Master Mix. PCR was conducted using the standard PCR protocol (**Table 3.18**).

3.2.2.14 Generation of bacterial mutant strains by lambda Red recombination

Amplification and purification of the insert:

Bacterial mutant strains were generated using lambda Red recombination (Datsenko and Wanner, 2000). At first, oligonucleotides for the amplification of the reporter genes or antibiotic resistance cassettes have been designed. The forward oligonucleotide included a homologous region of about 50 bp upstream from the start codon of the target gene(s) and the reverse oligonucleotide included a homologous region of about 50 bp downstream from the stop codon of the gene(s), respectively. To control the correct insertion oligonucleotides were designed, which bind about 20 bp up- and downstream of the insertion site. For the amplification of the insert a standard PCR mix (200 µl) was assembled (**section 3.2.2.1**) including 50-100 ng linearized template plasmid DNA followed by a standard PCR program as described previously in **section 3.2.2.1**.

Recombination:

At first, the respective *Salmonella* or *E. coli* background strain, in which the recombination should take place, was transformed with the plasmid pKD46 (**Table 3.2**) and grown o.n. at 30 °C. After this, an o.n. culture of these bacteria was prepared in 3 ml LB medium supplemented with 100 µg/ml ampicillin and incubated at 30 °C o.n. in the rotor wheel. The following day, 10 ml LB subcultures supplemented with 100 µg/ml ampicillin were inoculated using this o.n. cultures (ratio 1:20). To induce the recombinase of the pKD46 plasmid, the subcultures were additionally supplemented with 7 mM and 10 mM L (+)-arabinose. Bacterial cultures were incubated at 30 °C and 180 rpm until an OD_{600} of 0.5-0.8 was reached. Subsequently, bacterial cells were washed in ice cold $_{dd}H_2O$ and concentrated to 1/50 of the culture volume by four centrifugation and washing steps (10 min, 4 °C and 7000 x g). Between the centrifugation steps, bacterial cells were chilled on ice for 10 min. After the last centrifugation step an aliquot of 80 µl electro-competent bacteria was transformed (**section 3.2.2.7**) with ~4-6 µg of the prior amplified and purified (by precipitation, **section 3.2.2.12**) insert and grown o.n. on LB agar supplemented with the appropriate antibiotic(s) at 37 °C. To verify the success of the lambda Red recombination, a standard PCR (**section 3.2.2.1**) was conducted using the designed control oligonucleotides (**Table 3.3**).

3.2.2.15 Site specific recombination using Flp-recombinase

To eliminate antibiotic resistance cassettes flanked by FRT-sites, the Flp-recombinase system (Cherepanov and Wackernagel, 1995) encoded on plasmid pCP20 (**Table 3.2**) was used. Therefore, bacteria were transformed with 50-100 ng pCP20 (**section 3.2.2.7**). Bacteria harboring pCP20 were plated on selective LB agar and incubated at 30 °C o.n.. About 20 transformants were re-streaked on LB agar and incubated o.n. at 43 °C to induce the Flp-mediated recombination. Antibiotic sensitive colonies were then identified by double picking on LB agar with and without selective antibiotic(s) and incubation at 37°C o.n.

Antibiotic sensitive bacteria were re-streaked again on fresh LB agar and incubated o.n. at 37 °C in order to cure the strain from pCP20 plasmid.

3.2.2.16 Transposon mutagenesis assay

A transposon (Tn*10*) insertion library was constructed in *E. coli* 252R (Ec^{252R}) (**Table 3.1**) by using the suicide plasmid pJA1 (**Table 3.2**). For this purpose pJA1 carrying Tn*10* and the transposase was transformed at first into *E. coli* SM10 λ-pir (Ec^{SM10}) (**Table 3.1**) and then transferred to Ec^{252R} by conjugation. Transconjugants were selected on LB plates supplemented with 100 µg/ml kanamycin, 45 µg/ml rifampicin and 10 µM IPTG (Isopropyl-β-D-thiogalactopyranosid) to induce transposase-dependent random insertion of Tn*10* into the chromosome of Ec^{252R} at 37 °C o.n.. Single colonies were transferred into 96-well plates filled with LB (180 µl/well) and supplemented with 100 µg/ml kanamycin and incubated at 37°C o.n. while shaking for long time storage.

3.2.2.17 Single Primer PCR

For the identification of Tn*10* insertion sites by single primer random PCR, a protocol was followed as previously described (Karlyshev *et al.*, 2000). To this end, a PCR mix was prepared for a volume of 50 µl. This reaction mix included ~100 ng genomic DNA, 1 µM of the oligonucleotide T7 (**Table 3.3**) as well as 1x Dream Taq Mastermix (**Table 3.4**). Oligonucleotide T7 targeting the T7 transcriptional promoter, located adjacent to the kanamycin resistance cassette of the Tn*10*, and directed outside of Tn*10*. The standard PCR protocol was modified and consists of three different stages (**Table 3.19**). In the first stage a stringent annealing temperature is chosen to ensure specific binding of the single oligonucleotide T7. In the next stage a low annealing temperature is used to facilitate unspecific binding of the oligonucleotide to the chromosomal DNA in the adjacent region of Tn*10*, to generate with subsequent oligonucleotide extension an amplicon flanked with at both ends with sequences derived from the oligonucleotide T7. Finally in the last stage, using again a stringent annealing temperature, transposon specific amplicons, generated in the second stage, are exponential amplified.

Table 3.19: Single primer PCR protocol

Step 1	Step 2	Step3
94 °C 1 min		
20 cycles	30 cycles	30 cycles
94 °C 30 sec	94 °C 30 sec	94 °C 30 sec
50 °C 30 sec	30 °C 30 sec	50 °C 30 sec
72 °C 3 min	72 °C 2 min	72 °C 2 min
		final extension
		72 °C 7 min

PCR amplicons were loaded on a 1% agarose gel and a single DNA band with an appropriate size was extracted as described in **section 3.2.2.3**. Using the oligonucleotide InsideT7, which binds downstream of the oligonucleotide T7 promoter with a region of 18 bp, purified DNA was sent for sequencing (**section 3.2.2.5**).

3.2.2.18 qPCR to determine plasmid copy number and stability of $p^{Pcib\ gfp}$

The qPCR was performed using the FastStart Essential DNA Green Master reaction mix for SYBR Green I-based real-time PCR (Roche) on a LightCycler® 96 Instrument (Roche). Each reaction was run in triplicates in a reaction mix of 20 µl volume containing 10 µl of 2x FastStart Essential DNA Green Master, 0.3 µm forward primer, 0.3 µm reverse primer, 5-10 ng/µl of extracted total DNA from bacterial cultures of *S.* Tm^{wt} $p^{Pcib\ gfp}$ (passages 1-5; **section 3.2.3.4**) and RNase-free water.

To determine the copy number of $p^{Pcib\ gfp}$ per *S.* Tm bacteria oligonucleotides Ampli4_Fwd/Ampli4_Rev (amplicon: 86 bp) (**Table 3.3**), targeting the plasmid, and oligonucleotides PsicA_2_fwd/PsicA_2_rev (amplicon: 79 bp) (**Table 3.3**), targeting the *S.* Tm chromosome, were used. To determine qPCR reaction efficiency plasmid DNA was linearized, purified and quantified using Quant-IT Picogreen dsDNA assay kit (Life Technologies). The linear dynamic range and reaction efficiency was determined from a standard curve using a 1:10 dilution series of the linearized plasmid $p^{Pcib\ gfp}$ or linearized plasmid $p^{PsicA\ gfp}$ (encoding P_{sicA}) ranging from $2.5x10^7$ - $2.5x10^2$ copies. The optimal qPCR efficiency (97% for p^{Pcib} and 93% for $p^{PsicA\ gfp}$) was obtained using the following cycling protocol: pre-incubation step of 1 cycle 95° C for 10 min, following a 3-step amplification for 45 cycles at 95 °C for 10 s, 53 °C for 10 s and 72 °C for 10s. After each run a melting curve analysis was performed using the following cycling parameters: 95 °C for 10 s, 65 °C for 60 s and 5 °C temperature changes to the end temperature of 97 °C. Standard curves were taken for absolute quantification of genome ($p^{PsicA\ gfp}$) as well as for plasmid copy numbers ($p^{Pcib\ gfp}$)

and consequential relative plasmid copy number was calculated (plasmid copy number/genome copy number).

3.2.3 *In vitro* assays

3.2.3.1 Colicin killing-assay (halo-assay)

To test colicin production and sensitivity, a colicin producer strain was grown for 12 h in 3 ml liquid LB medium and 3 µl (to an OD_{600} of 0.25) were spotted (Ø 5 mm) on a LB agar containing 0.25 µg/ml MitC and grown o.n. at 37 °C. The next day, the plate was overlayed with 6 ml LB softagar containing 100 µl (to an OD_{600} of 1) of a sensitive tester strain grown for 12 h in 3 ml liquid LB. The plate was incubated for 24 h at 37 °C, following inspection and quantification of an inhibition zone (halo) around the colony of the producer strain. For halo-size the diameter of the colony is substracted from the total diameter (halo+colony).

3.2.3.2 Flow Cytometry

Bacteria were grown as described in **section 3.2.1.4**. For FACS, the culture was diluted in 1x PBS to a concentration of ~10^6 CFU. FACS data were recorded by a FACS Canto II running the FACSDiva software (Aria Becton Dickinson). Data analysis was done using the FlowJo software 8.8.4 (Tree Star, Inc.).

3.2.3.3 *In vitro* evolution assay

For the *in vitro* evolution assay the plasmid pColBM of Ec^{B1144} ($pColBM^{B1144}$) was transferred in Ec^{MG1655}, Ec^{Stx} and $Ec^{Stx\Delta SR}$ (**Table 3.1**) by conjugation, respectively. Successful conjugation of $pColBM^{B1144}$ was verified by PCR on bacterial lysates (**section 3.2.2.13**) using the oligonucleotides CBA CBI Primer For/CBA complete Rev and cma-check-up/cma-check-down (**Table 3.3**). Colicin production and sensitivity was confirmed using a colicin killing assay (**section 3.2.3.1**). To test for colicin BM production, transconjugants were overlayed with the colicin sensitive strain Ec^{MG1655}.

Evolution experiment I:

Strains (Ec^{MG1655} $pColBM^{B1144}$, Ec^{Stx} $pColBM^{B1144}$, $Ec^{Stx\Delta SR}$ $pColBM^{B1144}$; **Table 3.1**) were streaked from –80 °C on LB agar containing 100 µg/ml ampicillin (to select for maintenance of $pColBM^{B1144}$) and incubated o.n. at 37 °C. The next day, 10 individual colonies were picked and used to inoculate 10 ml LB medium supplemented with 100 µg/ml ampicillin (to select for maintenance of $pColBM^{B1144}$) and incubated ~15 h at 37 °C and 180 rpm. Cultures were diluted and plated on LB agar containing 0.25 µg/ml MitC to select for colicin resistant bacteria and incubated o.n. at 37 °C. Additionally, the o.n. culture was prepared for long-term storage and stored at -80 °C (**section 3.2.1.2**). Next, 10 re-grown colonies were picked and used to inoculate 10 ml LB media supplemented with 100 µg/ml ampicillin and incubated for

~15 h at 37 °C and 180 rpm. Again, 5 ml of this o.n. culture were prepared for long-term storage and stored at -80 °C. The rest from this o.n. culture was diluted as before and plated on LB agar containing 0.25 µg/ml MitC and incubated o.n. at 37 °C. This whole procedure was repeated five times in total.

After the last time a bacteria from the last set (Set 6; Ec^{MG1655} $pColBM^{B1144}$ S6, Ec^{Stx} $pColBM^{B1144}$ S6, $Ec^{Stx\Delta SR}$ $pColBM^{B1144}$ S6; **Table 3.1**) were streaked on LB agar containing 100 µg/ml ampicillin and incubated o.n. at 37 °C. The next day, clones were tested for colicin production (overlay with Ec^{MG1655}). Additionally the presence of $pColBM^{B1144}$, and possible mutations in the *cma* gene were controlled by PCR using oligonucleotides CBA CBI Primer For/CBA complete Rev (**Table 3.3**) and cma-check-up/cma-check-down (**Table 3.3**).

Evolution experiment II:

Ec^{MG1655} $pColBM^{B1144}$, Ec^{Stx} $pColBM^{B1144}$, $Ec^{Stx\Delta SR}$ $pColBM^{B1144}$ (**Table 3.1**) were streaked from -80 °C on LB agar containing 100 µg/ml ampicillin and incubated o.n. at 37 °C. The procedure was conducted as before however only one colony was used to inoculate the 15 h o.n. culture. After five times repeating the selection, the last bacteria of the generation G5 (Ec^{MG1655} $pColBM^{B1144}$ G5, Ec^{Stx} $pColBM^{B1144}$ G5, $Ec^{Stx\Delta SR}$ $pColBM^{B1144}$ G5) were streaked from -80 °C on LB agar containing 100 µg/ml ampicillin and incubated o.n. at 37 °C. Strains were tested for colicin as before. Additionally the presence of $pColBM^{B1144}$, and possible mutations in the *cma* gene were controlled by PCR using oligonucleotides CBA CBI Primer For/CBA complete Rev (**Table 3.3**) and cma-check-up/cma-check-down (**Table 3.3**).

3.2.3.4 Test for plasmid stability

S. Tm^{wt} $p^{Pcib\ gfp}$ was streaked on LB agar containing ampicillin (100 µg/ml) from -80 °C cryostock and incubated o.n. at 37 °C. The following day, a single colony was used to inoculate 10 ml LB liquid medium without antibiotics (flask). This culture was incubated o.n. at 37 °C with 180 rpm shaking. Subsequently, a sample of 1 ml to an OD_{600} of 0.4 was taken and used to set up a subculture in 10 ml LB liquid media (flask) in a ratio of 1:10. This subculture was incubated for about 3 h at 37 °C with 180 rpm. Passaging was then repeated for additional 4 times.

From each culture, samples were taken (OD_{600} of 1), diluted 10^{-6}, and plated on LB agars. After incubating o.n. at 37 °C a minimum of 100 colonies were picked and tested for presence of $p^{Pcib\ gfp}$. To check the variation of plasmid copy number of $p^{Pcib\ gfp}$ 1 ml of each culture was spun down (2 min, 19283 x g) and total DNA was extracted as described in **section 3.2.2.12**. Total DNA was used as template for qPCR (**section 3.2.2.18**) to determine plasmid stability and the copy number per genome.

3.2.4 Biochemical methods

3.2.4.1 Generation of samples for the immunoblot (western blot)

To extract total bacterial protein and bacterial proteins from the supernatant for immunoblot, bacteria were grown as described previously in **section 3.2.1.4**.

Extraction of total bacterial proteins

For the extraction of total bacterial proteins, a sample of 250 µl (to an OD_{600} of 1) was taken from the subculture and spun down for 10 min at 4 °C and 9838 x g. The pellet was resuspended in 1x protein loading buffer (**Table 3.13**), incubated for 10 min at 95 °C and immediately after this chilled on ice.

Extraction of bacterial proteins from the supernatant

Bacterial proteins from the supernatant were harvested by transferring 500 µl sample into a collection tube followed by a centrifugation at 9838 x g and 4 °C for 10 min. 400 µl of the supernatant were transferred into a new collection tube containing 100 µl of 5x protein loading buffer. The suspension was mixed, incubated for 10 min at 95 °C and immediately chilled on ice.

3.2.4.2 SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblot

SDS-PAGE was used to separate proteins (Laemmli UK, 1970). For this purpose, a polyacrylamide gel was generated consisting of a 5% stacking gel (**Table 3.12**) and, depending the size of the protein of interest, a 10-15% resolving gel (**Table 3.12**). Proteins were separated using the Mini-PROTEAN® Tetra System and the Power Pac 200 (Biorad). Subsequently, separated proteins were transferred onto a nitrocellulose membrane (GE Healthcare), using the PerfectBlue Semi-Dry Electro Blotter (PEQLAB Biotechnology) at 300 mM for 2 h. The membrane was blocked, in blocking solution (**Table 3.13**). The membrane was incubated with polyclonal primary antibody HA probe (Y11) [1:200] (Santa Cruz) or anti-ColM (rabbit polyclonal) [1:5000] (this study) diluted in blocking solution, and washed three times 5 min in 1x PBT (1X PBS and 0.1% Tween20). The membrane was then incubated for 30 min with the secondary anti-rabbit IgG (HRP) antibody [1:5000] (GE Healthcare) diluted in blocking solution. At last, the membrane was washed three times 5 min in 1x PBT, followed by detection with the ECL detection system or Immobilon Western Chemiluminescent HRP substrate (**Table 3.4**).

3.2.4.3 Coomassie Brilliant Blue R250 staining of SDS-polyacrylamide gels

After SDS-PAGE the polyacrylamide gel was transferred into a plastic box containing the fixing solution (**Table 3.14**) and heated in a microwave for 30 sec by avoiding boiling of the gel. Subsequently, the Coomassie Brilliant Blue R250 staining solution (**Table 3.14**) was applied and the gel was heated again for 30 sec in the microwave and further incubated for 3 min at RT while shaking. Next, the gel was de-stained by rinsing with $_{dd}H_2O$, heating for

30 sec in the microwave and another incubation step at RT on a shaker. This washing procedure was repeated until an optical signal noise ratio was reached.

3.2.4.4 Generation and affinity purification of recombinant $His_{(6x)}$-tagged proteins

For the extraction and purification of $His_{(6x)}$-tagged ColM (ColM-$His_{(6x)}$), $Ec^{BL21(DE3)\ \Delta fhuA}$ (**Table 3.1**) electro-competent cells were transformed with pSJB4 to generate $Ec^{BL21(DE3)\ \Delta fhuA}$ pSJB4 (**Table 3.2** and **Table 3.1**, respectively).

A single bacterial colony of $Ec^{BL21(DE3)\ \Delta fhuA}$ pSJB4 was used to inoculate 50 ml LB medium supplemented with 30 µg/ml kanamycin and 30 µg/ml chloramphenicol. The bacterial culture was incubated o.n. at 37 °C and 180 rpm. Using this o.n. culture, 1 L LB media supplemented with 30 µg/ml kanamycin and 30 µg/ml chloramphenicol was inoculated in a ratio of 1:20. When the subculture reached an OD_{600} of about 0.9, the culture was induced with 0.1 mM IPTG. After an incubation of 4 h at 180 rpm and 37 °C, bacterial cells were harvested by centrifugation (4030 x g, 4 °C, 30 min). The cell pellet was washed one time with 30 ml 1x PBS and an additional centrifugation step (4030 x g, 4 °C, 30 min). The supernatant was discarded and the bacterial cell pellet was stored at -80 °C. To test for production of ColM-$His_{(6x)}$ samples of 250 µl to an OD_{600} of 1 were taken before induction with IPTG and each hour after induction with IPTG, and further processed for immunoblot as described in **section 3.2.4.1**. Total bacterial protein was separated by SDS-PAGE and stained with Coomassie (**section 3.2.4.2** and **3.2.4.3**). The bacterial cell pellet was thawed and resuspended at RT with 25 ml lysis buffer (**Table 3.11**) and then lysed using the French Pressure Cell Press (SLM Aminco Instruments) at 1000 PSI. Afterwards, the bacterial lysate was spun down at 4030 x g, 4 °C, 30 min and a sample of 250 µl of the supernatant was taken and processed for immunoblot as described in **section 3.2.4.1**. Subsequently, the supernatant was filtered (0.22 µm filter) and loaded on a 5 ml HisTrap column equilibrated with loading buffer (**Table 3.11**). The purification and the later desalting steps were performed on an ÄKTA system (GE Healthcare). ColM-$His_{(6x)}$ were eluted with elution buffer (**Table 3.11**) and fractions containing the ColM-$His_{(6x)}$ were desalted by loading them on a 5 ml HiTrap desalting column and the application of the exchange buffer 1 (**Table 3.11**).

Antisera against ColM-$His_{(6x)}$ were obtained from immunized rabbits. Immunization was performed using standard protocols of Pineda Antibody-Service (Berlin, Germany).

Rabbit antisera were received from day 61, 90, 120 and 150 post immunization and checked for specificity by SDS-PAGE and immunoblot using ColM producer strain $Ec^{cba::cat}$, parental strain Ec^{252R} (ColMB producer) and Ec^{MG1655} as negative control.

3.2.5 Microscopy methods

3.2.5.1 Immunofluorescence staining of intrabacterial proteins

In order to stain intrabacterial proteins, bacteria were grown as described in **section 3.2.1.3**. Samples of 250 µl from an OD_{600} of 1 were taken, spun down for 5 min, at 4 °C and 6297 x g and cell pellets were resuspended and fixed in 1 volume icecold 1x PBS and three volume icecold 4% paraformaldehyde (PFA) in 1x PBS for 1 h. After fixation, bacteria were washed three times in icecold 1x PBS. Next, bacteria were immobilized on poly L-lysine coated glass slides (Superfrost Plus, Thermo Scientific). For this purpose, samples of 10 µl bacterial cell solution were spotted on the glass slides and dried o.n. at RT. Immobilized bacteria were additionally fixed for 5 min with 4% PFA in 1x PBS and bacterial cells were washed three times in 1x PBS. The next steps of the immunostaining of intrabacterial proteins were conducted as previously described (Schlumberger *et al.*, 2005). Bacteria were permeabilized at first for 5 min in icecold permeabilization buffer A (**Table 3.16**). Then, pre-permeabilized bacteria were equilibrated with icecold permeabilization buffer B (**Table 3.16**) and further permeabilized with 5 mg/ml lysozyme solved in icecold permeabilization buffer B for 30 min at 4°C. Bacteria were washed three times for 5 min in 1x PBS. Next, bacteria were incubated in blocking solution (10% normal goat serum diluted [Biozol] in 1x PBS) for 1 h at RT. Afterwards, bacteria were incubated for 1 h with the primary antibody (HA probe (Y11) [1:200] (**Table 3.17**), ADI-SPA-880 (Hsp70) [1:200] (**Table 3.17**), or anti-GFP [1:200] (**Table 3.17**)) diluted in blocking solution. Thereafter, bacteria were washed three times for 5 min in 1x PBS and incubated for 30 min with the respective secondary antibody (anti-rabbit IgG (DyLight^{TM}549) [1:400] (**Table 3.17**), anti-rabbit IgG (DyLight^{TM}649) [1:400] (**Table 3.17**), or anti-mouse IgG (Rhodamine Red^{TM}-X) [1:200] (**Table 3.17**)) diluted in blocking solution. Nucleic acids were stained with 4'6-Diamidin-2-phenyl (DAPI [1 µg/ml], Roth). Finally bacteria were washed three times with 1x PBS, dried at RT in the dark, mounted with Vectashield (Vector) and sealed with nail polish.

3.2.5.2 Confocal microscopy and image analysis

For microscopic image analysis a minimum of three images (corresponding to >100 bacteria counted) were taken using the Leica TCS SP5 confocal microscope (HCX PL APO CS, Leica Microsystems, Mannheim) and a 63x oil objective and a magnification of 1 or 2.4, respectively. ImageJ software, version 1.48v (Wayne Rasband, National Institute of Health, USA) (Schneider *et al.*, 2012) was used for image analysis. Initially, a mask was created using the DAPI channel. With this mask the bacterial shape was defined. The mask was superimposed to the channel of interest like the green (GFP-signal of the multicopy reporter or the prophage lysis gene reporter) and red (CollIb-HA- or anti-GFP-signal) channels. Cell

size, the integrated density and the mean grey values (MFI: mean fluorescence intensity) were determined for individual bacteria.
The corrected total cell fluorescence (CTCF) was calculated in order to correlate intrinsic GFP-levels with intrinsic CoIIb-HA, or with anti-GFP-signal, respectively.

$$CTCF = Integrated\ density_{bacteria} - (Area_{bacteria} \times MFI_{background})$$

The maximum value of MFI or CTCF value from *S.* Tm^{wt} served as detection limit.

3.2.5.3 Live cell microscopy using CellASIC® ONIX Microfluidic Platform

For live cell microscopy bacterial strains were grown as previously described in **section 3.2.1.6**.
The medium/buffer reservoirs (wells 1-5) of the microfluidic plate for bacteria (B04) were filled with liquid LB medium (300 µl/well) and the cell outlet well 6 was emptied. Well 7 (for waste) was also emptied but a drop of solution was left over in the bottom whole. The plate was vacuum sealed to the manifold using the CellASIC® ONIX Microfluidic Perfusion System (model: EV262) and placed onto a Spinning disk confocal microscope (TE300 eclipse, Nikon Instruments, Düsseldorf) with Perkin Elmer UltraVIEW spinning disk system and Hamamatsu Orca ER CCD or Orca Flash 4.0 CMOS camera and surrounded by a climate chamber at 37 °C. Subsequently, the plate was primed with LB medium of wells 1-5 simultaneously for 5 psi for 5 min. After this, the plate was removed from the microscope and different liquid test media were loaded to the wells (1-5; 300 µl/ml) and 100 µl cell suspension (10^6 cells/ml) was loaded in the cell inlet well 8. The plate was again vacuum sealed to the manifold and placed onto the microscope. Bacterial cells were loaded with 4 psi for 6 sec from the cell inlet well following an additional priming step to wash out un-trapped bacterial cells. Using the CellASIC™ ONIX FG Software the protocol for the medium flow over time was set up (**Table 3.20**).

Table 3.20: Protocol for medium flow over time (example)

Well	Medium	Pressure [psi]	Time [min]
1	LB	1	180
2	LB+DTPA (100 µM)	2	1
2	LB+DTPA (100 µM)	1	20
3	LB+DTPA (100 µM) + MitC (0.5 µg/ml)	2	1
3	LB+DTPA (100 µM) + MitC (0.5 µg/ml)	1	20
4	LB+DTPA (100 µM)	2	1
5	LB+DTPA (100 µM)	1	190

Every 10 min images were taken with a Nikon 100×/1.40 Plan Apo oil objective. Image analysis was done using the Volocity 6.0.1 software (Perkin-Elmer) and the ImageJ software, version 1.48v (Wayne Rasband, National Institute of Health, USA) (Schneider *et al.*, 2012).

3.2.6 Statistical analysis

For all statistical analysis the Graph Pad Prism Version 5.01 was used. As detailed in the results the following statistical tests were used:

The 1-way ANOVA test followed by the Tukey's post test. P-values less than 0.05 were considered as significant.

The 1-way ANOVA test followed by the Kruskal-Wallis test with Dunn's Multiple Comparison test. P-values less than 0.05 were considered as significant.

The Spearman-rank correlation coefficient [ρ] was calculated on a regression to determine linear correlation.

The 2-way-ANOVA was used followed by the Bonferroni posttest. P-values less than 0.05 were considered as significant**.**

3.2.7 Construction of plasmids and mutant strains

Bacterial mutant strains and bacterial plasmid generated and used in this study are listed in **Table 3.1 and Table 3.2**, respectively.

3.2.7.1 Construction of mutant strains by lambda Red recombination (Datsenko and Wanner, 2000)

Construction of *S.* Tm p2$^{cib\text{-}HA}$ (M1400): *S.* Tm^{wt} was transformed with plasmid pKD46. As template for the amplification of the HA (Hemagglutinin)-epitope tag, including a kanamycin resistance cassette flanked by FRT-sites, plasmid pSU315 (**Table 3.2**) and the oligonucleotides Colicin-HA-fwd/Colicin-HA-rev (**Table 3.3**) were used. The correct insertion in the resulting strain M994-1 was confirmed by PCR using the oligonucleotides Col-ÜE-XbaI/ Col-ÜE-XhoI (**Table 3.3**). A clean *S.* Tm^{wt} background strain was transduced with the *cib-HA-aphT* allele using P22-transduction. Correct insertion in *S.* Tm p2$^{cib\text{-}HA}$ was confirmed by PCR using oligonucleotides Col-ÜE-XbaI/ Col-ÜE-XhoI and the functional HA-tag was assessed by immunoblot using HA probe (Y11) antiserum (**Table 3.17**). Additionally, functionality of ColIb-HA in *S.* Tm p2$^{cib\text{-}HA}$ was confirmed using a colicin killing-assay.

Construction of Ec^{252R} pColBM$^{cba::cat}$ (MAD4-1): Ec^{252R} was transformed with pKD46 and pKD3 was used as template to amplify the chloramphenicol resistance cassette flanked by FRT-sites by PCR using the oligonucleotides cba-fwd-ko/cba-rev-ko (**Table 3.3**). The elimination of *cba* in Ec^{252R} pColBM$^{cba::cat}$ (MAD4-1) was verified by PCR with the oligonucleotides cba-check-up/cba-check-down (**Table 3.3**) (Manuel Diehl).

Construction of Ec^{252R} pColBM$^{cma::cat}$ (MAD5-1): To generate Ec^{252R} pColBM$^{cma::cat}$ (MAD5-1) the lambda Red recombinase system was used as described. Plasmid pKD3 was used as template to amplify the chloramphenicol resistance cassette flanked by FRT-sites by PCR using the oligonucleotides cma-fwd-ko/cma-rev-ko (**Table 3.3**). The elimination of *cma* in Ec^{252R} pColBM$^{cma::cat}$ (MAD5-1) was verified by PCR with the oligonucleotides cma-check-up/cma-check-down (**Table 3.3**) and by a colicin killing-assay (Manuel Diehl).

Construction of Ec^{252R} pColBM^{cmaHA} (SJB3): Ec^{252R} was transformed with the recombinase including plasmid pKD46 (**Table 3.1**). Next, plasmid pSU314 (**Table 3.2**) was used as template to amplify the HA (hemagglutinin)-tag sequence, including a FRT-site-flanked kanamycin resistance gene, with the oligonucleotides cma-HA-fwd/cma-HA-rev (**Table 3.3**). Correct insertion was verified by PCR using the oligonucleotides cma-check-up/cma-check-down (**Table 3.3**). The functionality of the *cma*-HA-tag was tested by immunoblot using HA probe (Y11) antiserum (**Table 3.17**).

Construction of Ec^{252R} pColBM^{cmiHA} (SJB4): Ec^{252R} was transformed with plasmid pKD46 (**Table 3.2**). Plasmid pSU314 (**Table 3.2**) was used as template to amplify the HA (hemagglutinin)-tag sequence, including a FRT-site-flanked kanamycin resistance gene, using oligonucleotides cmi-HA-fwd/cmi-HA-rev (**Table 3.3**). Correct insertion was verified by PCR using the oligonucleotides cmi check up forward/cmi check down rev (**Table 3.3**). The functionality of the *cmi*-HA-tag was checked by immunoblot using HA probe (Y11) antiserum (**Table 3.17**).

Construction of Ec$^{BL21(DE3)\ \Delta fhuA}$ (SJB7): Ec$^{BL21(DE3)}$ was transformed with plasmid pKD46 (**Table 3.2**). Plasmid pKD3 (**Table 3.2**) served as template for the FRT-site flanked chloramphenicol resistance cassette. By PCR using the oligonucleotides FhuA-KO-fwd/FhuA-KO-rev (**Table 3.3**) the chloramphenicol resistance cassette was amplified. Correct insertion was validated by PCR using oligonucleotides FhuA_seq-fwd1/FhuA_sequ-rev (**Table 3.3**). The functionality of the *fhuA* deletion was confirmed by a colicin M killing-assay (**section 3.2.3.1**).

Construction of Ec$^{DH5\alpha\ \Delta fhuA}$ (SJB8): Ec$^{DH5\alpha}$ was transformed with plasmid pKD46 (**Table 3.2**). Plasmid pKD3 (**Table 3.2**) served as template for the FRT-site flanked chloramphenicol resistance cassette. By PCR using the oligonucleotides FhuA-KO-fwd/FhuA-KO-rev (**Table 3.3**) the chloramphenicol resistance cassette was amplified. Correct insertion was validated by PCR using oligonucleotides FhuA_seq-fwd1/FhuA_sequ-rev (**Table 3.3**). The functionality of the *fhuA* deletion was confirmed by a colicin M killing-assay.

Construction of *S.* Tm p2$^{cib::sfgfp}$ (SJB15-2): *S.* Tm^{wt} was transformed with pKD46. The plasmid pWRG7 (**Table 3.2**) was used as template to amplify the *sfgfp* gene including a kanamycin resistance cassette flanked by FRT-sites with the oligonucleotides SFGFP_cib_fwd/SFGFP_cib_rev (**Table 3.3**) resulting in SJB11-14. Correct insertion was confirmed by PCR and sequencing using the oligonucleotides Check up_SFGFP_fwd/ Check up_SFGFP/RFP_rev (**Table 3.3**). Sequencing confirmed the correct sequence of the colicin Ib promoter (P_{cib}) and *sfgfp* gene for SJB11-14. The functionality of the *sfgfp* expression was assessed by FACS analysis (**section 3.2.3.2**). P22 transduction (**section 3.2.2.9**) was used to transfer the allele *cib imm::sfgfp-aphT* from SJB11-14 in a clean *S.* Tm^{wt} (SB300 pWKS30) background strain yielded in strain *S.* Tm p2$^{cib::sfgfp}$. The correct insertion was verified by PCR using oligonucleotides Check up_SFGFP_fwd/ Check up_SFGFP/ RFP_rev (**Table 3.3**). The functionality of *sfgfp* expression was assessed by FACS analysis.

Construction of Ec$^{MG1655\ \Delta fhuA}$ (SJB20): Ec^{MG165} was transformed with plasmid pKD46 (**Table 3.2**). Plasmid pKD3 (**Table 3.2**) served as template for the FRT-site flanked chloramphenicol resistance cassette. By PCR using the oligonucleotides FhuA-KO-

fwd/FhuA-KO-rev (**Table 3.3**) the chloramphenicol resistance cassette was amplified. Correct insertion was validated by PCR with the oligonucleotides FhuA_seq-fwd1/FhuA_sequ-rev (**Table 3.3**). The functionality of the *fhuA* deletion was confirmed by a colicin killing-assay.

Construction of *S.* Tm$^{lysST::sfgfp}$ p2$^{cib\text{-}HA}$ (SJB36-1): The ST64B lysis genes (*SL1344_1955-SL1344_1957=lysST)* were replaced by the *sfgfp* reporter gene, with the assistance of the lambda Red recombination system. *S.* Tm^{wt} was transformed with plasmid pKD46 (**Table 3.2**) and plasmid pWRG7 (**Table 3.2**) was used as template to amplify the *sfgfp* gene including a FRT-site-flanked kanamycin resistance cassette by PCR using the oligonucleotides ST64B-sfgfp-fwd/ ST64B-sfgfp-rev (**Table 3.3**) resulting in SJB24-15. Correct insertion was validated by PCR with the oligonucleotides Check_ST64_for/Check_ST64_rev (**Table 3.3**). Functionality of the reporter was assessed by FACS analysis. P22 transduction was used to transfer the allele *SL1344_1955-SL1344_1957::sfgfp-aphT* of SJB24-15 in a clean *S.* Tm^{wt} background strain. Correct insertion in the yielded strain *S.* Tm SJB26-2, was controlled by PCR with the oligonucleotides Check_ST64_for/Check_ST64_rev (**Table 3.3**) and functionality of the reporter was assessed using FACS analysis. Site specific-DNA recombination using Flp-recombinase was conducted to delete the FRT-site-flanked kanamycin resistance cassette of SJB26-2. The deletion of the kanamycin resistance cassette yielded in strain SJB35-1 and was confirmed by PCR using the oligonucleotides check_ST64_for/check_ST64_rev (**Table 3.3**). To transfer the plasmid p2$^{cib\text{-}HA}$, total DNA was extracted of *S.* Tm p2$^{cib\text{-}HA}$ (M1400) (**section 3.2.2.12**) and transformed in SJB35-1 resulting in strain **SJB36-1**. Transformants were selected on LB agar containing 30 µg/ml kanamycin. Plasmid transfer was verified by PCR using the oligonucleotides check_ST64_for/check_ST64_for and Col_lux_check_for/Col_lux_check_rev (**Table 3.3**).

Construction *S.* Tm$^{lysST::T7\ pol}$ (SJB34): In order to replace the ST64B lysis genes (*SL1344_1955-SL1344_1957*) by the T7 RNA polymerase gene (*T7 gene 1*) the lambda Red recombinase system was used. Therefore, *S.* Tm^{wt} was transformed at first with pKD46. The plasmid pJLG2 (**Table 3.2**) was used as template for the amplification of *T7 gene 1* by PCR using the oligonucleotides T7_ST64B_fwd/ST64B-sfgfp-rev (**Table 3.3**).
Successful recombination in SJB33 was verified by PCR using the oligonucleotides Check_ST64_for/Check_ST64_rev (**Table 3.3**). Correct sequence was controlled by sequencing using oligonucleotides Check_ST64_for/ T7 seq left 2/ T7 seq right2/ Check_ST64_rev (**Table 3.3**). P22 transduction was used to transfer the allele *SL1344_1955-SL1344_1957::T7 gene 1 aphT* of SJB33 into a new *S.* Tm background strain (*S.* Tm^{wt}) resulting in *S.* Tm$^{lysST::T7\ pol}$. Correct insertion was validated by PCR with the oligonucleotides Check_ST64_for/Check_ST64_rev (**Table 3.3**). To confirm functionality of the T7 RNA polymerase *S.* Tm$^{lysST::T7\ pol}$ was transformed with the plasmid pJLG1 (**Table 3.2**).

This plasmid encodes the T7 RNA polymerase promoter (P_{T7}) fused to a *sfgfp* gene. Functionality of the reporter was assessed using FACS analysis.

3.2.7.2 Generation of mutant strains by other techniques

Generation of Ec^{252R} $pColBM^{\Delta cba}$ (SJB5): Site specific-DNA recombination was used to delete the FRT-site-flanked chloramphenicol resistance cassette of Ec^{252R} $pColBM^{cba::cat}$. Therefore, Ec^{252R} $pColBM^{cba::cat}$ was transformed with Flp-recombinase encoding plasmid pCP20. Deletion of the chloramphenicol resistance cassette was confirmed by PCR using the oligonucleotides cba-check-up/cba-check-down (**Table 3.3**).

Generation of Ec^{252R} $pColBM^{\Delta cma}$ (SJB6): Site specific-DNA recombination was used to delete the FRT-site-flanked chloramphenicol resistance cassette of Ec^{252R} $pColBM^{cma::cat}$. Therefore, Ec^{252R} $pColBM^{cma::cat}$ was transformed with Flp-recombinase encoding plasmid pCP20. Deletion of the chloramphenicol resistance cassette was confirmed by PCR using the oligonucleotides cma-check-up/cma-check-down (**Table 3.3**).

Generation of Ec^{MG1655} $pColBM^{B1144}$ (SJB21-2): To generate Ec^{MG1655} $pColBM^{B1144}$ (SJB21-2) the plasmid pColBM of *E. coli* strain B1144 (**Table 3.1**) carrying an ampicillin resistance marker was transferred to Ec^{MG1655} by conjugation. This was verified by PCR using the oligonucleotides CBA-CBI-For/CBA-complete rev and CBA-CBI-For/CBA-remnant rev (**Table 3.3**). Production of colicin BM was determined using colicin BM sensitive Ec^{MG1655} and colicin M resistant $Ec^{MG1655\ \Delta fhuA}$ as test strains.

Generation of Ec^{Stx} $pColBM^{B1144}$ (SJB22-2): To generate Ec^{Stx} $pColBM^{B1144}$ (SJB22-2) the plasmid pColBM of *E. coli* strain B1144 (**Table 3.1**) was transferred to Ec^{Stx} (MBK13) by conjugation. This was verified by PCR using the oligonucleotides CBA-CBI-For/CBA-complete rev and CBA-CBI-For/CBA-remnant rev (**Table 3.3**). Production of colicin BM was determined using colicin BM sensitive Ec^{MG1655} and colicin M resistant $Ec^{MG1655\ \Delta fhuA}$ as test strains.

Generation of $Ec^{Stx\Delta SR}$ $pColBM^{B1144}$ (SJB23-2): To generate $Ec^{Stx\Delta SR}$ $pColBM^{B1144}$ (SJB23-2) the plasmid pColBM of *E. coli* strain B1144 (**Table 3.1**) was transferred to $Ec^{Stx\Delta SR}$ (MBK14) by conjugation. This was verified by PCR using the oligonucleotides CBA-CBI-For/CBA-complete rev and CBA-CBI-For/CBA-remnant rev (**Table 3.3**). Production of colicin BM was determined using colicin BM sensitive Ec^{MG1655} and colicin M resistant $Ec^{MG1655\ \Delta fhuA}$ as test strains.

Generation of $Ec^{252R\ Tn10}$ (MAD3): $Ec^{252R\ Tn10}$ (MAD3) was isolated from the Ec^{252R} transposon mutagenesis assay (**section 3.2.2.16**). The strain harbors Tn*10* at an unknown

location in the chromosome and does not exhibit a phenotype regarding colicin M production or sensitivity (Manuel Diehl).

3.2.7.3 Generation of plasmids

Generation of p^{empty} (pM955): To generate p^{empty} the *gfpmut3b* gene was deleted from plasmid pM946 (**Table 3.2**) by restriction hydrolysis with EcoRI, followed by a re-ligation step.

Generation of $p^{cmi\ 252R}$ (pMAD7): The *cmi* promoter region (P_{cmi}) and the *cmi* gene were amplified from extracted total DNA of Ec^{252R}. In order to this the oligonucleotides cmi-pro-fwd-XbaI/cmi-pro-rev-BamHI (**Table 3.3**) with respective binding sites of 95 bp downstream of the *cmi* stop codon and 135 bp upstream of the promoter region of *cmi*. P_{cmi}-*cmi* of Ec^{252R} was inserted into p^{empty} via BamHI and XbaI. Correct sequence was confirmed by sequencing using the oligonucleotides cmi-pro-fwd-XbaI/cmi-pro-rev-BamHI (Manuel Diehl).

Generation of $p^{cmi\ Cl139}$ (pMAD8): The *cmi* promoter region (P_{cmi}) and the *cmi* gene were amplified from extracted total DNA of Ec^{Cl139} using the oligonucleotides cmi-pro-fwd-XbaI/cmi-pro-rev-BamHI (**Table 3.3**) with respective binding sites of 95 bp downstream of the *cmi* stop codon and 135 bp upstream of the promoter region of *cmi*, respectively. P_{cmi}-*cmi* of Ec^{Cl139} was inserted into p^{empty} via BamHI and XbaI. Correct sequence was confirmed by sequencing using the oligonucleotides cmi-pro-fwd-XbaI/cmi-pro-rev-BamHI (Manuel Diehl).

Generation of $p^{PT7\ sfgfp}$ (pJLG1): To construct plasmid $p^{PT7\ sfgfp}$ the *sfgfp* gene of pWRG7 was amplified using the oligonucleotides pJLG1 SFGFP SD BamHI Fw /pJLG1 SFGFP EcoRI Rev (**Table 3.3**). The amplicon was inserted into p^{empty} (**Table 3.2**) using the enzymes BamHI and EcoRI. Correct sequence was confirmed by sequencing using the oligonucleotides pJLG1proof fwd/ pJLG1proof rev (**Table 3.3**) (Jana Glaser).

Generation of $p^{T7\ Pol}$ (pJLG2): To construct the plasmid $p^{T7\ Pol}$ genomic DNA was extracted from $Ec^{BL21\ (DE3)}$ (**Table 3.1**) and used as template to amplify the T7 polymerase gene (T7 *gene-1*) using the oligonucleotides T7 pol FW NotI/T7 pol Rev XhoI (**Table 3.3**). Following restriction hydrolysis by NotI and XhoI the fragment was inserted into p2795 (**Table 3.2**). Correct sequence in pJLG2 was verified by sequencing using the oligonucleotides pJLG2 seq Fw/T7 seq left 2/ T7 seq right2/pJLG2 seq Rev (**Table 3.3**) (Jana Glaser).

Generation of pSJB4: To generate pSJB4 encoding for the c-terminal ColM-$His_{(6x)}$-tag fusion, the open reading frame of $cma^{w/o\ stop\ codon}$ was amplified of *E. coli* ^{252R} genomic DNA by PCR using the oligonucleotides cma-histag_fwd/cma-histag_rev (**Table 3.3**). By restriction

hydrolysis with NheI and HindIII, *cma*$^{w/o\ stop\ codon}$ was inserted into hydrolyzed pET24c (Novagen) to yield pSJB4 (**Table 3.2**).

Generation of p$^{P_{cib}}$ (pSJB16): To generate p$^{P_{cib}}$ the *gfpmut2* gene was deleted from plasmid pM1437 (**Table 3.2**) by restriction hydrolysis with EcoRI followed by a ligation step. Correct sequence of P_{cib} was checked by sequencing using oligonucleotide check up_pSJB16 (**Table 3.3**).

Generation of p^{control} (pSJB17): To generate p^{control} the *gfpmut2* gene was deleted from plasmid pM968 (**Table 3.2**) by restriction hydrolysis with EcoRI, followed by a re-ligation step.

4 Results

4.1 Single cell analysis of colicin Ib (*cib*) gene expression in *S.* Tm

S. Tm^{SL1344} (*S.* Tm^{wt}) harbors a large (86.9 kb) conjugative plasmid (p2) carrying the colicin Ib locus. Thus, *S.* Tm^{wt} is able to produce colicin Ib (ColIb) and its immunity protein (ColIb Imm) to protect itself against self-killing. As previously shown, using western blot analysis and luciferase reporter constructs, ColIb (*cib*) is under the control of the SOS-response and is expressed in response to Fe^{2+}-limiting conditions both *in vitro* and in the inflamed intestine (Nedialkova *et al.*, 2014). In the same study it was additionally shown that *cib* expressing *S.* Tm^{wt} can outcompete commensal *E. coli* in inflammation-dependent Enterobacterial blooms in the mouse gut (Nedialkova *et al.*, 2014). In general it is known that colicins are produced only by small fraction of the (genetically identical) population and thus by individual bacteria (Mulec *et al.*, 2003). This is of importance as colicin-release by the producing bacteria depends on cell lysis and thus on death of the producing bacteria (Cascales *et al.*, 2007) which is referred to as “division of labor”.

Thus cell death of individual colicin producing bacteria (cooperative suicide) allows colicin-release and serves as common good for the remaining population. As a consequence this may eventually increase the overall fitness of a strain in competition with a colicin sensitive Enterobacterial community. Therefore it is of interest to analyze *cib* expression at the single cell level, to determine the fraction of individual *cib* expressing bacteria of *S.* Tm^{wt}. For this purpose, different *gfp*-reporters for *cib* expression were generated and characterized. Using these reporter tools it was possible to analyze *cib* expression of individual bacterial cells under different environmental conditions in a quantitative fashion.

This work was published in (Spriewald *et al.*, 2015).

4.1.1 Generation of *gfp*-reporters for the analysis of *cib* expression at the single cell level

To analyze *cib* expression in *S.* Tm^{SL1344} at the single cell level, at first the single-copy *sfgfp*-reporter *S.* Tm $p2^{cib::sfgfp}$ was generated. In *S.* Tm $p2^{cib::sfgfp}$ the colicin Ib activity gene (*cib*) and the *cib* immunity gene (*cib imm*) on plasmid pColIB9 (further named as p2) (**Figure 4.1A**) were exchanged against a bright and photo stable superfolder GFP (*sfgfp*) (Pedelacq *et al.*, 2006) (**Figure 4.1B**). Since p2 is only present in about one copy per *S.* Tm bacterial cell, the generated reporter is further termed as single-copy *gfp*-reporter. To characterize the reporter *S.* Tm $p2^{cib::sfgfp}$ was analyzed by FACS (**section 3.2.3.2**) under different environmental growth conditions (**Figure 4.1C**). Bacterial cells were grown for 4 h in liquid LB medium. To trigger the induction of the SOS-response only, bacteria were grown in liquid LB medium supplemented with 0.25 µg/ml MitC. For de-repression of *cib* promoter by Fe^{2+}-limitation, LB medium was supplemented with 100 µM DTPA. For a full de-repression of *cib* promoter bacteria were cultured in liquid LB with both supplements at the same time. FACS data demonstrate that *sfgfp* expression of the single-copy reporter is slightly induced by induction of the SOS-response and Fe^{2+}-limitation alone. Maximal expression was achieved by addition of both supplements simultaneously. These data are in accordance with results achieved by bulk assays using western blot analysis and luciferase reporter (Nedialkova *et al.*, 2014). In conclusion, the single-copy reporter of *S.* Tm $p2^{cib::sfgfp}$ has the capacity of reporting *cib* expression, but the sfGFP signal intensity is fairly low due the low copy number of p2.

To improve the signal intensity an additional multi-copy reporter ($p^{P_{cib\ gfp}}$) was generated, due to the overall low GFP fluorescence intensity of the single copy reporter. Therefore, *gfpmut2* was fused to the *cib* promoter region (P_{cib}) (**Figure 4.1D**) and this was inserted on a pBAD24 backbone plasmid encoding the ori of pBR322 (Guzman *et al.*, 1995) a derivative of the plasmid pMB1 (ColE1-type) (Bolivar *et al.*, 1992). Hence, this *gfp*-reporter plasmid harbors the replication control mechanism of ColE1-type plasmids and derivatives. But interestingly, pBAD24 plasmids lack the gene encoding for the repressor of primer protein (ROP) (Cronan, 2006). This RNA-binding protein negatively regulates the medium copy number characteristic for pBR322 by stabilizing the RNAI/RNAII complex, which inhibits primer formation and thus plasmid replication (Cesareni *et al.*, 1982, Lacatena *et al.*, 1984). The lack of ROP results in high copy numbers similar to the copy number of pUC plasmids (Cronan, 2006). Notably, $p^{P_{cib\ gfp}}$ copy number is thus comparable to group A colicin plasmids, as these colicins are usually encoded on small high copy number plasmids (Cascales *et al.*, 2007).

S. Tm^{wt} transformed with $p^{P_{cib\ gfp}}$ has the advantage that, in addition to *gfp* expression, *cib* and *imm* are still intact and thus expressed from their native location. Additionally, the GFP

signal intensity is expected to be higher due to the multi-copy number plasmid. However, the high copy number may also affect gene regulation of *cib* by introducing “multi-copy-effects”, due to the additional copies of the repressor binding sites (FUR- and LexA-Box) on $p^{Pcib\ gfp}$ (**Figure 4.1D**).

Interestingly, FACS data show the same supplement-dependent *cib* expression pattern as it was observed for the single-copy reporter (**Figure 4.1C**). As expected, in contrast to the single-copy reporter the induction of *cib* resulted in overall higher GFP signal intensity (**Figure 4.1E**).

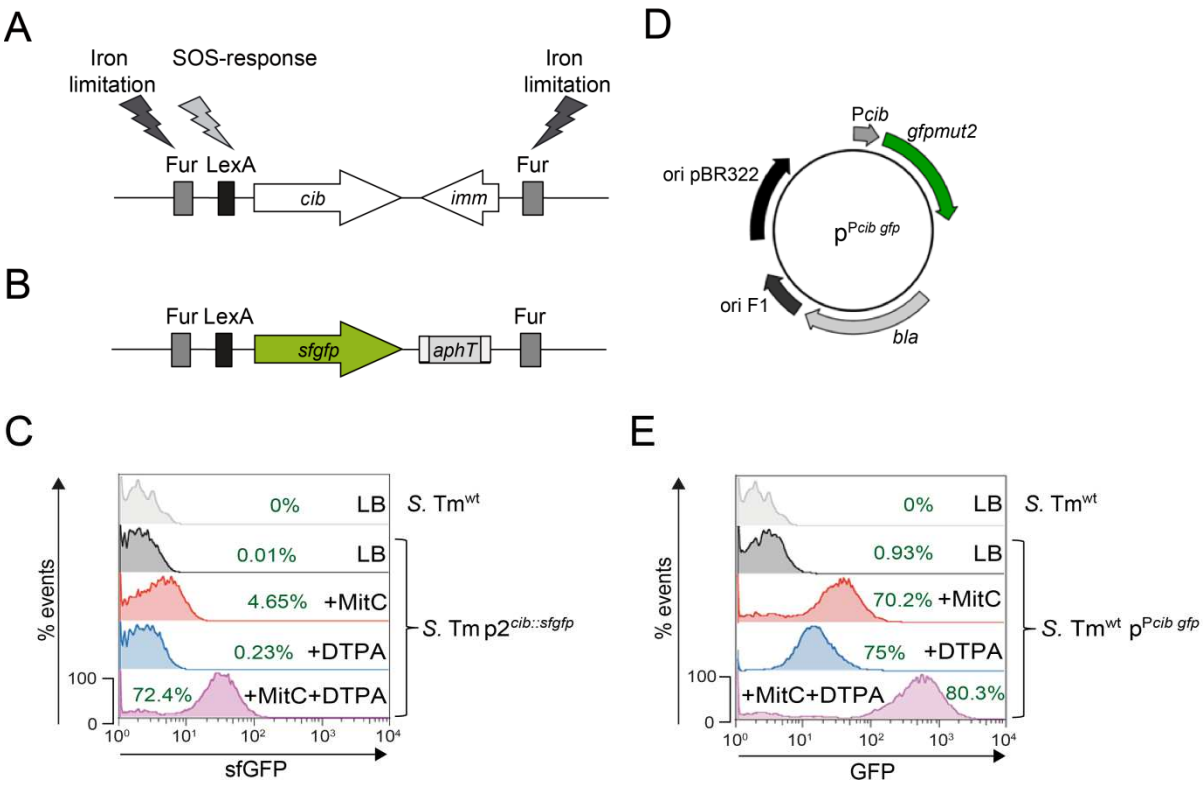

Figure 4.1: Characterization of *gfp* expression by the single- and multi-copy *gfp*-reporter. (A) Organization of the CollB locus encoded on the p2 plasmid in *S.* Tm^{SL1344} and **(B)** on the single-copy reporter in *S.* Tm $p2^{cib::sfgfp}$. **(C)** Flow cytometry analysis for sfGFP signal intensities of *S.* Tm $p2^{cib::sfgfp}$. **(D)** Schematic view of the gene structure of the multi-copy reporter $p^{Pcib\ gfp}$. **(E)** Flow cytometry analysis for GFP signal intensities *S.* Tm^{wt} $p^{Pcib\ gfp}$. **(C and E)** Bacterial strains were cultivated for 4 h in either liquid LB medium (grey), LB supplemented with 0.25 µg/ml MitC (red), LB supplemented with 100 µM DTPA (blue), or in LB containing both inducer agents (purple). *S.* Tm^{wt} grown for 4 h in liquid LB medium served as negative control to determine the fraction (%) of $sfGFP^+$ and GFP^+ bacteria. Adapted from (Spriewald *et al.*, 2015).

4.1.2 The influence of different inducer concentrations on *cib* expression pattern in *S.* Tm^{wt}

It was hypothesized that colicin production following the strategy of division of labor would be of advantage for the entire population. In this scenario a small fraction of colicin producing

bacteria would sacrifice itself to kill closely related environmental competitors. Consequently, this would result in a growth benefit for the majority of the colicin producing population, as this was shown for colicin-dependent growth benefit of *S.* Tm over commensal *E. coli* in the inflamed gut (Nedialkova *et al.*, 2014). According to this hypothesis, a bimodal gene expression pattern of *cib* upon induction would be expected. So far I have not observed bimodal expression with the high supplement concentrations, applied before and in bulk assays to support maximal *cib*-induction (Nedialkova *et al.*, 2014). Thus, *gfp* expression of the single-copy reporter was determined by FACS, using decreasing supplement concentrations. *S.* Tm p2$^{cib::sfgfp}$ was grown 4 h with either decreasing concentrations of DTPA (200 µM, 100 µM, 50 µM, 25 µM, 12 µM, 6 µM) or MitC (1 µg/ml, 0.5 µg/ml, 0.25 µg/ml, 0.2 µg/ml, 0.1 µg/ml, 0.05 µg/ml, 0.01 µg/ml) and subsequently analyzed by FACS (**Figure 4.2AB top**). Again only a shift in the entire population of GFP$^+$ bacteria was detected (unimodal gene expression) but no bimodal gene expression, which would have been characterized by two peaks of different fluorescence intensity. Nevertheless, as the promoter region of *cib* harbors two repressor binding sites (FUR- and LexA-box), both inducer compounds might be required simultaneously to favor a bimodal expression. Accordingly, *S.* Tm p2$^{cib::sfgfp}$ was grown 4 h in LB with both supplements but this time one supplement was kept at a constant concentration and the other one was titrated in a decreasing manner. However, no bimodal expression pattern was observed. To exclude, that the rather low sfGFP signal intensity of *S.* Tm p2$^{cib::sfgfp}$ is the reason for unimodal expression pattern the experiment was repeated with *S.* Tm^{wt} p$^{P\mathit{cib\ gfp}}$ (**Figure 4.2CD**). FACS data of *S.* Tm^{wt} p$^{P\mathit{cib\ gfp}}$ revealed higher GFP fluorescence intensity but a similar expression pattern compared to *S.* Tm p2$^{cib::sfgfp}$. The influence of different time points of induction on *sfgfp* expression was also investigated and similar results were obtained (data not shown). Consequently, *cib* is expressed rather in a unimodal than bimodal fashion.

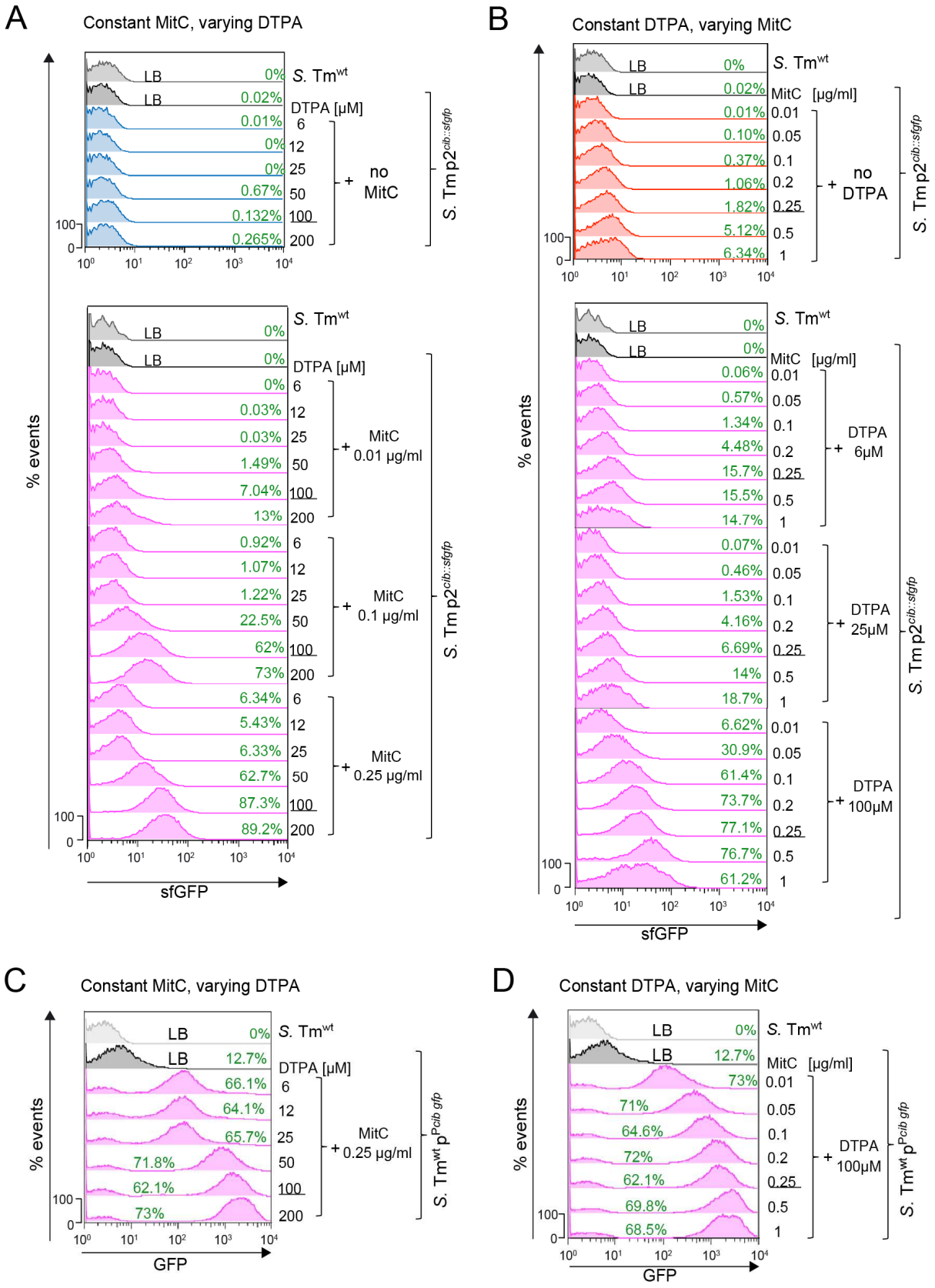

Figure 4.2: Impact of varying exogenous supplement concentrations on *cib expression* in *S.* Tm p2$^{cib::sfgfp}$ and *S.* Tm^{wt} p$^{Pcib\ gfp}$. (A-B) GFP fluorescence intensity of *S.* Tm p2$^{cib::sfgfp}$ (single-copy reporter) was analyzed by flow cytometry after 4 h growth in liquid LB media supplemented with either **(A)** increasing concentrations of DTPA (0 µM, 6 µM, 12 µM, 25 µM, 50 µM, 100 µM and 200 µM) and addition of a constant MitC concentration (either 0 µg/ml, 0.01 µg/ml, 0.05 µg/ml, 0.1 µg/ml, 0.2 µg/ml, 0.25 µg/ml, 0.5 µg/ml or 1 µg/ml) or **(B)** increasing MitC concentrations (0 µg/ml,

0.01 µg/ml, 0.05 µg/ml, 0.1 µg/ml, 0.2 µg/ml, 0.25 µg/ml, 0.5 µg/ml and 1 µg/m) while the DTPA was kept at a constant concentration (0 µM, 6 µM, 12 µM, 25 µM, 50 µM, or 100 µM). Underlined values highlight MitC and DTPA concentrations used before for the reporter characterization (Figure 4.1) and in bulk assays (Nedialkova *et al.*, 2014). **(C)** *S.* Tm^{wt} $p^{P_{cib\ gfp}}$ was cultured 4 h in liquid LB medium supplemented with increasing DTPA concentrations (0 µM, 6 µM, 12 µM, 25 µM, 50 µM, 100 µM and 200 µM) and a constant MitC concentration of 0.25 µg/ml and **(D)** with increasing MitC concentrations (0 µg/ml, 0.01 µg/ml, 0.05 µg/ml, 0.1 µg/ml, 0.2 µg/ml, 0.25 µg/ml, 0.5 µg/ml and 1 µg/m) and a constant DTPA concentrations of 100 µM. GFP fluorescence intensities were analyzed by FACS. To determine the fraction of GFP^+ (%) bacteria *S.* Tm^{wt} (no GFP) was grown as negative control in LB (grey) (Spriewald *et al.*, 2015).

4.1.3 Plasmid stability of $p^{P_{cib\ gfp}}$ and its influence on intrinsic *cib* expression in *S.* Tm^{wt}

As the GFP signal intensity of the single-copy reporter *S.* Tm $p2^{cib::sfgfp}$ was overall quite low, the multi-copy reporter $p^{P_{cib\ gfp}}$, with its high GFP signal intensities, would be a useful tool for further analyses. For time course experiments as well as live cell microscopy or animal experiments it would be relevant to determine the plasmid stability of $p^{P_{cib\ gfp}}$, as the plasmid loss could lead to not reliable results. In order to investigate plasmid stability of the multi-copy plasmid over time without selective pressure, *S.* Tm^{wt} $p^{P_{cib\ gfp}}$ was cultivated in liquid LB without antibiotics for 5 successive passages and thus for about 24 generations (**section 3.2.3.4**). Furthermore, samples were taken, following replica plating to determine plasmid loss. Additionally, total DNA was extracted (**section 3.2.2.11**) and culture samples were analyzed by qPCR to determine plasmid copy number variations over time. Replica plating results exhibited an excellent plasmid stability of >99% which was also confirmed by qPCR analysis (**Figure 4.3**).

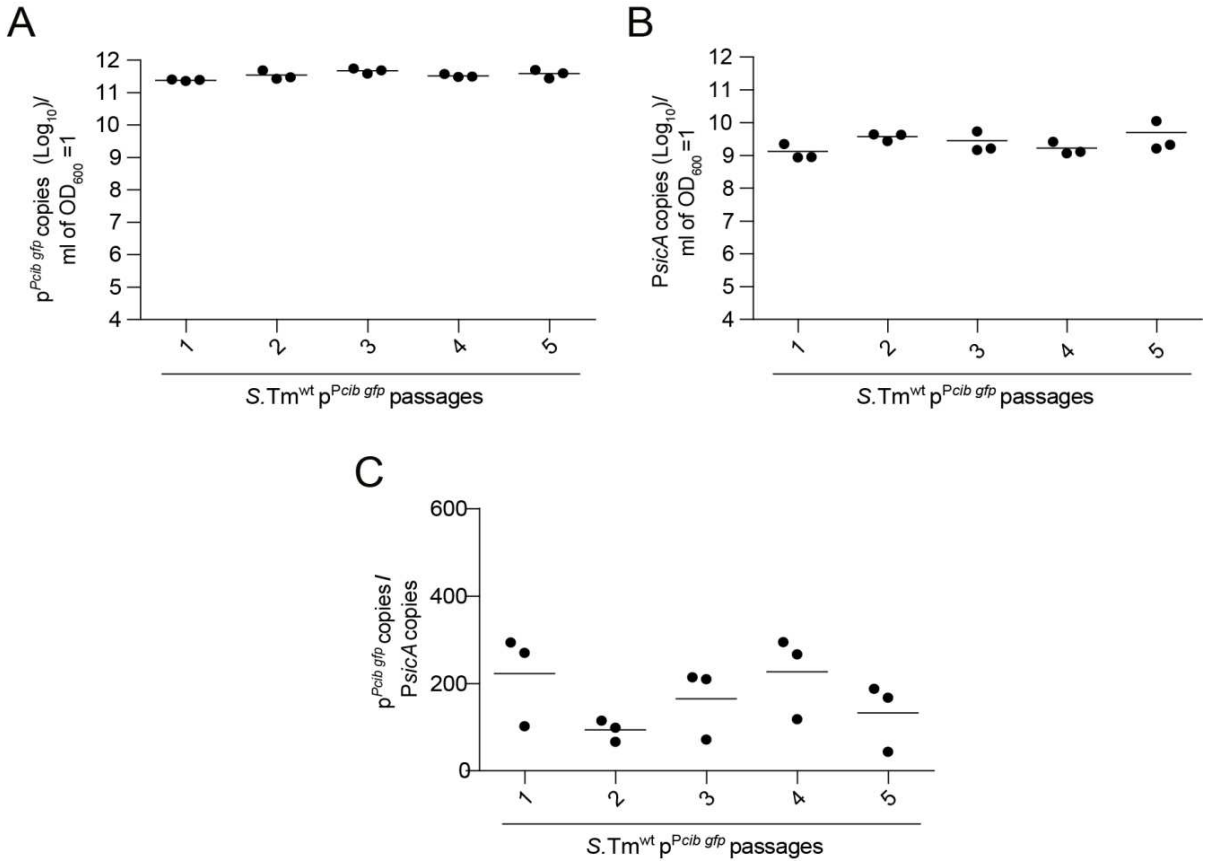

Figure 4.3: Quantitative PCR (qPCR) to determine plasmid stability and copy number of the reporter plasmid p$^{Pcib\ gfp}$. S. Tm^{wt} p$^{Pcib\ gfp}$ was grown in 5 successive passages in LB without antibiotics. After each passage, samples (1 ml for an OD_{600} of 1) were taken, total DNA was extracted and **(A)** plasmid (oligonucleotides Ampli4_Fwd/Rev) and **(B)** genome copy numbers (oligonucleotides PsicA_1_fwd/rev) were determined by qPCR. **(C)** Plasmid (p$^{Pcib\ gfp}$) copy numbers per genome as calculated from the values of (A) and (B). Bars represent the mean. Statistical analysis was done using 1-way ANOVA with Tukey's post test and no significant difference was found ($p>0.05$). Adapted from (Spriewald *et al.*, 2015).

p$^{Pcib\ gfp}$ reports *cib* expression through a P$_{cib}$ *gfpmut2* fusion on an external plasmid, and *S.* Tm^{wt} p$^{Pcib\ gfp}$ express intrinsic *cib* in addition to *gfpmut2*. This is a useful tool to correlate intrinsic *cib* expression with *gfpmut2* expression from p$^{Pcib\ gfp}$ in *S.* Tm^{wt}. To this end a c-terminal ColIb Hemagglutinin (HA) fusion was generated to detect ColIb-HA via intracellular immunostaining (**Figure 4.4A**). To exclude differences in production and/or functionality of ColIb-HA compared to the wildtype ColIb a halo-assay was conducted (**section 3.2.3.1**). *S.* Tm p2$^{cib\text{-}HA}$ and a wildtype control strain *S.* Tm^{wt} were spotted on LB agar containing 0.25 µg/ml MitC and overlayed with the colicin sensitive strain Ec^{MG1655} to compared ColIb production and toxicity (**Figure 4.4B**). The halo-assay revealed no significant differences between ColIb-HA and wildtype ColIb production and functionality.

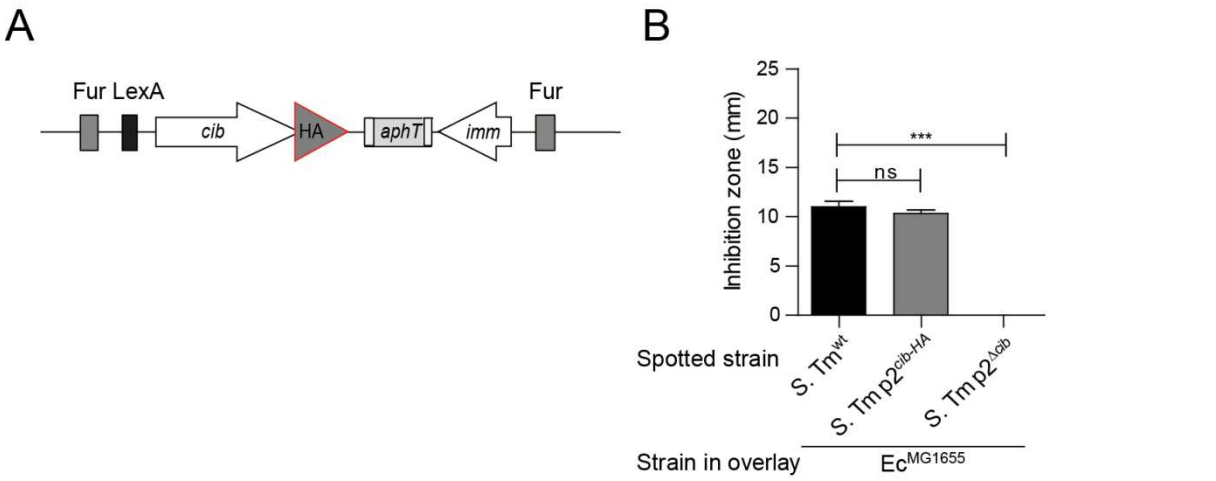

Figure 4.4: Verification of the ColIb-HA functionality in *S.* Tm p2$^{cib\text{-}HA}$. (A) Organization of the ColIb-HA locus encoded on the p2 plasmid in *S.* Tm p2$^{cib\text{-}HA}$. Adapted from (Spriewald *et al.*, 2015). **(B)** Halo-assay to determine colicin production and functionality of ColIb-HA in *S.* Tm p2$^{cib\text{-}HA}$ compared to wildtype ColIb produced by *S.* Tm^{wt}. O.n. cultures of *S.* Tm^{wt} and *S.* Tm p2$^{cib\text{-}HA}$ as well as *S.* Tm p2$^{\Delta cib}$ lacking the entire *cib* locus were spotted on LB agar plates containing 0.25 µg/ml MitC. The next day, agar plates were overlayed with LB softagar mixed with o.n. culture of colicin sensitive Ec^{MG1655}. The experiment was done in triplicates and the halo sizes were measured 24 h after the overlay. Statistical analysis was done using 1-way ANOVA with Tukey's post test (***p<0.001). Bars show mean and StD. Additional data not shown in (Spriewald *et al.*, 2015).

To detect intrabacterial ColIb-HA in *S.* Tm p2$^{cib\text{-}HA}$, an immunofluorescent staining protocol for intrabacterial proteins (**section 3.2.5.1**) was employed (Schlumberger *et al.*, 2005). For this purpose, the bacterial cell wall was permeabilized with lysozyme. The lysozyme permeabilization efficiency was optimized using a specific antibody (ADI-SPA-880 (Hsp 70)) against the conserved and ubiquitously expressed chaperon, DnaK (**Figure 4.5**). Besides this, specificity of the HA probe (Y11) antiserum was confirmed (**Figure 4.6**).

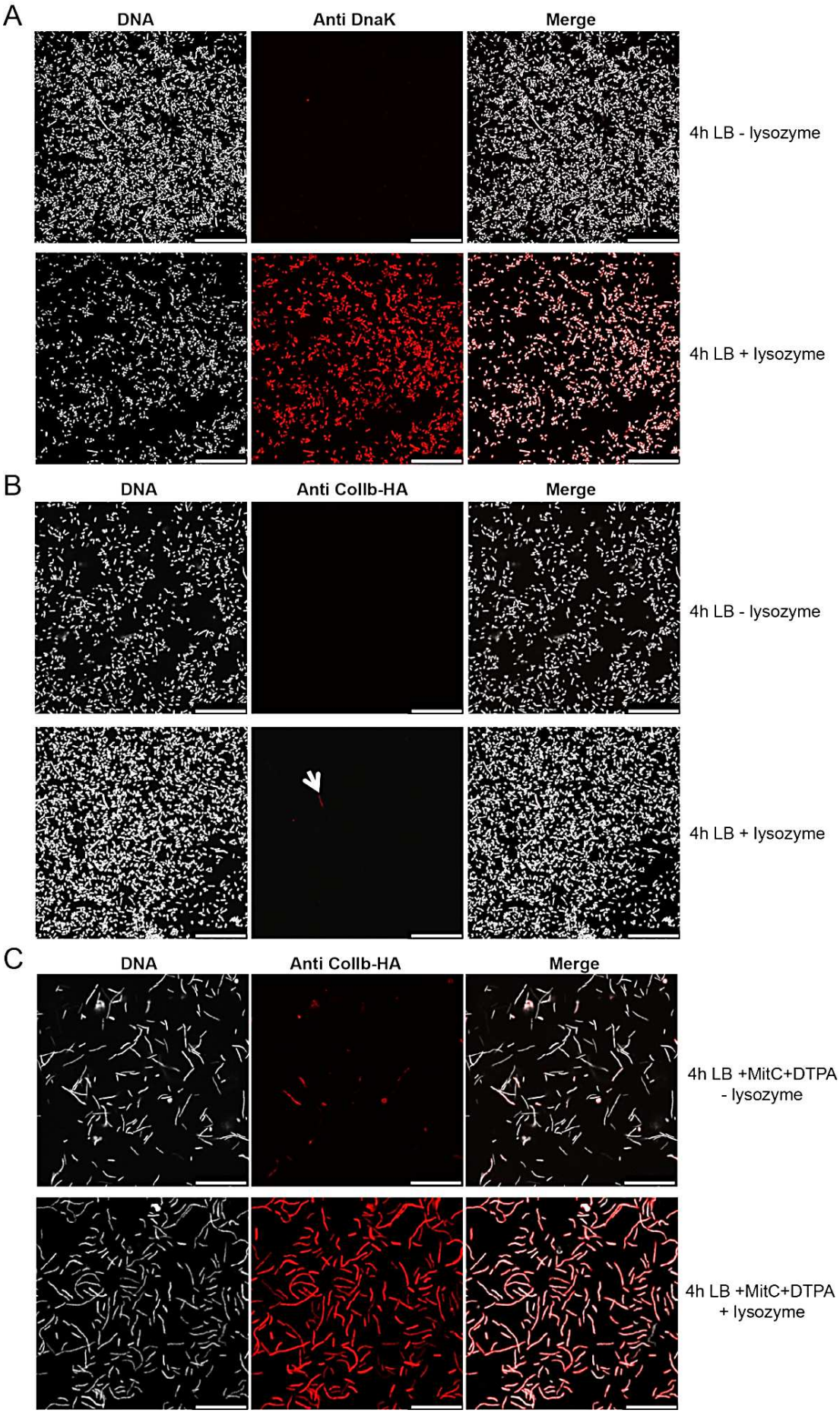
A
DNA
Anti DnaK
Merge
4h LB - lysozyme
4h LB + lysozyme
B
DNA
Anti CollB-HA
Merge
4h LB - lysozyme
4h LB + lysozyme
C
DNA
Anti CollB-HA
Merge
4h LB +MitC+DTPA
- lysozyme
4h LB +MitC+DTPA
+ lysozyme

Figure 4.5: Intrabacterial detection of DnaK and CoIIb-HA proteins in individual bacteria by immunofluorescence. *S.* Tm p2$^{cib\text{-}HA}$ was grown in LB for 12 h or 4 h or 4 h in LB supplemented with 0.25 µg/ml MitC, 100 µM DTPA or both. Bacteria were fixed and either treated with lysozyme (A,B,C lower panels) or not (A,B,C upper panels). **(A)** Lysozyme permeabilization efficiency was validated through intrabacterial staining of the constitutively produced, cytosolic protein DnaK. **(B-C)** Intrabacterial CoIIb-HA was detected using HA-specific antiserum (HA probe (Y11)). Depicted images show examples of intrabacterial staining of CoIIb-HA in individual *S.* Tm p2$^{cib\text{-}HA}$ p^{control} bacteria, grown for 4 h in **(B)** LB medium, showing only a small fraction of CoIIb-HA producing bacteria (arrow), and **(C)** in LB medium supplemented with MitC and DTPA, with maximal *cib* expression. Under these conditions CoIIb-HA was also detected in a small fraction of *S.* Tm p2$^{cib\text{-}HA}$ without lysozyme treatment (C upper panel). Scale bar 25 µm. Adapted from (Spriewald *et al.*, 2015).

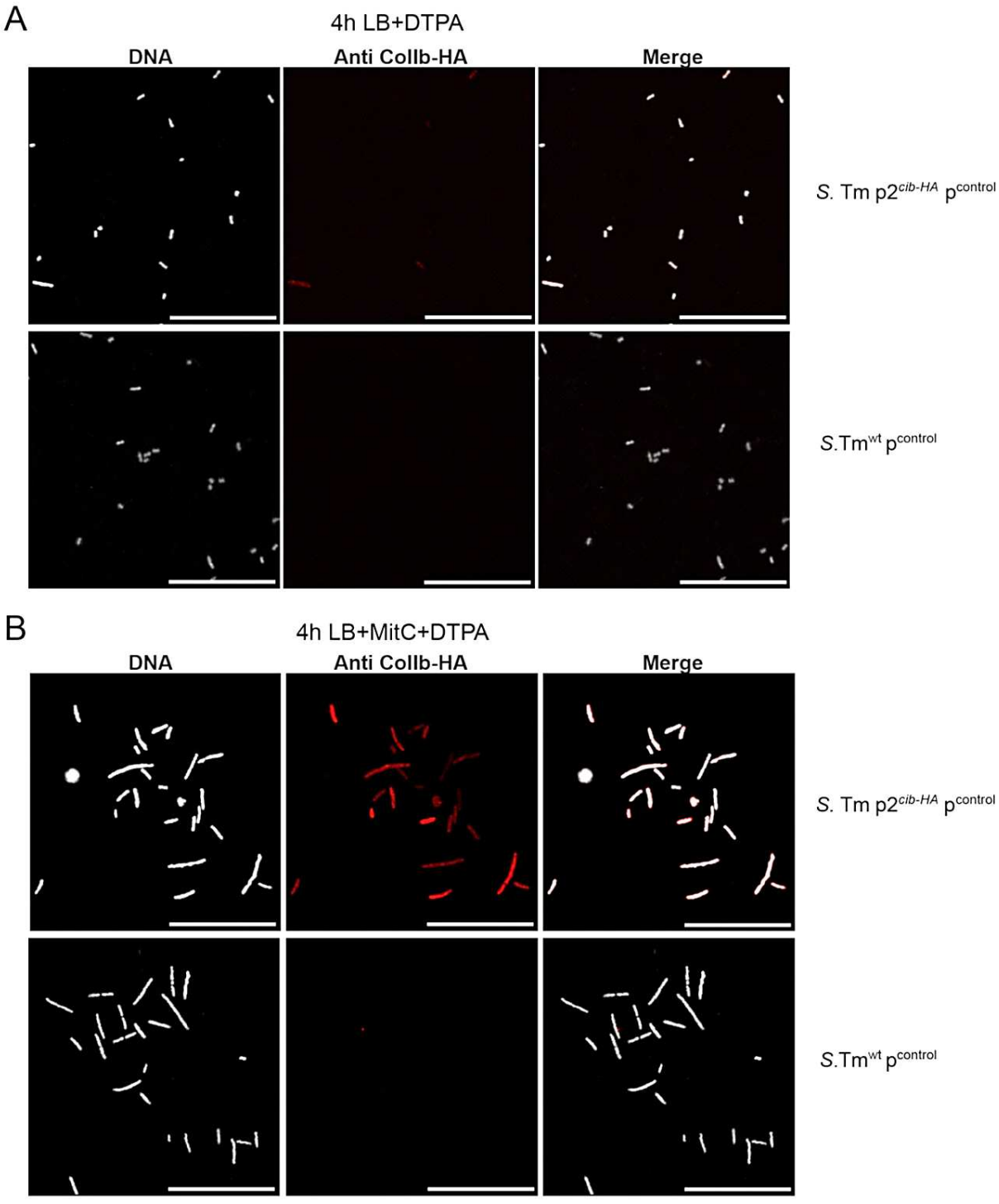

Figure 4.6: Confirmation of binding specificity of HA probe (Y11) antiserum against CoIIb-HA within individual bacteria. *S.* Tm^{wt} p^{control} and *S.* Tm p2$^{cib\text{-}HA}$ p^{control} were grown in LB medium for 12 h or 4 h and for 4 h in LB supplemented with 0.25 µg/ml MitC, 100 µM DTPA **(A)** or both **(B)**. Bacteria were fixed, lysozyme-permeabilized and stained with HA-specific antiserum (HA probe (Y11)) to detect intrabacterial CoIIb-HA (red). Total DNA was stained with DAPI (grey). Scale bar 25 µm. Adapted from (Spriewald *et al.*, 2015).

As shown by qPCR (**Figure 4.3**), the multi-copy plasmid is present in >100 copies per cell, consequently each cell of *S.* Tm^{wt} p$^{P\textit{cib gfp}}$ harbors unnaturally more than 100 copies of the P_{cib} promoter and *gfp*. This may influence native *cib* regulation. For example, it has been shown, that eukaryotic GFP can be toxic for bacterial cells (Liu *et al.*, 1999). Even if bacteria are only stressed by *gfp* expression, this could also lead to changes in intrinsic *cib* expression. To exclude any side effects on native *cib* expression by the colicin promoter (P_{cib}) or *gfp* of p$^{P\textit{cib gfp}}$, a variety of plasmid derivatives of the multi-copy reporter were constructed (**Figure 4.7**). These p$^{P\textit{cib gfp}}$ derivatives are the plasmid p$^{P\textit{cib}}$ lacking only *gfp*, p^{gfp} lacking the promoter P_{cib} and p$^{P\textit{rpsM gfp}}$, a derivative expressing *gfp* constitutively under the control of the ribosomal *rpsM* promoter. The plasmid p^{control}, lacking the entire P_{cib} *gfp* fusion, was generated as negative control.

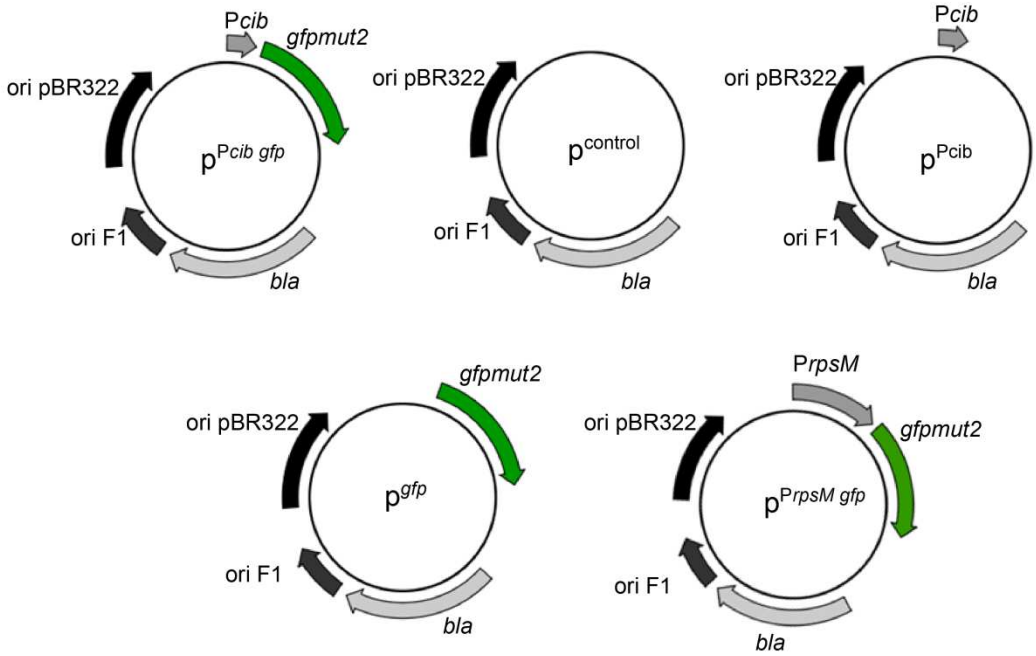

Figure 4.7: Schematic view of multi-copy reporter p$^{P\textit{cib gfp}}$ plasmid and its derivatives. The multi-copy reporter plasmid p$^{P\textit{cib gfp}}$, the empty backbone vector p^{control}, p^{Pcib} lacking the *gfp*, p^{gfp} lacking the *cib* promoter (P_{cib}) and plasmid p$^{P\textit{rpsM gfp}}$ for constitutive *gfp* expression are shown (Spriewald *et al.*, 2015).

Each plasmid derivative was transformed into *S.* Tm^{wt} p2$^{cib\text{-}HA}$ and *cib-HA* expression was examined by intrabacterial immunofluorescent staining. Bacteria were cultured for 4 h in liquid LB media containing 100 µg/ml ampicillin (LBA) without supplements or in LBA supplemented with 0.25 µg/ml MitC, or 100 µM DTPA or supplemented with even both inducing agents. As already mentioned, it was shown that induction of the SOS-response can take place spontaneously during a late logarithmic growth phase and colicins can also be induced in this growth phase in a small fraction of the population (e.g. 3%), as observed for ColK (Mulec *et al.*, 2003, Mrak *et al.*, 2007, Nanda *et al.*, 2014). Thus, bacterial samples were also taken from cultures grown for 12 h (late logarithmic growth phase) in liquid LBA media. Subsequently, bacteria were stained for total DNA and intrabacterial ColIb-HA in individual bacteria (**section 3.2.5.1**). Microscopic images were taken by confocal microscopy and ColIb-HA fluorescence intensities (DyLight^{TM}549), and GFP signal intensities were further analyzed using ImageJ software. *S.* Tm^{wt} transformed with the control plasmid p^{control} served as negative control to quantify the fraction (%) of *cib-HA*- and *gfp*-expressing bacteria (**Figure 4.8**).

Bacteria grown for 4 h (logarithmic growth phase) without any supplements exhibit rather low ColIb-HA and GFP levels which were close to the detection limit. Overall, quantification of ColIb-HA levels showed no significant differences between strains transferred with the reporter p$^{P\textit{cib gfp}}$ compared to the derivatives lacking the *cib* promoter (p$^{\textit{gfp}}$), *gfp* (p$^{P\textit{cib}}$) or both elements (p^{control}). Only control plasmid p$^{P\textit{rpsM gfp}}$, generating high intrabacterial GFP-levels, positively influenced the ColIb-HA levels.

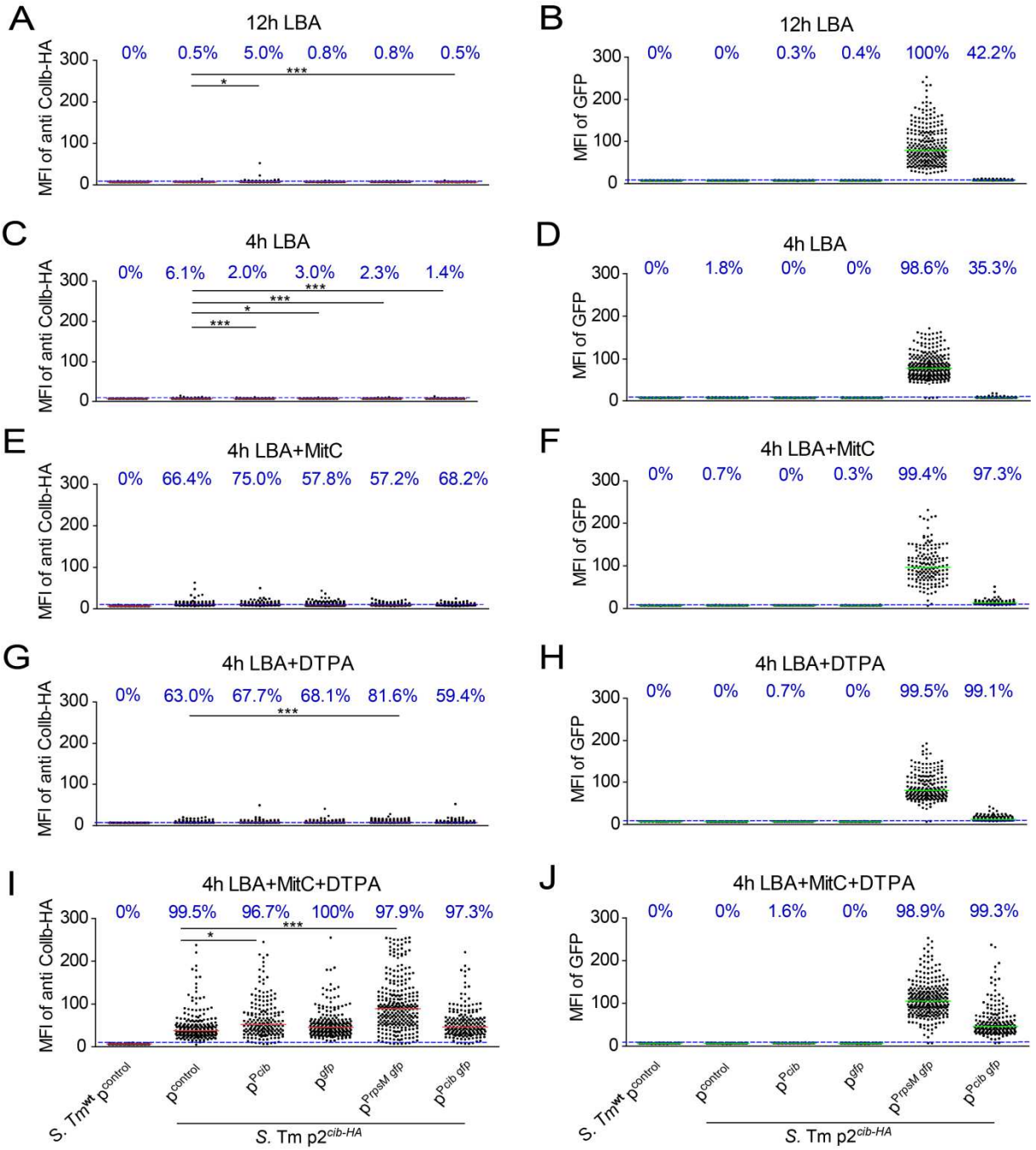

Figure 4.8: Influence of reporter p$^{Pcib\ gfp}$ and its derivative plasmids on intrinsic *cib-HA*-expression in individual bacteria of S. Tm p2$^{cib\text{-}HA}$. S. Tm p2$^{cib\text{-}HA}$ was transformed either with p$^{Pcib\ gfp}$ or its derivatives (p^{control}, p^{Pcib}, p^{gfp}, p$^{PrpsM\ gfp}$) and grown over night for 12 h (stationary phase) in LBA (LB+ampicillin) or subcultured and grown for 4 h (late logarithmic phase) in LBA without or with supplements (0.25 µg/ml MitC, 100 µM DTPA) as indicated. Bacteria were fixed, immobilized on glass slides and lysozyme-permeabilized. Intrabacterial CollB-HA was detected in individual bacterial using HA-specific antiserum (HA probe (Y11)) and a DyLight^{TM}549-conjugated secondary antibody (anti-rabbit IgG (DyLight^{TM}549)). Bacterial DNA was stained with DAPI. By confocal microscopy bacteria were imaged and fluorescence of DAPI, GFP and DyLight^{TM}549 (CollB-HA) was recorded. Mean fluorescence intensities (MFI) **(A, C, E, G, I)** of CollB-HA and **(B, D, F, H, J)** GFP ascertained using ImageJ. MFI of Individual bacteria are represented by dots. Bars represent the median and blue values represent the fraction above the detection limit (dotted line; background fluorescence of *S.* Tm^{wt} p^{control}). Statistical analysis was done using Kruskal-Wallis test with Dunn's post test (* $p < 0.05$). Adapted from (Spriewald *et al.*, 2015).

4.1.4 Correlation of GFP- and intrinsic ColIb-HA- signal intensities of *S.* Tm p2$^{cib\text{-}HA}$ p$^{Pcib\ gfp}$

Having ascertained that p$^{Pcib\ gfp}$ does not have any major impact on intrinsic *cib-HA* expression, the correlation between *gfp*- and intrinsic *cib-HA*-expression in *S.* Tm p2$^{cib\text{-}HA}$ p$^{Pcib\ gfp}$ was determined. This would demonstrate that the multi-copy reporter p$^{Pcib\ gfp}$ is a reliable tool to report intrinsic *cib-HA* expression in individual bacterial cells.

Using the intrabacterial immunofluorescence staining method, it is not possible to differentiate between folded (=functional) and unfolded ColIb-HA proteins. GFP fluorescence can only be detected when the GFP protein is correctly folded. A delayed GFP-maturation would lead to a bias in the correlation. To exclude this possibility the fluorescence signal intensities of mature folded GFP (GFP^{mature}) was correlated with signal intensities of total GFP (GFP^{total}) by intrabacterial staining against GFP using a GFP specific antiserum (anti-GFP) and a DyLight^{TM}649-conjugated secondary antibody (anti-rabbit IgG (DyLight^{TM}649)). *S.* Tm p2$^{cib\text{-}HA}$ p$^{Pcib\ gfp}$ was cultured under different environmental conditions and bacteria were stained for total bacterial DNA and intrabacterial GFP. Microscopic images were then taken and analyzed using the ImageJ software. Using *S.* Tm^{wt} p^{control} as negative control it was now possible to show that under all tested conditions GFP^{mature} and GFP^{total} (DyLight^{TM}649) signal intensities correlate well in *S.* Tm p2$^{cib\text{-}HA}$ p$^{Pcib\ gfp}$ with an overall Spearman rank correlation coefficient of ρ=0.95 (**Figure 4.9**).

To finally correlate the *gfp* expression gained by the multi-copy reporter p$^{Pcib\ gfp}$ with intrinsic *cib-HA* expression (ColIb-HA; DyLight^{TM}549) of individual *S.* Tm p2$^{cib\text{-}HA}$ p$^{Pcib\ gfp}$ bacteria, the corrected total cell fluorescence (CTCF) values for GFP and anti-ColIb-HA were determined. The CTCF values of GFP and ColIb-HA (DyLight^{TM}549) of individual *S.* Tm p2$^{cib\text{-}HA}$ p$^{Pcib\ gfp}$ bacteria were plotted against each other. Results revealed a good correlation of mature GFP signal levels and ColIb-HA (DyLight^{TM}549) signal intensities (overall Spearman-rank correlation coefficient ρ=0.87; **Figure 4.10**).

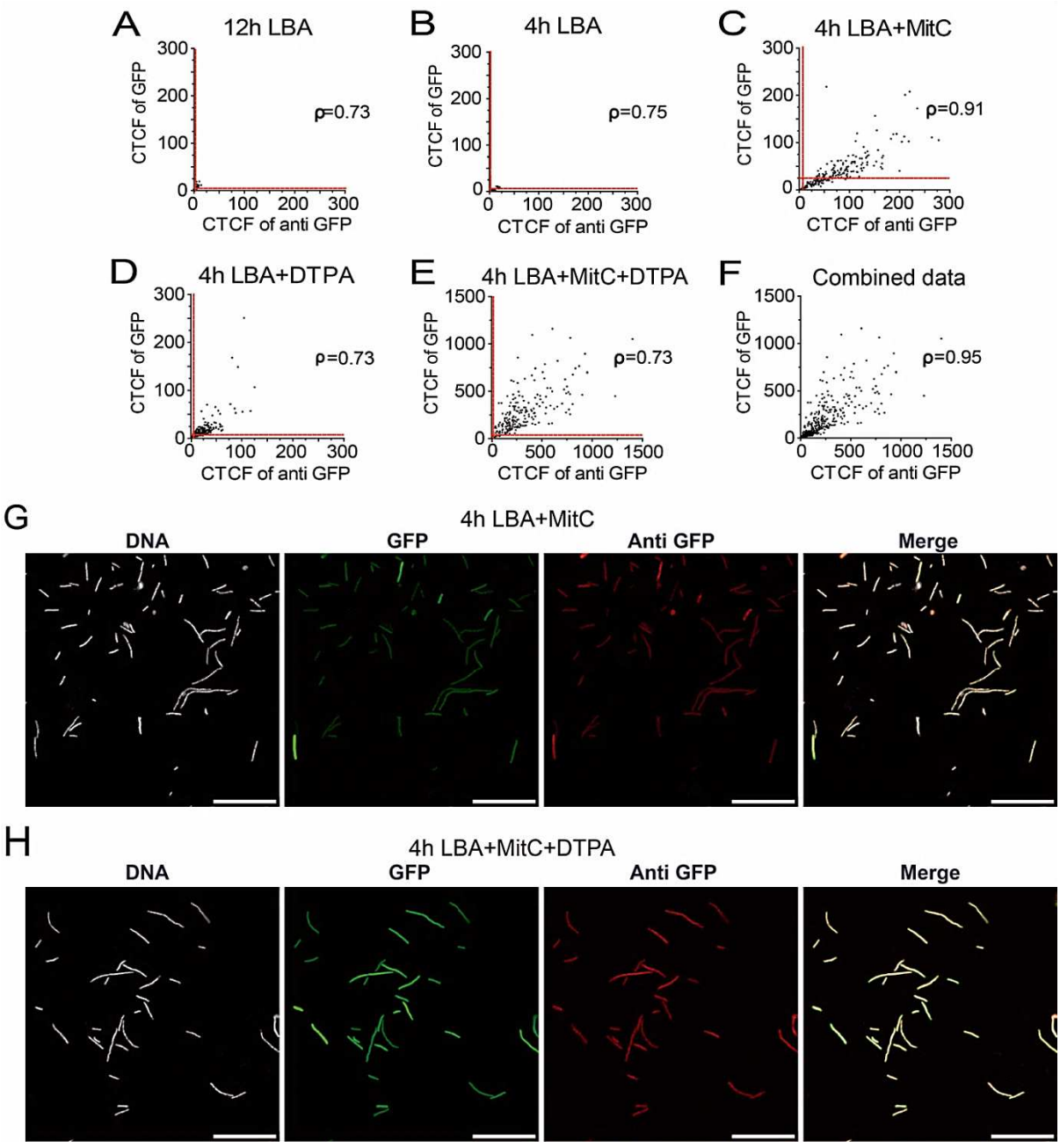

Figure 4.9: Correlation of the fluorescence signal of GFP^{mature} with anti-GFP^{total} immunofluorescent staining signal of p$^{Pcib\ gfp}$. *S.* Tm$^{cib\text{-}HA}$ p2$^{Pcib\ gfp}$ bacterial cells were cultured in LB medium supplemented with 100 µg/ml ampicillin (LBA) for **(A)** 12 h, **(B)** 4 h, and **(C)** for 4 h in LBA supplemented with 0.25 µg/ml MitC, **(D)** LBA supplemented with 100 µM DTPA and **(E)** LBA supplemented with both. Fixed bacteria were lysozyme-permeabilized and stained for intrabacterial total GFP (GFP^{total}) with a GFP-specific antiserum and a DyLight^{TM}649-conjugated secondary antibody. Confocal microscopic images were taken. Using ImageJ software the corrected total cell fluorescence signal intensity (CTCF) of maturated and fluorescent GFP (GFP^{mature}) and intrabacterial stained total GFP^{total} (DyLight^{TM}649) was determined for each single bacterium. CTCF of GFP^{mature} and GFP^{total} were correlated using the Spearman-rank correlation (ρ). **(F)** The combination of all conditions presents a good correlation. The detection limit determined by *S.* Tm^{wt} p^{control} is depicted as red line. **(G-H)** Examples of microscopic images (scale bar 25 µm). Adapted from (Spriewald *et al.*, 2015).

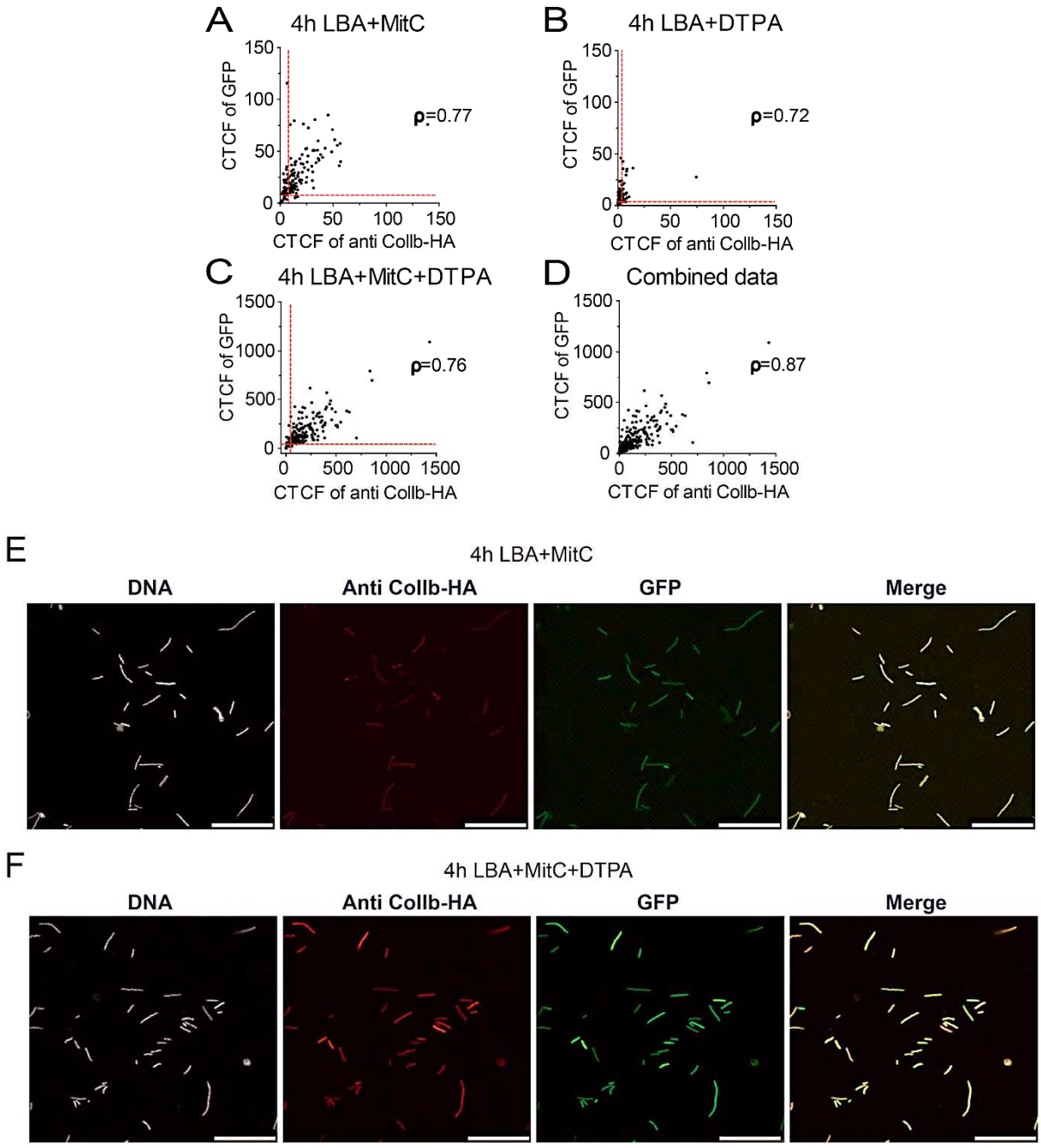

Figure 4.10: Correlation of GFP-levels obtained by the reporter p$^{Pcib\ gfp}$ with intrinsic CoIIb-HA levels under different conditions in individual bacteria. Confocal microscopic images of *S.* Tm p2$^{cib\text{-}HA}$ p$^{Pcib\ gfp}$ obtained in the experiment described in Figure 4.8 were used to calculate the corrected total cell fluorescence intensity (CTCF) of GFP- and CoIIb-HA (DyLight^{TM}549)-levels of each single cell. **(A-D)** CTCF values of GFP and CoIIb-HA (DyLight^{TM}549) of individual bacteria were analyzed by Spearman-rank correlation (ρ). The combined data of all conditions exhibit a high correlation coefficient (ρ=0.87). The detection limit determined by *S.* Tm^{wt} p^{control} is depicted as red line. **(E, F)** Examples of microscopic images (scale bar 25 µm) are shown. Adapted from (Spriewald *et al.,* 2015).

4.2 Interaction of prophages and colicin Ib gene expression at the single cell level in *S.* Tm

It has been previously shown, at the bulk level (Nedialkova *et al.*, 2014) and at the single cell level (Spriewald *et al.*, 2015), that *cib* is expressed by *S.* Tm^{SL1344} (*S.* Tm^{wt}) under SOS-induced or Fe^{2+}-limiting conditions and that maximal *cib* expression is seen by combined induction of the SOS-response and the Fe^{2+}-limitation. In general, group B colicin loci (e.g. ColIb locus) do not encode a lysis gene for the colicin release as it is the case for group A colicins. Therefore it is unknown how group B colicins, including ColIb, are released. Recently, it was shown at the bulk level (using western blot analysis and luciferase reporters) that prophage lysis genes of *S.* Tm^{wt} prophages contribute to the release of ColIb (Nedialkova *et al.*, 2015). In this part of the thesis these findings were analyzed at the single cell level to answer the question, if individual bacteria expressing *cib* also induce prophage lysis genes to release ColIb, and lyse to sacrifice themselves as common good for the whole bacterial population.

4.2.1 Investigation of the fate of individual bacteria expressing *cib*

It is known that *S.* Tm^{SL1344} harbors four different temperate phages (the lambdoid phages Gifsy-1, Gifsy-2 and ST64B and the P2-like phage SopEϕ) within its genome (Alonso *et al.*, 2005). Temperate phages can either follow the lytic life cycle as virulent phages or they permanently integrate their DNA into the host-chromosome as prophages, which can be then induced to enter the lytic life cycle. This prophage-induction is correlated with the induction of the SOS-response (Nanda *et al.*, 2014). Recently, our work has been shown that among the four prophages of *S.* Tm^{SL1344}, the P27-like phage ST64B mainly contributes to ColIb release upon SOS-response, as tested by bulk assays using western blot analyses and luciferase reporters (Nedialkova *et al.*, 2015).

To uncover the fate of individual *cib* expressing bacteria with and without functional prophages the multi-copy reporter $p^{P_{cib\ gfp}}$ was transformed into *S.* Tm^{WT} and in *S.* $Tm^{\Delta Ph}$ lacking all four prophages. As prophage-harboring *S.* Tm^{wt} bacteria should lyse in the course of SOS-response induction, it was hypothesized that less GFP^+ bacteria would be observed after inducing the culture with SOS-response inducer MitC compared to *S.* $Tm^{\Delta Ph}$.

To test this hypothesis strains were cultured under different environmental conditions and analyzed by flow cytometry. *S.* $Tm^{\Delta Ph}$ $p^{P_{cib\ gfp}}$ and *S.* Tm^{WT} $p^{P_{cib\ gfp}}$ were cultured in 96-well plates (**section 3.2.1.5**) for 4 h in LB medium or in LB medium supplemented with either 0.5 µg/ml MitC or 100 µM DTPA or both agents. Every hour, the OD_{600} was determined to follow the growth and lysis of the strains (**Figure 4.11A**). Samples (10^6 cfu) were taken for

flow cytometry analysis (**Figure 4.11BC** and **Figure 4.12**). As controls *S.* Tm^{WT} and *S.* $Tm^{\Delta Ph}$ strains without the multi-copy *gfp*-reporter were also cultured under the same conditions. As shown previously (Nedialkova *et al.*, 2015), all strains grew well and in a similar manner in LB medium and in LB medium supplemented with DTPA. With the addition of MitC to the growth medium the prophage-harboring strains *S.* Tm^{WT} and *S.* Tm^{WT} $p^{P_{cib\ gfp}}$ start to lyse about 2 h after the SOS-response was induced by MitC whereas the strains *S.* $Tm^{\Delta Ph}$ and *S.* $Tm^{\Delta Ph}$ $p^{P_{cib\ gfp}}$ lacking the prophages did not show any cell lysis (**Figure 4.11A**).

FACS measurements revealed a time dependent increase in *gfp* expression for both reporter strains, *S.* Tm^{WT} $p^{P_{cib\ gfp}}$ and *S.* $Tm^{\Delta Ph}$ $p^{P_{cib\ gfp}}$, under all *cib*-inducing conditions. Interestingly, after about 2 h the prophage deficient reporter strain *S.* $Tm^{\Delta Ph}$ $p^{P_{cib\ gfp}}$ progressively exhibited higher GFP-signal levels compared to *S.* Tm^{WT} $p^{P_{cib\ gfp}}$. This effect was more pronounced when cultures were treated with MitC (**Figure 4.11C** and **Figure 4.12**).

This confirmed the hypothesis that, preferentially, GFP^+ bacteria lyse in case of *S.* Tm^{WT} but not in case of *S.* $Tm^{\Delta Ph}$.

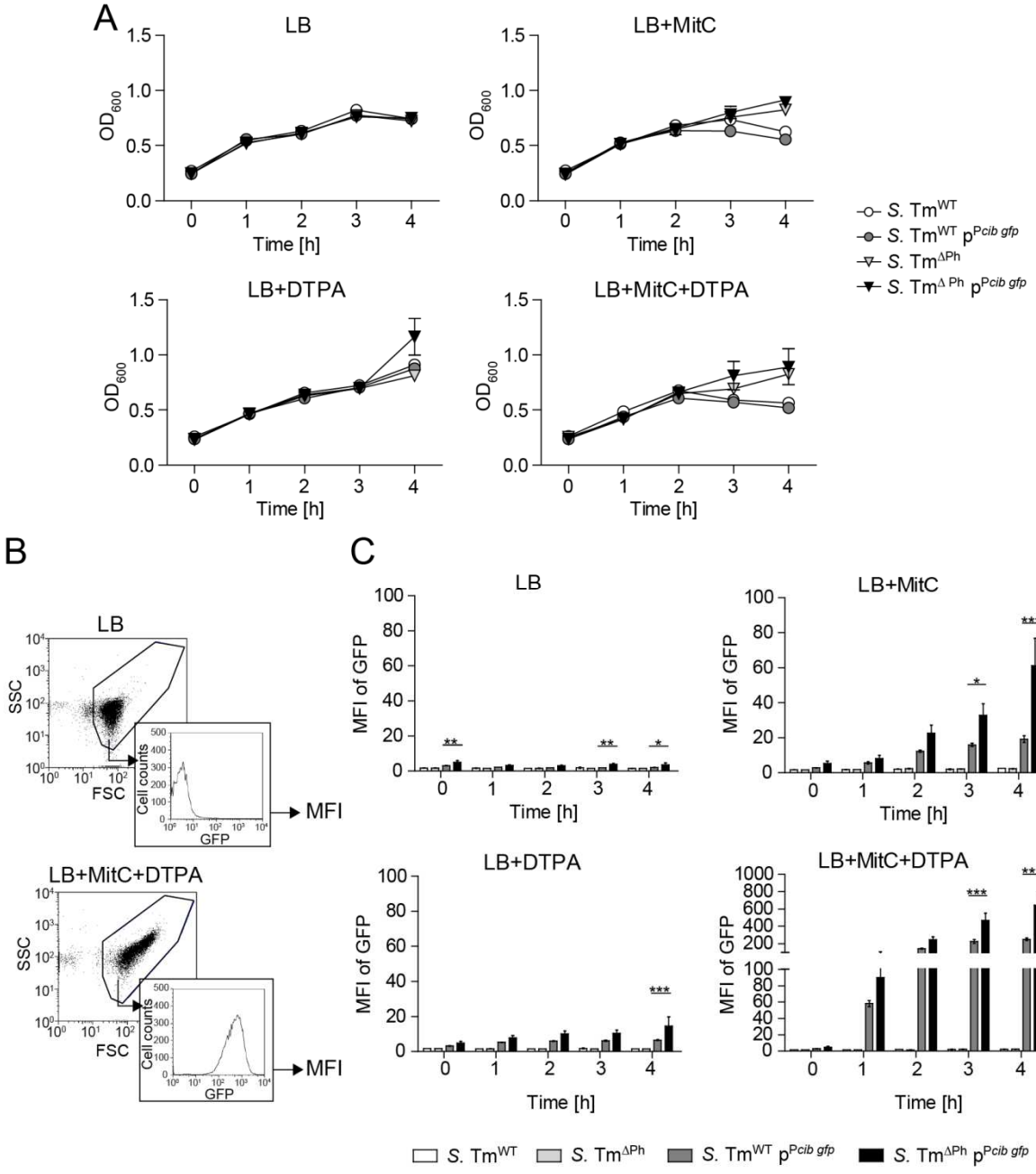

Figure 4.11: GFP- levels of *S.* Tm^{WT} p$^{Pcib\ gfp}$ compared to prophage-deficient *S.* Tm$^{\Delta Ph}$ p$^{Pcib\ gfp}$. *S.* Tm^{WT}, *S.* Tm$^{\Delta Ph}$, *S.* Tm^{WT} p$^{Pcib\ gfp}$ and *S.* Tm$^{\Delta Ph}$ p$^{Pcib\ gfp}$ were subcultured in a 96-well plate and grown for 4 h in LB or LB supplemented with 0.5 µg/ml MitC or 100 µM DTPA or both. Every hour OD_{600} was measured and samples (10^6 CFU) were taken for further flow cytometry measurements. **(A)** Growth curves of *S.* Tm^{WT} p$^{Pcib\ gfp}$ and *S.* Tm$^{\Delta Ph}$ p$^{Pcib\ gfp}$ and their respective negative control strains *S.* Tm^{WT} and *S.* Tm$^{\Delta Ph}$ without the *gfp*-reporter p$^{Pcib\ gfp}$ (to exclude growth defects due to p$^{Pcib\ gfp}$) each curve represents three independent experiments. Bars shown mean and StD. Statistical analysis using 2-way-ANOVA with Bonferroni post-test (***p<0.001) showed no significance in the growth in LB for all tested strains and in LB+DTPA (except *S.* Tm$^{\Delta Ph}$ p$^{Pcib\ gfp}$ at time point 4 h, due to one outlier). In the presence of MitC significance was observed between *S.* Tm^{WT}, *S.* Tm$^{\Delta Ph}$ at time point 4 h (***) and between *S.* Tm^{WT} p$^{Pcib\ gfp}$ and *S.* Tm$^{\Delta Ph}$ p$^{Pcib\ gfp}$ at time point 3 h (*) and 4 h (***). In the presence of MitC and DTPA significance was observed between *S.* Tm^{WT}, *S.* Tm$^{\Delta Ph}$ at timepoint 4 h (**) and between *S.* Tm^{WT} p$^{Pcib\ gfp}$ and *S.* Tm$^{\Delta Ph}$ p$^{Pcib\ gfp}$ at time point 3 h (*) and 4 h (***). **(B)** FACS plot examples of *S.* Tm$^{\Delta Ph}$ p$^{Pcib\ gfp}$ at time point 4 h. Using FSC-SSC plots bacteria were gated and the mean fluorescence intensity (MFI) of the gated bacteria were plotted in (C). **(C)** MFI of the *gfp*-reporter strains *S.* Tm^{WT} p$^{Pcib\ gfp}$ and *S.* Tm$^{\Delta Ph}$ p$^{Pcib\ gfp}$ and their control strains *S.* Tm^{WT} and *S.* Tm$^{\Delta Ph}$, respectively. Bars show means and StD. The experiment was performed in triplicates and analyzed by 2-way-ANOVA with Bonferroni post-test (***p<0.001).

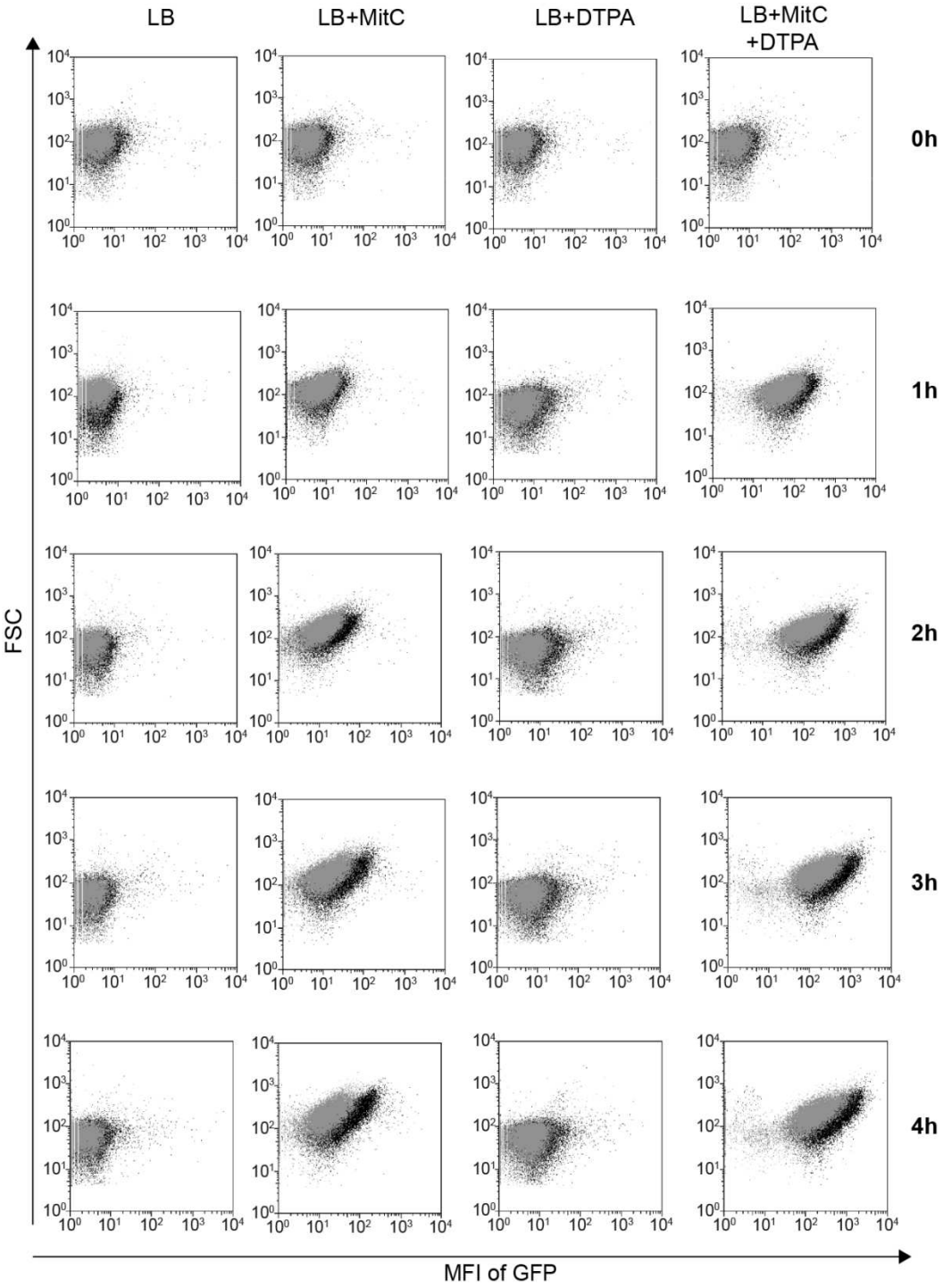

Figure 4.12: FACS plot examples of GFP-levels of *S.* Tm^{WT} p$^{P cib\ gfp}$ compared to *S.* Tm$^{\Delta Ph}$ p$^{P cib\ gfp}$. MFI of *S.* Tm^{WT} p$^{P cib\ gfp}$ (grey) compared to *S.* Tm$^{\Delta Ph}$ p$^{P cib\ gfp}$ (black) are plotted against the cell size (FSC) of one experiment of Figure 4.11. showing size-independent higher GFP levels of *S.* Tm$^{\Delta Ph}$ p$^{P cib\ gfp}$.

Using the CellASIC® ONIX Microfluidic Platform (Millipore) it is possible to follow *cib* expression in individual bacteria and their resulting cell fate over time during different environmental conditions. In contrast to methods were agar patches are used as growth medium, with this instrument bacteria are only able to grow in 2-dimensions, a flow of fresh liquid medium is applied, and the growth medium can be changed quickly, when necessary.

S. Tm^{WT} $p^{P_{cib\ gfp}}$ and *S.* $Tm^{\Delta Ph}$ $p^{P_{cib\ gfp}}$ were grown individually in B04 plates (CellASIC® ONIX Microfluidic Platform, Millipore) with different supplements for a time course of about 6 h (**Figure 4.13A**). Initially, single bacteria were trapped in the cell chamber of the microfluidic system and grown for 3 h in liquid LB medium, to let bacteria adapt to the conditions. The microcolonies were exposed to Fe^{2+}-limiting conditions by changing the growth medium to LB supplemented with 100 µM DTPA. Thereby, *S.* Tm would start producing high amounts of intracellular GFP while prophages remain inactive. About 20 min later, the medium was exchanged by liquid LB supplemented with 100 µM DTPA and 0.5 µg/ml MitC, leading to maximal induction of *cib* expression (*gfp* expression) and, concomitantly, the induction of prophages. As a consequence of prophage induction, lysis of individual bacteria is expected in *S.* Tm^{WT} $p^{P_{cib\ gfp}}$. In contrast, *S.* $Tm^{\Delta Ph}$ $p^{P_{cib\ gfp}}$ strain lacking all prophages would fail in prophage-dependent cell lysis under these conditions (Nedialkova *et al.*, 2015). 20 min later, the growth medium was changed back to LB supplemented with 100 µM DTPA and this condition was kept until the end of the experiment. The experimental protocol is depicted in **Figure 4.13A**.

As expected, following the cell fate of individual cells of *S.* Tm^{WT} $p^{P_{cib\ gfp}}$ lysis was observed ~2 h after induction with MitC (**Figure 4.13B; Movie S1**). In contrast, no bacterial cell lysis was observed for *S.* $Tm^{\Delta Ph}$ $p^{P_{cib\ gfp}}$ lacking all prophages (**Figure 4.13C; Movie S2**). Furthermore, live cell imaging revealed enhanced *gfp* expression in *S.* $Tm^{\Delta Ph}$ $p^{P_{cib\ gfp}}$ compared to *S.* Tm^{WT} $p^{P_{cib\ gfp}}$, and pronounced elongation in *S.* $Tm^{\Delta Ph}$ $p^{P_{cib\ gfp}}$ compared to *S.* Tm^{WT} $p^{P_{cib\ gfp}}$ (**Figure 4.13BC**) in response to MitC treatment.

Importantly, phage-dependent lysis was shown for the first time at the single cell level, confirming the results obtained at the population level, using western blot analysis and luciferase reporters (Nedialkova *et al.*, 2015).

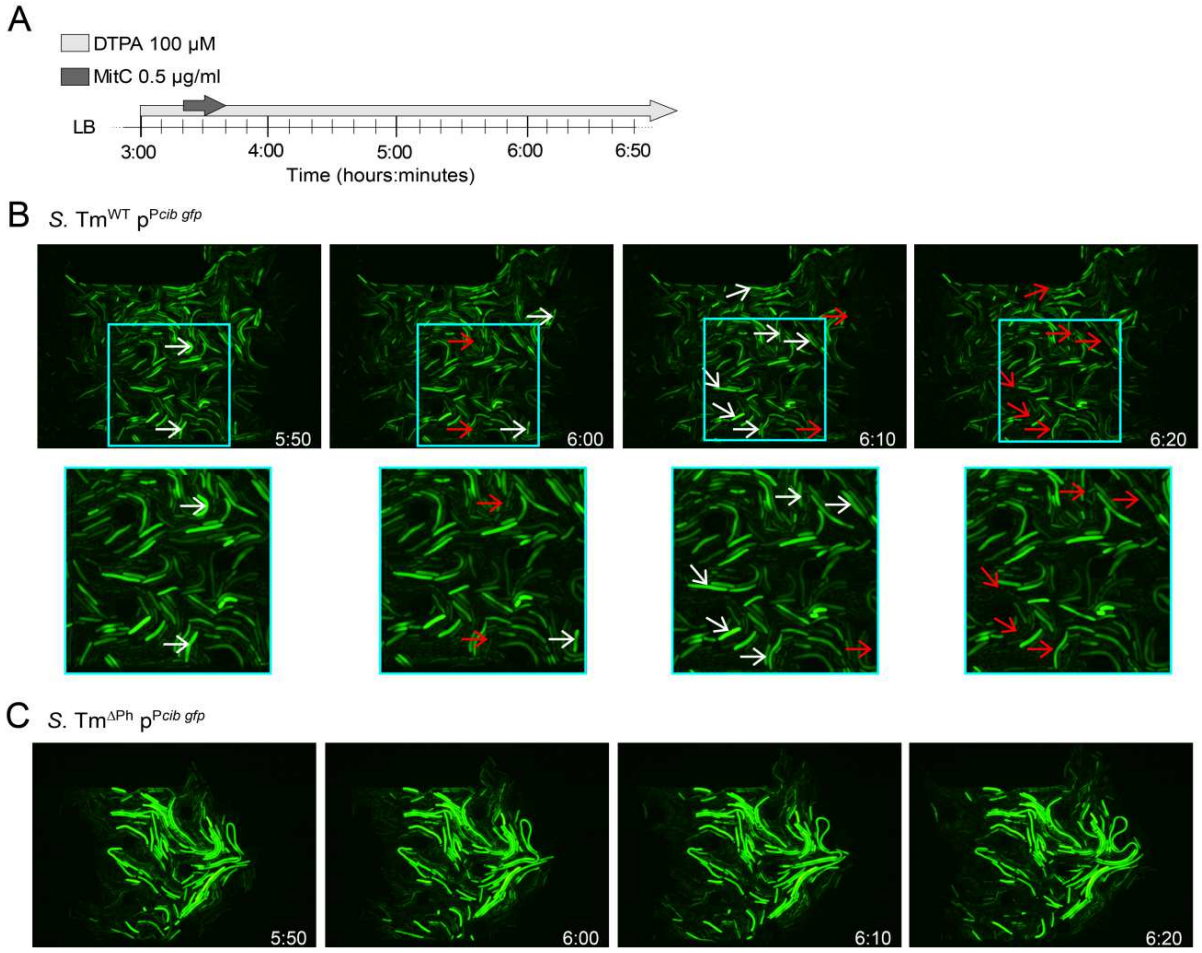

Figure 4.13: Live cell microscopy of *S.* Tm^{WT} p$^{Pcib\ gfp}$ and *S.* Tm$^{\Delta Ph}$ p$^{Pcib\ gfp}$. (A) Timeline depicting the experimental protocol. First, microcolonies were grown for 3 h in LB, in the Onix microfluidic system (Millipore). Afterwards, media was switched first to LB medium supplemented with 100 µM DTPA (Fe^{2+}-limiting conditions). After 20 min the SOS-response was induced by applying LB supplemented with 100 µM DTPA and 0.5 µg/ml MitC. Again after 20 min, media was switched back to LB supplemented with 100 µM DTPA only, for the remaining time. **(B)** Microscopic images (only GFP channel) of *S.* Tm^{WT} p$^{Pcib\ gfp}$ microcolony, 2½ h-3½ h after SOS-response induction with MitC. Arrows highlight lysing bacterial cells (white: about to lyse; red: lysed). **(C)** Examples of microscopic images (only GFP channel) of growth of the prophage deficient strain *S.* Tm$^{\Delta Ph}$ p$^{Pcib\ gfp}$, 2½ h-3½ h after treatment with MitC.

Live cell images also disclosed that bacterial cell length of *S.* Tm$^{\Delta Ph}$ p$^{Pcib\ gfp}$ become longer over time compared to *S.* Tm^{WT} p$^{Pcib\ gfp}$, as observed in the FACS dot plots of **Figure 4.12**. It is known that, among other effects, bacteria as *S.* Tm respond with stopping cell division to the SOS-response induction. This is due to the expression *sulA*, which is also regulated by the SOS-response. SulA binds in the following to the FtsZ monomer of the dividing bacterial cell. This leads to the inhibition of the FtsZ-polymerization. As a consequence the formation of the FtsZ-ring and the final cell division is inhibited (Kamensek *et al.*, 2010, Justice *et al.*, 2008). Therefore as only the pinching of mother and daughter cells is inhibited but not DNA replication or protein synthesis one can hypothesize that the longer the cell the more copy numbers of *gfp* and thus the higher the MFI of GFP signals. The hypothesis that differences in the GFP-signal intensity between both reporter strains rely only on the cell size was

excluded by gating bacteria of the same cell size and determining their GFP fluorescence intensities (data not shown).

4.2.2 Only a fraction of *cib* expressing individual bacteria also express prophage lysis genes

So far it could be shown at the single cell level that almost the entire *S.* Tm population expressed *cib* after SOS-response induction and/or Fe^{2+}-limitation condition and not, as expected, in a bimodal fashion (**section 4.1**) (Spriewald *et al.*, 2015). In addition, bacterial cell lysis could be observed upon induction of the SOS-response in individual, *cib*-expressing *S.* Tm^{wt} bacteria and, furthermore, that this bacterial cell lysis is dependent on the presence of functional prophages (**section 4.2.1**). It was now important to determine the fraction of all *cib* expressing bacteria that would also lyse to release colicin. This could reveal the costs and benefits of ColIb production and explain how *S.* Tm^{wt} gains a ColIb-dependent growth benefit over commensal *E. coli* in the inflamed gut (Nedialkova *et al.*, 2014, Nedialkova *et al.*, 2015). Therefore, prophage-dependent lysis genes are assumed to be only induced in a fraction of *cib* expressing bacteria to release ColIb.

To address this hypothesis, the reporter strain *S.* $Tm^{lysST::sfgfp}$ $p2^{cib\text{-}HA}$ was generated in order to determine the fraction of *cib* expressing individual bacteria which also express ST64B prophage lysis genes to release ColIb. This strain encodes a *sfgfp* gene instead of the ST64B prophage lysis genes of *S.* Tm^{wt} (*SL1344_1955-SL1344_1957; lysST*) for reporting lysis gene expression. Of note, in this reporter strain the ST64B lysis genes are inactive. Additionally, this strain carries *cib-HA* on the p2 plasmid to detect ColIb-HA by intrabacterial immunofluorescence staining and lysis gene (*sfgfp*) expression simultaneously. *S.* $Tm^{lysST::sfgfp}$ $p2^{cib\text{-}HA}$ was cultured in 96-well plates for 2 h and 4 h, respectively, in liquid LB medium or LB supplemented with 0.5 µg/ml MitC, or 100 µM DTPA or both inducers. Bacteria samples were fixed, lysozyme-permeabilized and stained for intrabacterial ColIb-HA using HA-specific antiserum and a DyLight™649 conjugated secondary antibody. Afterwards, images were taken by confocal microscopy and analyzed using ImageJ software. Mean fluorescence intensity above the detection limit, set by the background fluorescence of *S.* Tm^{wt} (no sfGFP, no ColIbHA) grown under the same conditions, was determined for sfGFP- and ColIb-HA (DyLight™649)-fluorescence signals of individual bacteria. In addition, the fraction (%) of *cib-HA* and *sfgfp* (lysis genes)-expressing individual bacteria were also determined (**Figure 4.14**).

The experiment revealed that ST64B lysis genes are expressed only upon induction of the SOS-response by MitC, whereas DTPA had no influence. In addition, lysis gene expression increases over time in individual bacteria. These results are in accordance with the ST64B phage lysis gene expression pattern obtained in bulk experiments (Nedialkova *et al.*, 2015).

By calculating the fraction (%) of *sfgfp* expressing bacteria, it was found that after 4 h only a fraction (~20-30%) of the whole population expressed the ST64B lysis genes (**Figure 4.14B**) whereas the entire population of *S.* Tm$^{lysST::sfgfp}$ p2$^{cib\text{-}HA}$ responded to MitC or/and DTPA with *cib-HA* expression(**Figure 4.14A**).

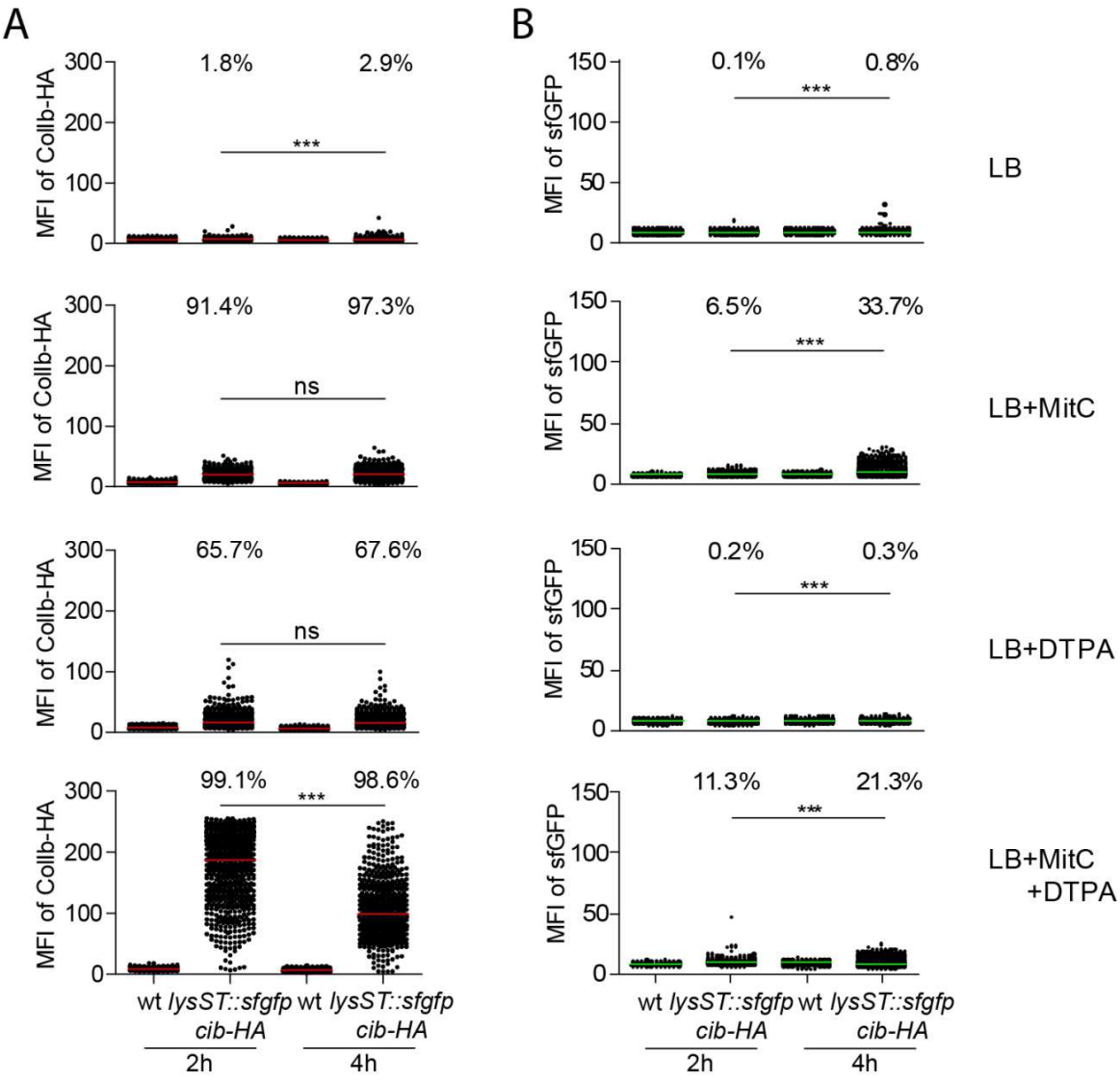

Figure 4.14: ST64B prophage lysis genes are only expressed in a fraction of *S.* Tm. *S.* Tm$^{lysST::sfgfp}$ p2$^{cib\text{-}HA}$ was cultured in 96-well plates for 2 h and 4 h in liquid LB medium or liquid LB medium supplemented with 0.5 µg/ml MitC or 100 µM DTPA or both inducer compounds. Bacteria were fixed, lysozyme-permeabilized and stained for total DNA with DAPI and for intrabacterial CoIIb-HA. *S.* Tm^{wt} (no sfGFP, no CoIIbHA), grown under the same conditions, was used as detection limit. By confocal microscopy images were taken and analyzed for the MFI of sfGFP- and CoIIb-HA (DyLight^{TM}649)-levels upon the detection limit. The fraction (%) of *cib-HA* and *sfgfp* (lysis genes) expressing individual bacteria was determine using *S.* Tm^{wt} bacteria as negative control. Each dot represents the measured MFI of an individual bacterium. Statistical analysis was done using Kruskal-Wallis test with Dunn's post test (***$p < 0.001$).

To investigate which fraction of the *cib-HA* expressing bacteria also express the ST64B prophage lysis genes the MFI values of sfGFP and CoIIb-HA (DyLight^{TM}649) of all conditions tested in the experiment represented in **Figure 4.14** were plotted as XY-graph (**Figure 4.15**).

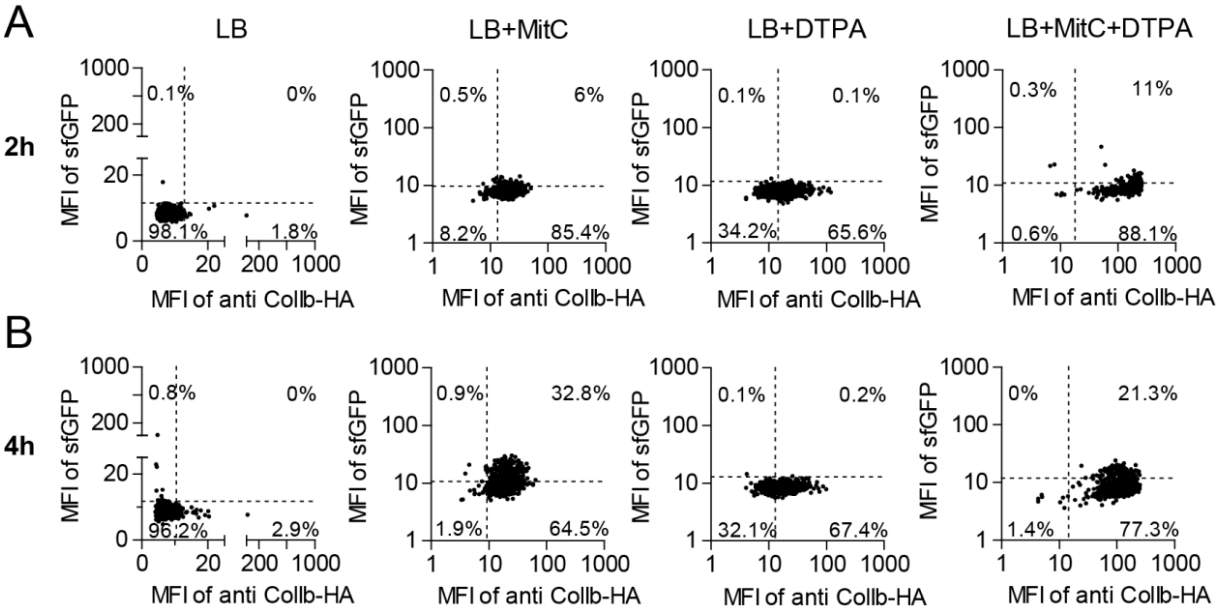

Figure 4.15: Correlation of *cib-HA* expression of individual *S.* Tm cells with *sfgfp* expression of the prophage lysis gene reporter. The MFI values of sfGFP and CoIIb-HA (DyLight^{TM}649) given by individual *S.* Tm$^{lysST::sfgfp}$ p2^{cib-HA} under different conditions, determined in the experiment of Figure 4.14AB were plotted as XY-graph. Each dot represents the MFI values of an individual object. **(A)** 2 h and **(B)** 4 h after treatment with or without supplements. The detection limit determined by no-fluorescent *S.* Tm^{wt} is depicted as dotted line.

These results showed that the majority of *sfgfp* (*lysST*) expressing bacteria also express *cib-HA* at the same time (**Figure 4.15**). However, only a fraction of *cib-HA* expressing *S.* Tm$^{lysST::sfgfp}$ p2^{cib-HA} also induces expression of prophage lysis genes (**Table 4.1**).

Table 4.1: Fraction of *cib* expressing *S.* Tm$^{lysST::sfgfp}$ p2^{cib-HA} co-expressing ST64B lysis genes.

Time	MitC	DTPA	CoIIb$^+$ [of total]	ST64B lysis$^+$ [of total]	CoIIb$^+$ and ST64B lysis$^+$ [of total]	ST64B lysis$^+$ [of CoIIb$^+$]
2 h	-	-	1.8%	0.1%	**0%**	**0%**
4 h	-	-	2.9%	0.8%	**0%**	**0%**
2 h	**+**	-	91.4%	6.5%	**6%**	**5.5%**
4 h	**+**	-	97.3%	33.7%	**32.8%**	**31.9%**
2 h	-	**+**	65.7%	0.2%	**0.1%**	**0.1%**
4 h	-	**+**	67.6%	0.3%	**0.2%**	**0.1%**
2 h	**+**	**+**	99.1%	11.3%	**11%**	**10.9%**
4 h	**+**	**+**	98.6%	21.3%	**21.3%**	**21%**

Due to the rather weak sfGFP signal intensity of *S.* Tm$^{lysST::sfgfp}$, a T7-polymerase based reporter strain (*S.* Tm$^{lysST::T7\ pol}$ p$^{PT7\ sfgfp}$) was constructed. Therefore, the ST64B prophage lysis genes (*SL1344_1955-SL1344_1957*) of *S.* Tm^{wt} were exchanged against the gene encoding a T7-polymerase, using the lambda Red recombination. This strain was further transformed with a low copy plasmid encoding *sfgfp* under the control of a T7 promoter (p$^{PT7\ sfgfp}$). Thus, instead of *lysST* the T7-polymerase is expressed under inducing conditions.

This in turn leads to amplification of *sfgfp* expression from $p^{P\,T7\,sfgfp}$, which results in enhanced sfGFP signal intensity.

To analyze the expression pattern of *lysST* compared to *cib* in *S.* Tm, the two reporter strains *S.* $Tm^{lysST::T7\,pol}$ $p^{P\,T7\,sfgfp}$ and *S.* Tm^{wt} $p^{P\,cib\,gfp}$, were cultured in 96-well plates for 4 h in liquid LB medium or LB supplemented with 0.5 µg/ml MitC, or 100 µM DTPA or both. Flow cytometry analysis revealed that SOS-inducing and thus *lysST*-inducing conditions result in the emergence of two phenotypically different subpopulations in case of *S.* $Tm^{lysST::T7\,pol}$ $p^{P\,T7\,sfgfp}$. A $sfGFP^+$ population and a $sfGFP^-$ subpopulation. In contrast, under the same conditions *cib* is expressed in the entire population (**Figure 4.16**) as shown previously (Spriewald *et al.*, 2015). In fact, this shows that in contrast to the overall unimodal expression of *cib*, upon SOS-inducing conditions *lysST* is expressed bimodally.

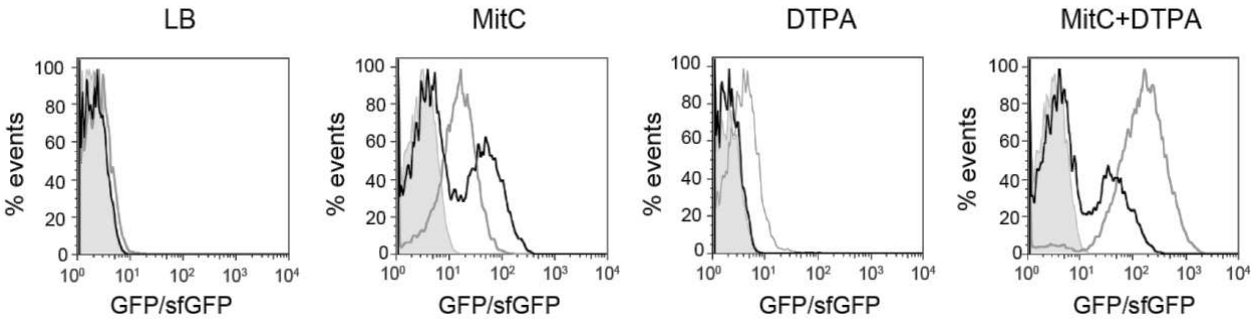

Figure 4.16: In contrast to the unimodal expression of *cib*, *lysST*-expression is bimodal upon induction of the SOS-response. *S.* Tm^{wt} $p^{P\,cib\,gfp}$ (grey) and *S.* $Tm^{lysST::T7\,pol}$ $p^{P\,T7\,sfgfp}$ (black) were cultured in 96-well plates for 4 h in LB medium or LB medium supplemented with 0.5 µg/ml MitC or 100 µM DTPA or both. GFP/sfGFP fluorescence signal intensities were analyzed by flow cytometry. *S.* Tm^{wt} (grey; filled histogram) grown for 4 h under the same conditions was used as GFP negative control.

In conclusion, although ColIb is produced in the entire *S.* Tm population by induction of the SOS-response and Fe^{2+}-limitation, only a fraction of $ColIb^+$ bacteria also induce ST64B prophage lysis genes and lyse to release ColIb. Therefore, bimodal gene expression of *S.* Tm prophage lysis genes secures survival of the overall population and is an explanation of how *S.* Tm gains a ColIb-dependent growth benefit over commensal *E. coli* in the inflamed gut (Nedialkova *et al.*, 2015).

4.3 Adaptation of *E. coli* strains to auto-cytotoxic effects of colicin M production

Upon screening for colicin producing *E. coli* strains, we identified a strain, *E. coli* 252R (Ec^{252R}), which produced a bacteriocin against which it was not protected. This was surprising, as, in general, bacteriocin producers protect themselves against "self-killing" by the expression of cognate immunity factors. Using a transposon mutagenesis screen the bacteriocin colicin M was identified as the cause for this phenotype. The gene coding for colicin M (*cma*) is encoded together with the colicin B gene (*cba*) on the same locus on a large conjugative plasmid and is co-regulated from the same SOS-box upstream of *cba*. Usually bacteria are protected against colicin M (ColM)- or colicin B (ColB)-mediated self-killing by the production of the respective immunity proteins. The immunity genes of ColB and ColM (*cbi* and *cmi*, respectively) are also encoded on the same locus, but transcribed in the reverse direction. In this thesis I have shown, that ColM immunity protein of Ec^{252R} is produced and functional. However, production of ColM immunity protein generally only partially protects against self-killing by ColM. Further analysis of different *E. coli* strains, originally classified as ColM producers revealed two other *E. coli* strains (Ec^{Cl139}, Ec^{B195}) which also showed a self-killing phenotype. Interestingly, I could also show that only strains which encode an intact *cba* gene are also able to express *cma*. This suggests that ColM producers might face an immense fitness cost due to high "self-toxicity" of ColM initially upon acquisition of a ColBM plasmid by conjugation. Thus, mutations which lead to resistance against ColM (e.g. by disrupting ColM production) are positively selected. Based on these data, it was hypothesized that Ec^{252R} might represent an isolate which just had acquired a functional pColBM plasmid and has not yet "evolved" towards either ColM tolerance or downregulation of ColM production. This hypothesis was investigated how bacteria cope with the cytotoxic effect of ColM, by *in vitro* evolution experiments.

4.3.1 Under stress conditions *E. coli* 252R (Ec^{252R}) produces a colicin against which it susceptible

E. coli 252R (Ec^{252R}) is a mouse isolate which was identified to kill itself upon exposure to MitC. This phenotype was discovered using a halo-assay (**section 3.2.1)** where Ec^{252R} was grown as a colony in the presence of the DNA-damaging antibiotic MitC (0.25 µg/ml). Overlayed with itself, Ec^{252R} exhibited a lysis zone around the colony. Additionally, the compound released by Ec^{252R} also killed other *E. coli* strains such as the commensal *E. coli* Ec^{MG1655} (**Figure 4.17**). Interestingly, the self-killing phenomenon was only observed in the presence of MitC (**Figure 4.17**).

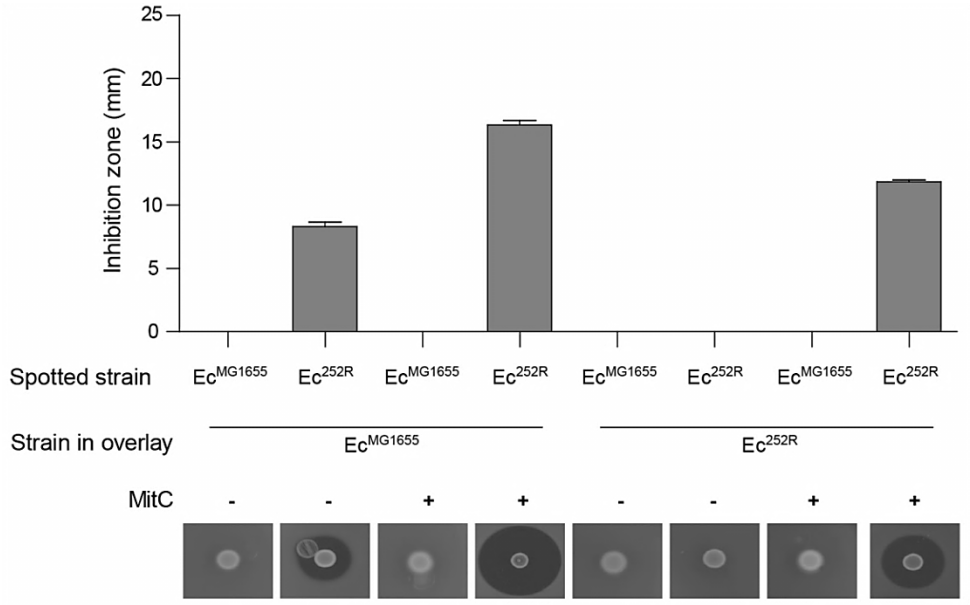

Figure 4.17: Halo-assay to determine bacteriocin release. Production and release of bacteriocin of Ec^{252R} were tested in comparison to commensal Ec^{MG1655}. Additionally, each strain was tested for a suicidal behavior. Initially, Ec^{252R} and Ec^{MG1655} were spotted and incubated o.n. on LB agar plates supplemented with 0.25 µg/ml MitC, to induce stress-dependent toxin secretion, and on plain LB agar plates. Both strains were cultivated o.n. in liquid LB and the next day mixed with LB softagar and used to overlay the agar plates. After 24 h, the lysis-zone (halo) was measured (size of the overall diameter substracted by the colony size). The experiment was done in triplicates. Bars represent means and StD.

To identify the bacteriocin in Ec^{252R} leading to the self-killing phenotype, a transposon mutagenesis screen (**section 3.2.2.16**) was conducted. Ec^{252R} was transformed with the plasmid pJA1 carrying Tn*10* and a transposase under the control of the *tac* promoter (Badarinarayana *et al.*, 2001). A library of 1536 mutants was generated in 96-well plates. Transconjugants were screened for the loss of the self-killing phenotype (similar to **section 3.3.1.1**). The mutant Ec^{P10A10} and Ec^{P13C5} (**Table 3.1**) were found to be non-producers but still sensitive to $Ec^{252R\ Tn10}$. Out of these 1536 mutants only two mutants (Ec^{P10A10}, Ec^{P13C5}) exhibited reduced self-killing (**Figure 4.18A**). Further analysis revealed that both mutants exhibit a reduction in toxicity against Ec^{MG1655} but only Ec^{P10A10} lost the self-killing phenotype. Mutant Ec^{P13C5}, however, showed only a reduction in the self-killing properties compared to the parental Ec^{252R} strain (**Figure 4.18B**).

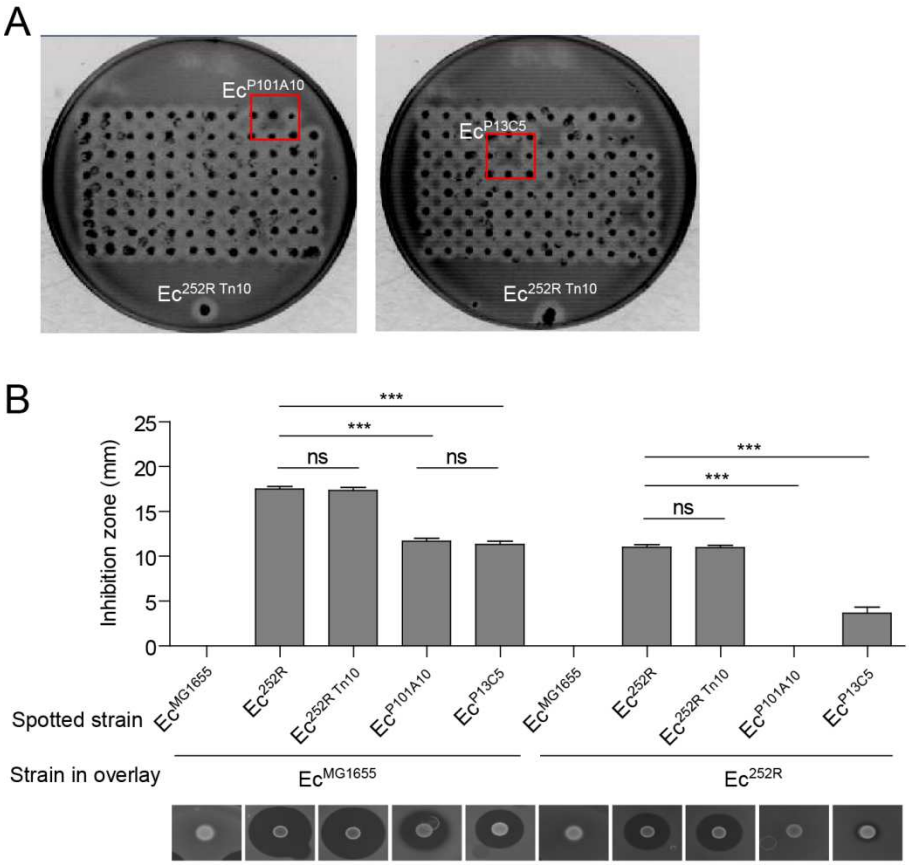

Figure 4.18: Characterization of the Ec^{252R} transposon mutant strains. (A) To screen the Ec^{252R} transposon mutant library (>1,500) for loss of self-killing-phenotype, strains were grown o.n. in LB medium in 96-well plates as described in section 3.2.2.17 and transferred using a replicator on LB agar plates supplemented with 0.016 µg/ml MitC and 100 µg/ml kanamycin. As control strain Ec$^{252R\ Tn10}$ was cultivated and also spotted on each agar plate. The next day, an o.n culture of Ec$^{252R\ Tn10}$ (carrying Tn*10* in an unknown location) was mixed with LB softagar and agar plates were overlayed with this mix. After o.n. incubation two mutants (Ec^{P10A10}, Ec^{P13C5}) which showed robust growth but did not produce a halo against Ec$^{252R\ Tn10}$ were identified. **(B)** Both bacterial mutant strains were tested with a halo-assay using LB agar plates containing 0.25 µg/ml MitC. 24 h after the overlay with the test strain Ec^{MG1655} or Ec^{252R}, the halo size of each strain was determined. The experiment was performed in triplicates. Bars represent means and StD. Statistical analysis was done using 1-way ANOVA with Tukey's post test (***p<0.001).

To identify the insertion site of Tn*10* in Ec^{P10A10} and Ec^{P13C5}, single primer PCR as described by Karlyshev *et al.* (Karlyshev *et al.*, 2000) was employed (**section 3.2.2.18**). Single primer PCR products for each mutant were obtained and sent for sequencing. NCBI BLAST analysis revealed that Tn*10* was inserted at the beginning of a colicin M gene (*cma*) in Ec^{P10A10} (**Figure 4.19**).

Usually *cma* is located together with its immunity gene (*cmi*), the colicin B gene (*cba*) and the colicin B immunity gene (*cbi*) at the same locus on a large conjugative plasmid (further named pColBM) (Christenson and Gordon, 2009). To confirm that Ec^{252R} encodes *cma* and harbors a pColBM plasmid, the oligonucleotides cma-check-up/cma-check-down and cba-check-up/cba-check-down (**Table 3.3**) were designed (binding 60 bp upstream and

downstream of the respective genes) based on nucleotide sequences published for pColBM plasmids (Accession no. NG_039430). PCR results, followed by sequencing of the amplicons for *cma* and *cba*, verified that Ec^{252R} harbors a plasmid encoding the entire ColBM locus (**Figure 4.19**). In parallel, the entire genome of Ec^{252R} was shotgun sequenced by the Wellcome Trust Sanger Institute (Cambridge, United Kingdom) by Illumina MiSeq sequencing, supporting these findings.

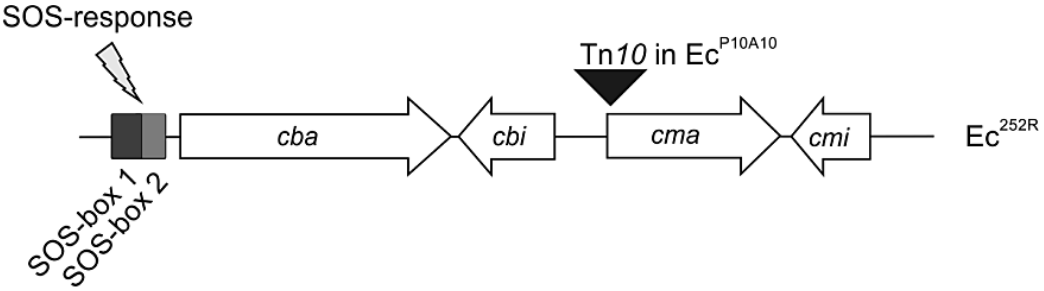

Figure 4.19: Scheme of the colicin BM locus of Ec^{252R} and location of the promoter. (**A**) Colicin B (*cba*) and colicin M activity gene (*cma*) are both regulated from the same promoter by two overlapping SOS-boxes upstream of *cba*, whereas, the respective immunity genes for ColM (*cmi*) and ColB (*cbi*) are transcribed in the opposite direction. The black triangle depicts the insertion site of Tn*10* in Ec^{P10A10}.

For the Ec^{252R} mutant, Ec^{P13C5} NCBI BLAST analysis showed that the transposon Tn*10* was inserted in a putative prophage region (putative gene *virE*), which was excluded to be a prophage lysis gene. Notably, it was previously shown (also in **section 4.2)** that the group B colicin ColIb is released via prophage lysis (Nedialkova *et al.*, 2015). Due to the reduced killing properties of the Ec^{P13C5} mutant against itself and against sensitive Ec^{MG1655}, and the fact that the insertion of Tn*10* in Ec^{P13C5} is located in a prophage region of Ec^{252R}, prophages may also play a role for ColM (at least) and/or ColB release.

To investigate more precisely if ColB or ColM are responsible for the self-killing phenotype of Ec^{252R}, targeted deletions for *cba* (Ec^{252R} $pColBM^{cba::cat}$) and *cma* (Ec^{252R} $pColBM^{cma::cat}$) were generated by gene replacement with a chloramphenicol resistance cassette (*cat*), respectively. To exclude any polar effect on gene expression compromised by the chloramphenicol resistance cassette (*cat*) in frame deletions of *cba* and *cma* (Ec^{252R} $pColBM^{\Delta cba}$ and Ec^{252R} $pColBM^{\Delta cma}$) were also generated. All strains were tested in a halo-assay compared to the parental strain Ec^{252R} for colicin production (overlayed by Ec^{MG1655}) and a self-killing phenotype (overlayed by themselves) both under normal conditions (LB; **Figure 4.20A**) and under SOS-response induced conditions (LB+0.25 µg/ml MitC; **Figure 4.20B**). FhuA is the outer membrane receptor for ColM and a *fhuA* mutant strain is resistant to ColM-dependent killing but still sensitive to ColB-dependent killing. To address ColB-dependent killing only, mutant strains were also overlayed with

$Ec^{MG1555\ \Delta fhuA}$. In addition, ColM production was detected by western blot analysis (**Figure 4.20C**) using an affinity-purified ColM specific antiserum (anti-ColM (rabbit polyclonal)) (**section 3.2.4.4**). To this end, Ec^{252R}, its mutant derivatives and a different ColBM producer strain Ec^{Cl139} were grown o.n. and used to inoculate subcultures (1:20) of liquid LB or LB supplemented with 0.25 µg/ml MitC. After 4 h, incubation ColM was detected by the anti-ColM (rabbit polyclonal) antiserum in the cell lysates and culture supernatants.

Mutant characterization in the halo-assay revealed, that under both conditions (LB and LB+MitC) the mutant strains Ec^{252R} pColBM$^{cba::cat}$ and Ec^{252R} pColBM$^{cma::cat}$ are reduced in total in colicin production compared to the wildtype strain Ec^{252R}. This was expected, as both mutant strains only produce one of the two colicins present in Ec^{252R}. Interestingly, the mutant Ec^{252R} pColBM$^{cba::cat}$ killed Ec^{MG1655} more efficiently compared to mutant Ec^{252R} pColBM$^{cma::cat}$ in the overlay. As the in frame deletion strains Ec^{252R} pColBM$^{\Delta cba}$ and Ec^{252R} pColBM$^{\Delta cma}$ showed no significant differences in the halo-assay compared to the strains carrying the antibiotic resistance marker (Ec^{252R} pColBM$^{cba::cat}$ and Ec^{252R} pColBM$^{cma::cat}$), polar effects of the *cat* gene in Ec^{252R} pColBM$^{cba::cat}$ and Ec^{252R} pColBM$^{cma::cat}$ could be excluded. By $Ec^{MG1555\ \Delta fhuA}$ in the overlay it was possible to show that killing by ColM was abolished once the receptor for ColM (FhuA) was deleted. Moreover, equal ColB-dependent killing was observed under SOS-induced condition of the ColB-producing mutant strains Ec^{252R} pColBM$^{cma::cat}$ and Ec^{252R} pColBM$^{\Delta cma}$. This assay could confirm the deletion of *cba* in Ec^{252R} pColBM$^{\Delta cba}$ and Ec^{252R} pColBM$^{cba::cat}$ as no killing of sensitive Ec^{MG1655} or $Ec^{MG1655\ \Delta fhuA}$ was detected. The deletion of *cma* Ec^{252R} pColBM$^{cma::cat}$ and Ec^{252R} pColBM$^{\Delta cma}$ could be confirmed by western blot analysis (**Figure 4.20C**).

Interestingly, the halo-assays revealed that under SOS-induced conditions the self-killing phenotype was lost in the mutant strains Ec^{252R} pColBM$^{cma::cat}$ and Ec^{252R} pColBM$^{\Delta cma}$, whereas in Ec^{252R} pColBM$^{cba::cat}$ and its corresponding strain Ec^{252R} pColBM$^{\Delta cba}$ the self-killing phenotype was only reduced compared to the wildtype Ec^{252R}. Therefore, ColM is the responsible toxin leading to the self-killing phenotype of Ec^{252R} and this ColM-mediated phenotype is pronounced in the presence of ColB.

Interestingly, ColM was not detected in supernatant samples. Probably, the concentration of produced ColM is too low in the supernatant and is under the detection limit for western blot analysis.

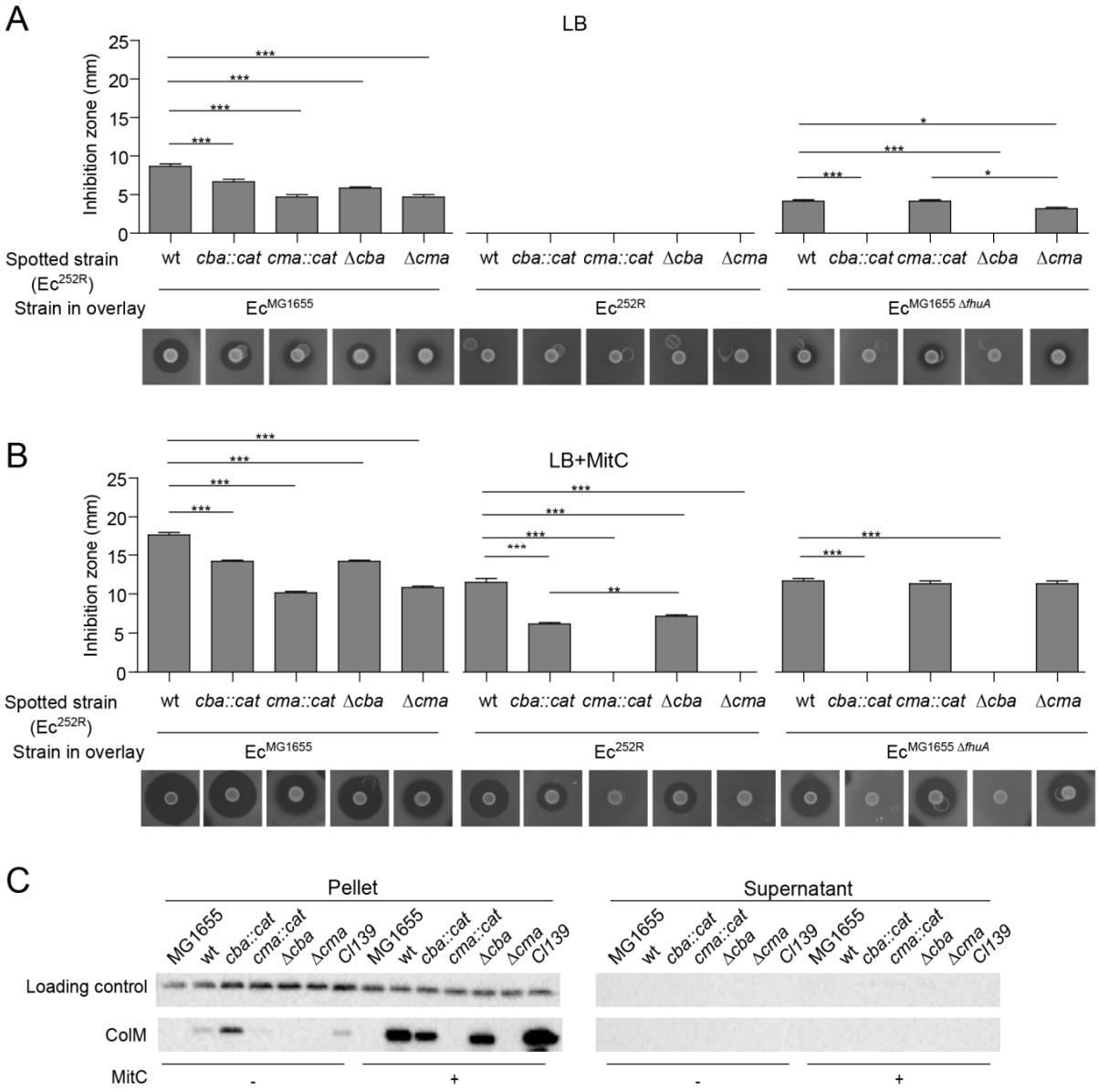

Figure 4.20: Characterization of *cma* and *cba* mutant strains in an halo-assay. *E. coli* strains Ec^{252R} wildtype (wt) and its mutant derivatives Ec^{252R} pColBM$^{cba::cat}$, Ec^{252R} pColBM$^{cma::cat}$, Ec^{252R} pColBM$^{\Delta cba}$ and Ec^{252R} pColBM$^{\Delta cma}$. The bacterial strains were grown o.n. in media and spotted the following day on **(A)** LB agar plates and **(B)** LB agar plates supplemented with 0.25 µg/ml MitC to induce *cba* and *cma* expression. The next day the agar plates were overlayed with LB softagar mixed with o.n. culture of Ec^{MG1655} (to analyze ColM- and ColB-dependent killing), or Ec^{252R} wildtype (to analyze self-killing), or the ColM receptor-deficient strain $Ec^{MG1655\ \Delta fhuA}$ (to analyze ColM-dependent killing), respectively. After 24h halo size was measured. The experiment was done in triplicates and statistical analysis was done using 1-way ANOVA with Tukey's post test (***$p<0.001$), if not indicated differences were not statistically significant. Bars represent means and StD. **(C)** Western blot analysis for ColM production. Ec^{252R} (wt) and its mutant derivative strains Ec^{252R} pColBM$^{cba::cat}$, Ec^{252R} pColBM$^{cma::cat}$, Ec^{252R} pColBM$^{\Delta cba}$ and Ec^{252R} pColBM$^{\Delta cma}$ as well as Ec^{MG1655} (served as negative control= nctrl) and Ec^{Cl139} (Cl139) used as a second non-related ColM-producer, were grown 4 h in subcultures (1:20 diluted from 12 h cultures) in LB or LB supplemented with 0.25 µg/ml MitC, as indicated. Samples from bacterial lysate (pellet) and culture supernatant were taken and ColM was detected using an affinity-purified anti-ColM (rabbit polyclonal) antiserum. An unknown protein recognized by the anti-ColM (rabbit polyclonal) antiserum was used as loading control.

4.3.2 The self-killing phenotype is not explained by the presence of a non-functional immunity protein

It is generally accepted that each colicin-producing strain produces a cognate colicin immunity protein which protects against self-intoxication (Cascales *et al.*, 2007). Immunity genes are usually encoded on the same locus downstream of the colicin activity gene and transcribed in the same direction (enzymatic colicins) or in the opposite direction (pore-forming colicins). For Colicin K and for other pore forming colicins the immunity protein is expressed constitutively (Cascales *et al.*, 2007). Since ColM is responsible for the self-killing phenotype, we assumed that Ec^{252R} might not express *cmi* or that the ColM immunity protein (CMI) is not functional. In order to investigate this, the mutant strains Ec^{252R} pColBM^{cmaHA} and Ec^{252R} pColBM^{cmiHA}, expressing either an HA-tagged *cma* or *cmi*, respectively, were generated to detect the ColM-HA and CMI-HA proteins using a HA-specific antiserum (**Table 3.17**). Initially, these mutants were tested for an altered self-killing phenotype and differences in ColM production using halo-assays (**Figure 4.21A**). Subsequently, 12 h cultures of wildtype Ec^{252R} and the mutant strains were used to inoculate subcultures (1:20) in liquid LB or LB supplemented with 0.25 µg/ml MitC. As it was unknown under which conditions *cmiHA* would be expressed, additional subcultures in LB supplemented with 100 µM DTPA or LB supplemented with 0.25 µg/ml MitC and 100 µM DTPA to test additionally for Fe^{2+}-limitation conditions. Samples from bacterial lysate (OD_{600} of 1), as well as culture supernatant, were harvested and ColM-HA and CMI-HA were quantified by western blot using HA-specific antiserum (**Table 3.17**) (**Figure 4.21B**). In addition, ColM was also detected using an affinity-purified anti-ColM (rabbit polyclonal) antiserum (**Figure 4.21C**).

As shown in the halo-assays in **Figure 4.21A,** the HA-tag of CMI-HA in Ec^{252R} pColBM^{cmiHA} does not influence the killing of sensitive Ec^{MG1655} or the self-killing phenotype compared to the wildtype Ec^{252R}. However, the HA-tag of ColM-HA in Ec^{252R} pColBM^{cmaHA} seems to interfere with the toxicity or the expression level of ColM, as this mutant is reduced in colicin killing of sensitive Ec^{MG1655} and in its self-killing properties.

Western blot analysis depicted in **Figure 4.21B** showed that ColM-HA is only produced in the presence of MitC. Iron limitation does not affect *cmaHA* expression. Levels of ColM-HA were reduced when detected with affinity-purified anti-ColM (rabbit polyclonal) antiserum in Ec^{252R} pColBM^{cmaHA} compared to Ec^{252R} or Ec^{252R} pColBM^{cmiHA} (**Figure 4.21C**). In contrast to the *cmaHA* expression pattern, *cmiHA* was expressed at basal levels if grown in LB. Moreover in SOS-inducing conditions or under Fe^{2+}-limitation, *cmiHA*-expression is increased compared to plain LB medium.

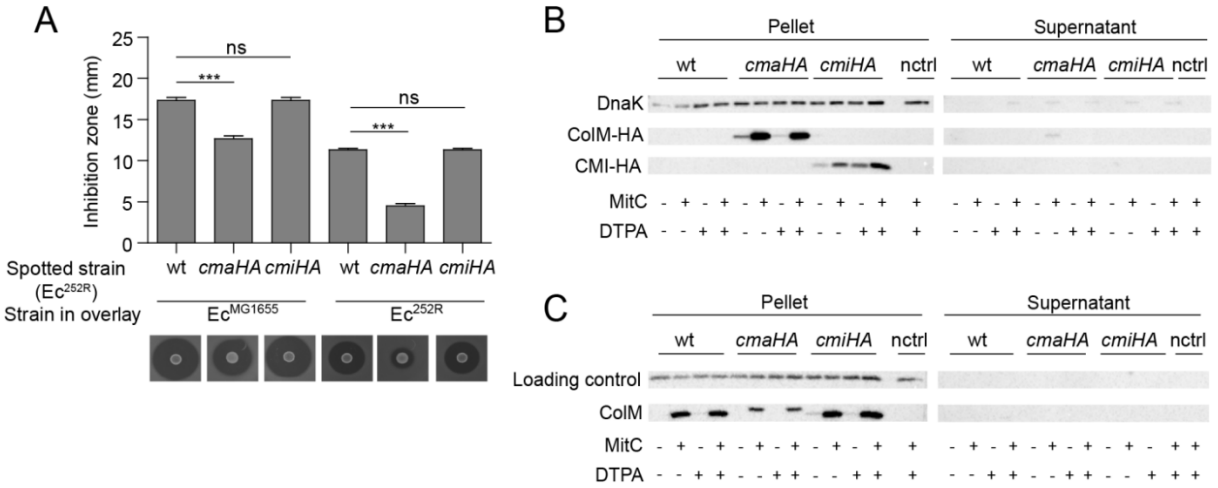

Figure 4.21: Analysis of *cmaHA* and *cmiHA* gene expression in Ec^{252R}. (A) Standard halo-assay performed to analyze the interference of ColMB dependent killing of sensitive Ec^{MG1655} and Ec^{252R} self-killing with the HA-tag of *cma* and *cmi*. Wildtype (wt) Ec^{252R}, Ec^{252R} pColBM^{cmaHA} and Ec^{252R} pColBM^{cmiHA} were spotted on LB agar plates, containing 0.25 µg/ml MitC to induce colicin gene expression. The next day agar plates were overlayed with either Ec^{252R} or Ec^{MG1655} .24h later halo size was measured. The experiment was done in triplicates and statistical analysis was done using 1-way ANOVA with Tukey's post test (***p<0.001). Bars represent means and StD. **(B)** Western blot analysis of ColM-HA and CMI-HA production in Ec^{252R}, Ec^{252R} pColBM^{cmaHA} and Ec^{252R} pColBM^{cmiHA} under different environmental conditions. Samples of Ec^{MG1655}, grown under the same conditions, were used as negative control (nctrl). Bacterial strains were grown in LB media or LB supplemented with 0.25 µg/ml MitC (SOS-response induction), or 100 µM DTPA (Fe^{2+}-limitation), or supplemented with both. As loading control DnaK was detected. **(C)** Western blot analysis of ColM and ColM-HA under different environmental conditions. Samples of (B) were also detected by affinity-purified anti-ColM antiserum. As loading control an unknown protein detected by the ColM-specific antiserum is shown.

These results showed that CMI of Ec^{252R} is expressed. However, we could not rule out that the protein is non-functional. Thus, a comparative sequence analysis of Ec^{252R} *cmi* with *cmi* sequence of Ec^{Cl139} and three other ColM resistant pColBM harboring *E. coli* strains (Ec^{B203}, Ec^{B1011}, Ec^{B1144}; **Table 3.1**) was performed and also compared with published *cmi* sequences of two other pColBM harboring *E. coli* strains from the database (*E. coli* R170 [Acc.no. FJ664740.1] and *E. coli* H354 [Acc.no. FJ664743.1]). The analysis revealed a single point mutation (G233T) in the *cmi* sequence of Ec^{252R} leading to a D26Y conversion in the protein sequence compared to the other CMI sequences (**Figure 4.22A)**. This mutation is located in the part connecting the membrane anchor and head (hinge region) of ColM immunity protein (**Figure 4.22B**).

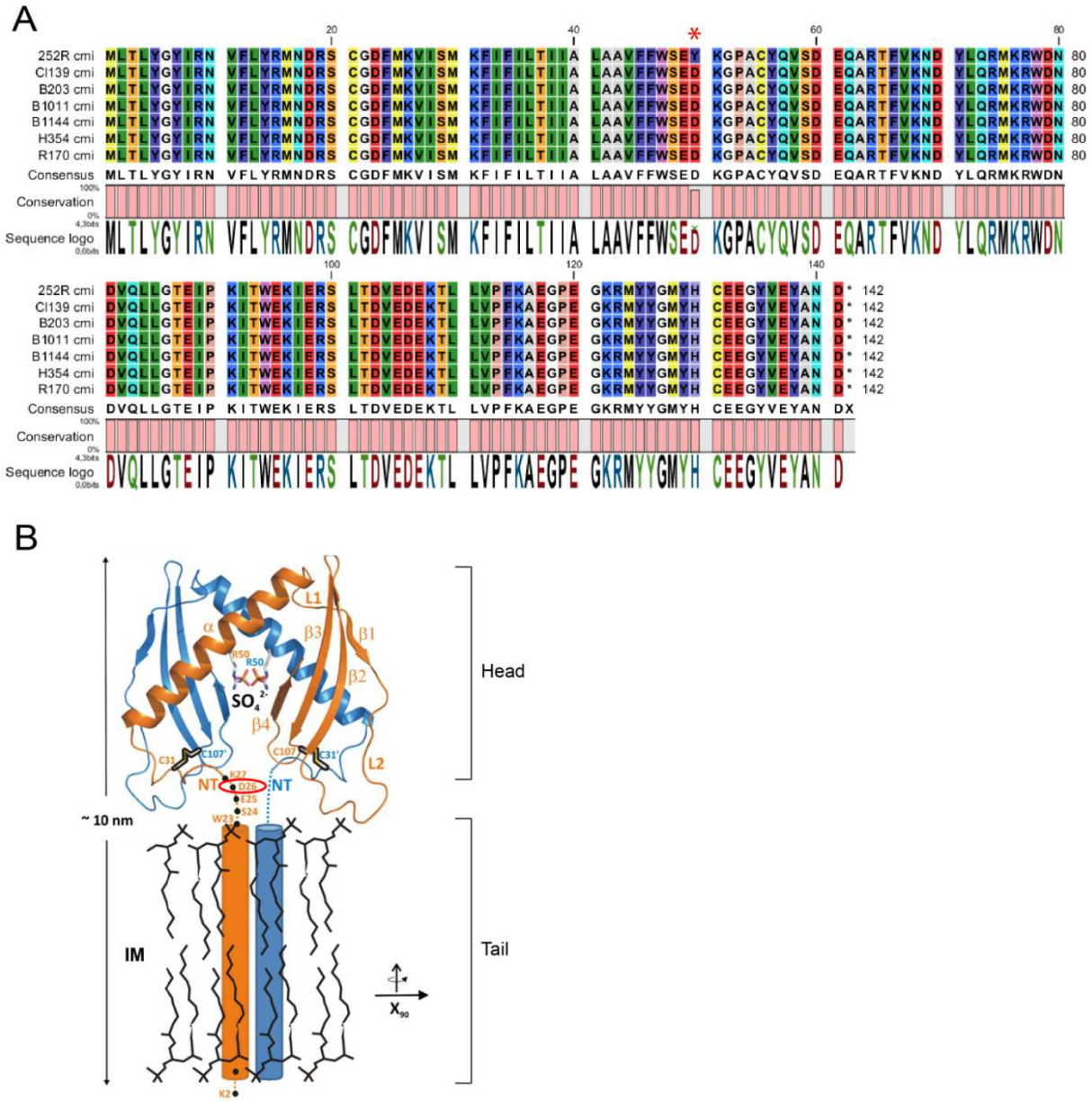

Figure 4.22: Protein sequence of CMI in Ec^{252R} compared to other *E. coli* strains. (A) CMI protein sequence of Ec^{252R} was aligned with sequences of Ec^{Cl139}, Ec^{B203}, Ec^{B1011}, Ec^{B1144} (Table 3.1) and with published CMI sequences of Ec^{H354} and Ec^{R170} (Acc.no. FJ664740.1 and FJ664743.1, respectively). The alignment reveals an amino acid exchange (D26Y marked by *) in the protein sequence of CMI. **(B)** Protein structure of a CMI dimer (monomers are shown in orange and blue, respectively; PDB-entry: 2XGL) (Usón *et al.*, 2012). CMI dimer is anchored in the inner membrane (IM) with the N-terminal helices (tail) and the head structure is directed in the periplasm. The position of the mutation in CMI of Ec^{252R} is highlighted with a red circle.

In order to test if the mutation affects *cmi* functionality ColBM producer strain Ec^{Cl139} (**Figure 4.20C**) was used and characterized by halo-assays for ColB and ColM production in comparison to Ec^{252R} under normal (LB) and stressed (LB with 0.25 µg/ml MitC) conditions and also tested for the presence of a self-killing phenotype (**Figure 4.23A-D**). Characterization of Ec^{Cl139} revealed a reduced killing capacity against Ec^{MG1655} compared to Ec^{252R}, but this was not due to ColB production, as killing of Ec$^{MG1655\ \Delta fhuA}$ was similar between

Ec^{252R} and Ec^{Cl139}. Moreover, according to the western blot results of the experiment, shown in **Figure 4.20C,** ColM production is enhanced in Ec^{Cl139} compared to Ec^{252R}. Furthermore, Ec^{Cl139} is partially resistant to its ColM toxicity (**Figure 4.23**). To investigate, if the mutation in *cmi* of Ec^{252R} leads to loss of impairment of ColM immunity, the sequence of Ec^{252R} *cmi* and from a ColM immune strain (Ec^{Cl139}) was cloned in a plasmid, yielding in $p^{252R\ cmi}$ (encodes *cmi* of Ec^{252R}) and $p^{Cl139\ cmi}$ (encodes *cmi* of Ec^{Cl139}) respectively. Notably, both *cmi* sequences were cloned together with their putative promoter region, to ensure that each *cmi* is under the control of its own and native promoter. ColM sensitive strain Ec^{MG1655} was then transformed with either $p^{252R\ cmi}$, $p^{Cl139\ cmi}$ or the control plasmid p^{empty} (lacking any *cmi* gene) and tested by a halo-assay for ColM-susceptibility using Ec^{252R} $pColBM^{cba::cat}$ as ColM producer strain (**Figure 4.23E**). The results revealed that both CMI of Ec^{252R} and CMI of Ec^{Cl139} confer protection and no significant difference was observed. This demonstrated that the point mutation in *cmi* of Ec^{252R} has no effect on its protective function and we thus concluded that other mechanisms, besides non functional ColM immunity must be underlying the self-killing phenotype of Ec^{252R}. Notably, expression of *cmi* from a plasmid was insufficient to protect Ec^{MG1655} against ColM-dependent killing. Therefore, I concluded that expression of the ColM immunity protein is generally insufficient to completely protect a ColM-producer against self-killing.

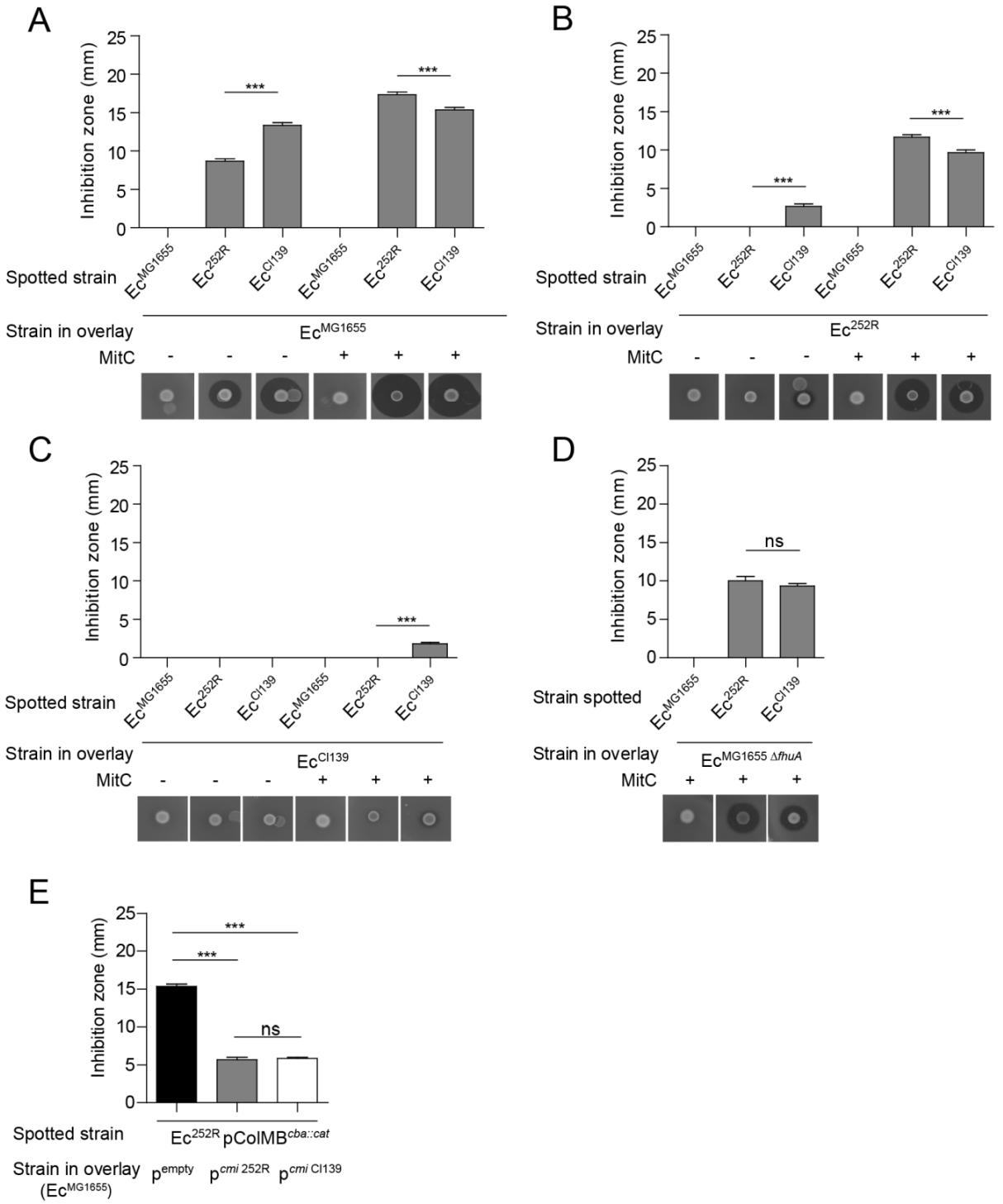

Figure 4.23: Characterization of ColM and ColB production of Ec^{252R} and Ec^{Cl139} and protection by CMI. Halo assays to determine **(A)** colicin production, **(B)** killing by Ec^{252R}, **(C)** killing by Ec^{Cl139} and **(D)** ColB-dependent killing. **(E)** Halo-assay using LB agar plates supplemented with 0.25 µg/ml MitC to determine the protection capacity of the ColM immunity protein (*cmi*) of Ec^{252R} compared to the *cmi* of Ec^{Cl139}. Ec^{252R} pColBM$^{cba::cat}$ was grown o.n. in LB media and spotted as ColM producer on LB agar plates containing 0.25 µg/ml MitC to induce ColM production. The next day, plates were overlayed with LB softagar mixed with o.n. cultures of either Ec^{MG1655} p^{empty}, Ec^{MG1655} $p^{cmi\ 252R}$ or Ec^{MG1655} $p^{cmi\ Cl139}$. The first served as control for a ColM sensitive strain. Both halo-assays were done in triplicates and statistical analysis was performed using 1-way ANOVA with Tukey's post test (***p<0.001).Bars represent means and StD.

4.3.3 Characterization of a collection of *cmi*-positive *E. coli* strains with respect to ColM-dependent self-killing

A literature research revealed that mutations in the ColBM locus occur rather frequently (Christenson and Gordon, 2009). Christenson and Gordon (2009) found that, out of 128 *E. coli* strains tested positive for *cma* and *cba* genes, 69% encoded both colicin genes, 3% lost the entire *cba* locus and about 28% encoded a full length *cma* but different truncated versions of *cba* (named as *cba*-remnant) (**Figure 4.24**).

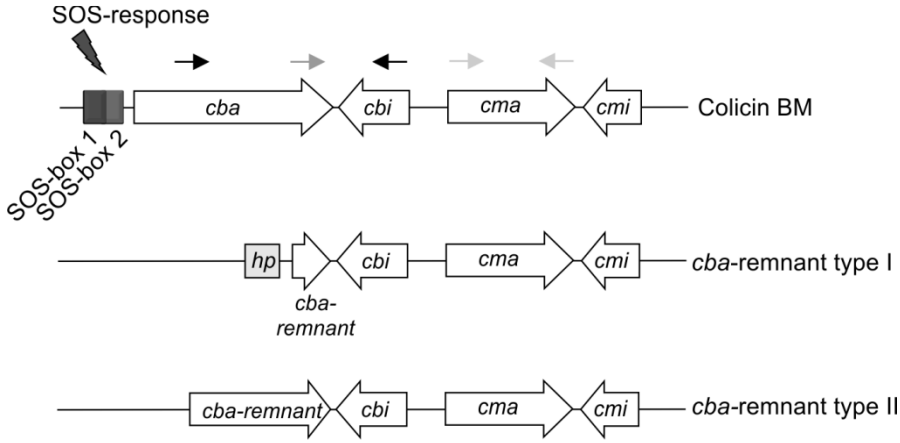

Figure 4.24.: Scheme of the gene structure for colicin BM strains and its colicin B remnant derivatives (adapted from Christenson *et al.*, 2009). The arrows indicate the binding sites and direction of the oligonucleotides used to identify full length *cba* (CBA complete Rev (→) and CBA CBI Primer For (←)) or *cma* (Colicin M Start Rev (→) and Colicin M Start For (←)) and the *cba-remnant* type I and II versions (CBA remnant Pri. Rev (→) and CBA CBI Primer For (←)). Hp is a conserved hypothetical protein found upstream of strains encoding the short *cba-remnant* type I gene. Strains carrying a longer *cba*-remnant version are classified as *cba*-remnant type II.

In this thesis 35 different *E. coli* strains (human isolates from Czech Republic; **Table 4.2.**) tested positive for *cma* by PCR, were characterized by halo-assays for colicin production and a self-killing phenotype. Moreover, strains were analyzed for ColM production by western blot analysis (**Figure 4.25 and Table 4.2**). PCR was used to validate full length *cma* and/or *cba* genes and the presence of the *hp* gene (conserved hypothetical protein) as described (Christenson and Gordon, 2009). All results are summarized in **Table 4.2**.

Table 4.2: Characterization of the 35 human *E. coli* strains

Strain ID	Other bacteriocins	Self-killing	Ec^{MG1655} halo size [mm]	$Ec^{MG1655\ \Delta fhuA}$ halo size [mm]	Significance ***p<0.0001	PCR			Western blot: ColM in pellet		Western blot: ColM in supernatant	
			MitC	MitC		full length *cma*	full length *cbi+cba*	*cba*-remnant	LB	MitC	LB	MitC
B522	-	-	13,3±0,3	12,7±0,3	ns	+	+	-	-	+	-	-
B1314	-	-	14,3±0,3	12±0,6	**	+	+	-	-	+	-	-
B1327	-	-	16,3±0,9	11,3±0,3	***	+	+	-	-	+	-	-
B7	micV	-	12±1,2	5,7±1,2	***	+	+	-	-	+	-	+
B195	-	+	15,8±0,2	9,7±0,3	***	+	+	-	-	+	-	+
B1011	-	-	15,3±0,9	7,3±1,3	***	+	+	-	-	+	-	+
B1144	-	-	15±1,2	14,7±1,5	***	+	+	-	-	+	-	+
B1161	micH47	-	22,7±0,3	14,8±0,2	***	+	+	-	-	+	-	+
B203	-	-	14±0,5	11±6	***	+	+	-	-	+	-	-
B383	ColE1, micV	-	17±0,6	10,7±0,3	***	+	+	-	-	+	-	-
B1218	-	-	20,7±0,3	6±0,6	***	+	-	type II	-	+	-	+
B72	ColE1	-	19,2±0,6	19±0	ns	+	-	type I	-	-	-	-
B54	ColE1	-	6,7±0,3	-	***	+	no *cba*	-	-	-	-	-
B2025	-	-	-	-	-	+	-	type I	-	-	-	-
B411	-	-	-	-	-	+	-	type I	-	-	-	-
B516	-	-	-	-	-	+	-	type I	-	-	-	-
B526	-	-	-	-	-	+	-	type I	-	-	-	-
B537	-	-	-	-	-	+	-	type I	-	-	-	-
B1216	-	-	2,7±0,3	3±0	ns	+	-	type I	-	-	-	-
B1311	-	-	-	-	-	+	-	type I	-	-	-	-
B1069	-	-	-	-	-	no *cma*	-	type I	-	-	-	-
B1156	micM	-	-	-	-	no *cma*	-	type I	-	-	-	-
B236	-	-	-	-	-	+	-	type I	-	-	-	-
B255	ColN	-	-	-	-	+	-	type I	-	-	-	-
B338	-	-	-	-	-	+	-	type I	-	-	-	-
B593	-	-	5,2±0,4	-	***	+	-	type I	-	-	-	-
B459	-	-	8,8±0,4	6,7±0,3	*	+	-	type I	-	-	-	-
B1688	-	-	5,3±0,3	-	***	+	-	type I	-	-	-	-

Strain ID	Other bacteriocins	Self-killing	Ec^{MG1655} halo size [mm]	$Ec^{MG1655\ \Delta fhuA}$ halo size [mm]	Significance ***p<0.0001	PCR			Western blot: ColM in pellet		Western blot: ColM in supernatant	
			MitC	MitC		full length *cma*	full length *cbi+cba*	*cba*-remnant	LB	MitC	LB	MitC
B3253	-	-	4,3±0,3	-	***	+	-	type I	-	-	-	-
B147	I2[(*)], micV	-	10,7±0,3	11±0	ns	no *cma*	no *cba*	-	-	-	-	-
B202	ColE1	-	3,3±0,3	3±0	ns	+	-	type I	-	-	-	-
B358	-	-	-	-	-	+	-	type I	-	-	-	-
B805	-	-	1,3±0,3	-	ns	+	-	type I	-	-	-	-
B848	ColE1	-	-	-	-	+	-	type I	-	-	-	-
B852	ColE1	-	-	-	-	+	no *cba*	-	-	-	-	-

mic: microcin; [(*)]: bacteriocin-like; ns: not significant; **+**: positive; **-**: negative

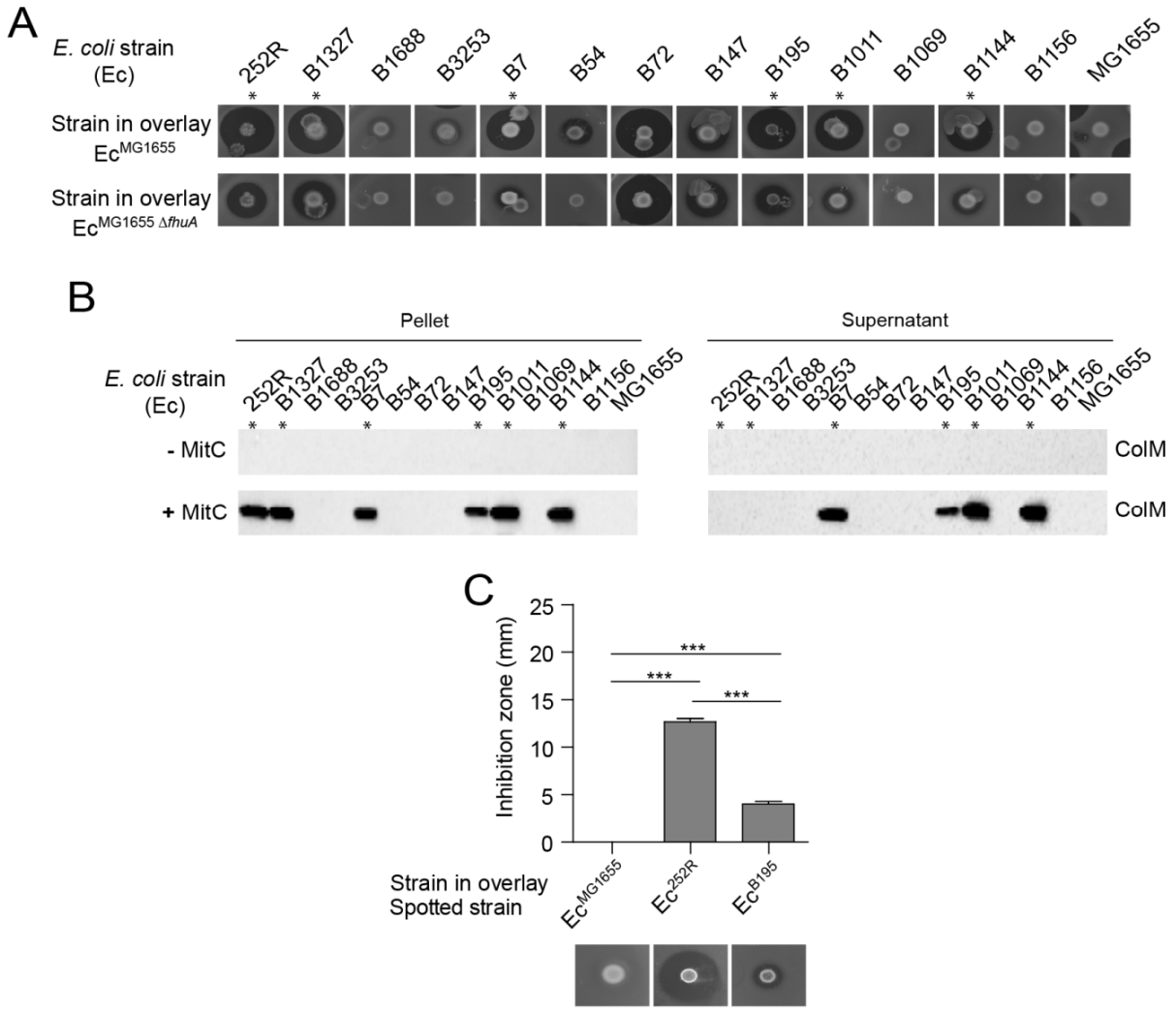

Figure 4.25: Characterization of the 35 *E. coli* human isolates (selection). (A) Examples of the halo-assay to determine colicin production. O.n cultures of the strains were spotted on LB agar plates supplemented with 0.25 µg/ml MitC. The next day strains were overlayed with LB softagar mixed with o.n. cultures of Ec^{MG1655} or $Ec^{MG1655\ \Delta fhuA}$. Halo sizes were measured after 24 h incubation. **(B)** Examples of western blot analysis of the tested strains. **(C)** Halo-assay showing the suicidal phenotype of Ec^{B195} in comparison to Ec^{252R}. O.n. cultures of both bacterial strains together with the negative control strain Ec^{MG1655} were spotted on LB agar plates containing 0.25 µg/ml MitC. The next day, plates were overlayed with LB softagar mixed with o.n. cultures of the spotted strains. Subsequently, each spotted bacteria was overlayed with itself. After an incubation of 24 h halo sizes were measured. All halo-assays in the course of the characterization were done in triplicates and statistical analysis was performed using 1-way ANOVA with Tukey's post test (***p<0.001).Bars represent means and StD. Colicin BM producer strains are marked by *****.

Out of the 35 tested strains, only Ec^{B195} strain was found to exhibit a selfkilling phenotype (**Figure 4.25C**). Only 10 strains out of the 35 *E. coli* strains harbor intact *cba* and *cma* genes. In three strains the *cba* gene was absent but a full-length *cma* was detected, while three other strains lack the *cma* gene but encode the *cba*-remnant gene version and the *hp* gene. Majority (18 strains) were *cma*-positive but carried the *cba*-remnant gene version together with the *hp* gene and are classified as *cba*-remnant type I. Ec^{B1218} was the only exception as

this strain was tested positive by PCR for a full length *cma* gene and a long *cba-remnant* version (<1000 kb) compared to the other strains and is classified as *cba*-remnant type II (**Figure 4.24**). This was revealed using oligonucleotides binding upstream and downstream *cba* adjacent region (CBA check up/CBA check down; **Table 3.3**).

Overall, the characterization of the 35 strains revealed an interesting correlation: only bacteria with full length *cma* and *cba* genes were able to produce ColM in response to MitC. Examples of the characterization by halo-assay and western blot analysis are depicted in **Figure 4.25B**.

4.3.4 Evolutionary experiment to monitor the course of ColM production and resistance after uptake of pColBM by a colicin sensitive *E. coli* strain

The characterization of Ec^{252R} revealed, so far, that ColM production is the reason for its self-killing phenotype. Although Ec^{252R} also produces the corresponding ColM immunity protein, it is not sufficiently protected against ColM-mediated killing. In contrast, other ColM producers are protected against self-killing although they harbor the same immunity protein gene (**Figure 4.23E**). This raised the question whether ColM-producing bacteria need to adapt to the high toxicity of produced ColM. In particular, a colicin sensitive strain can acquire the colicin BM plasmid (pColBM) from a colicin producer strain by conjugation. This new colicin producer then needs to adapt to the toxicity of ColM production, as the CMI is not sufficient to fully protect the strain against self-killing. The new pColBM producer strain might develop during an evolutionary process into a fully evolved, and thus fully protected, ColM producer. Adaptation to ColM toxicity might occur subsequently, e.g. by mutations in the ColM uptake machinery for instance by mutations in ColM receptor (*fhuA*), or by mutations at the ColBM locus (**Figure 4.26**).

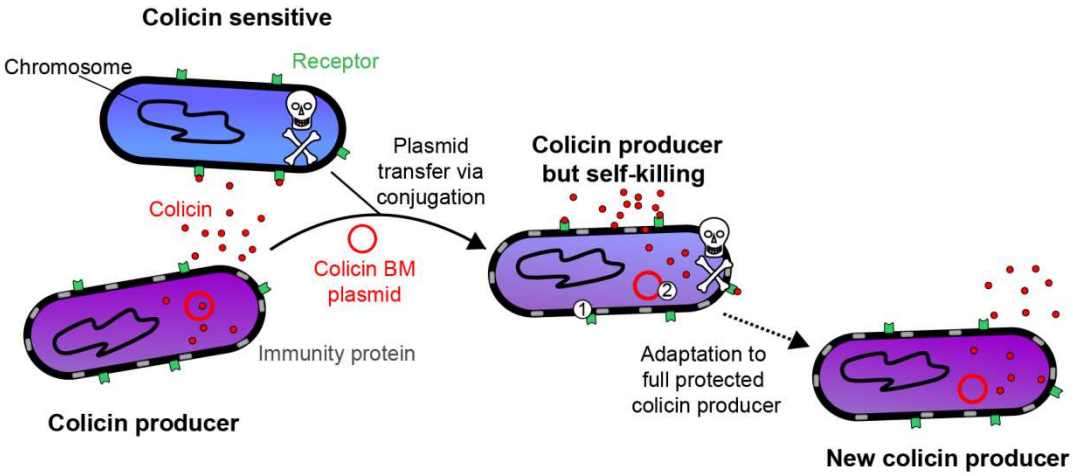

Figure 4.26: Scheme of pColBM uptake and adaptation. According to the literature group B colicin plasmids as pColBM can be transferred by conjugation from a colicin producer to a colicin sensitive bacterial strain. Due to the insufficient protection against ColM by its immunity protein, the pColBM transconjugant strain needs now to adapt to the colicin-dependent toxicity e.g. (1.) by accumulating mutations in the uptake machinery components (e.g. *fhuA*) or the colicin receptor or (2.) by mutations interfering with colicin gene expression resulting in a new colicin producer which is fully protected against ColM.

To confirm the hypothesis that originally colicin sensitive bacterial strains, which have recently acquired a pColBM plasmid, adapt successfully to the toxicity of ColM, an *in vitro* evolution assay was devised. The idea was to transfer a pColBM plasmid from an *E. coli* donor strain, which produces ColM and ColB and is, at the same time, fully protected against toxicity of both colicins, to a "naive" colicin BM sensitive *E. coli* strain (e.g. Ec^{MG1655}) (**Figure 4.27**). This transconjugant strain would be passaged several times under SOS-induced condition, to trigger ColM production and to initiate the putative "evolution process".

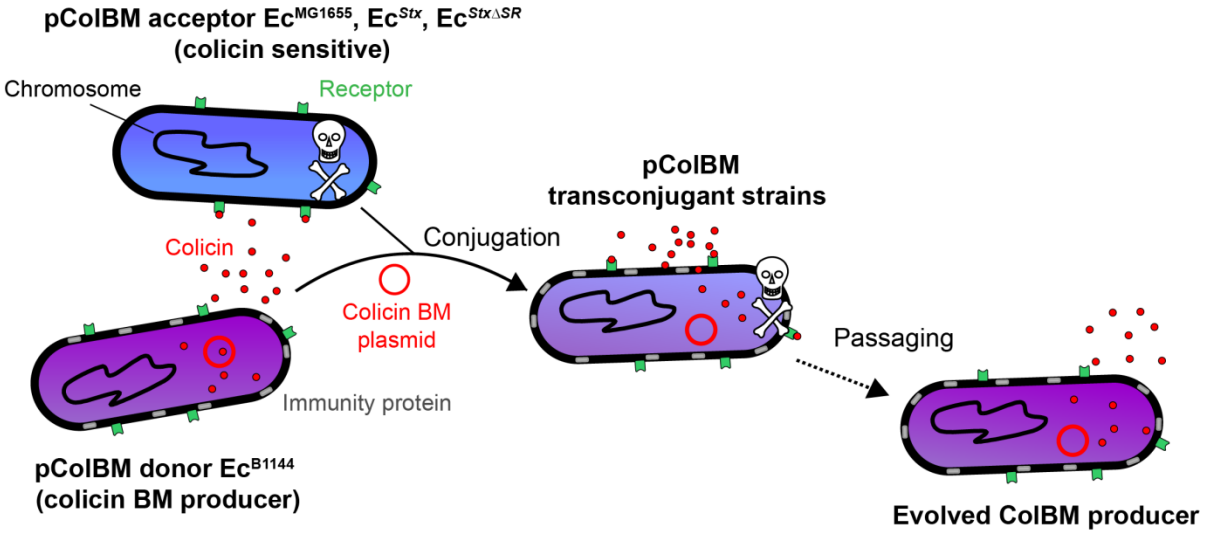

Figure 4.27: Scheme of evolution experiment. As a colicin group B plasmid, pColBM can be transferred by conjugation to a colicin sensitive *E. coli* strain (Ec^{MG1655}, Ec^{Stx} or $Ec^{Stx\ \Delta SR}$) resulting in pColMB transconjugant *E. coli* strains. These transconjugant strains will be exposed to SOS-inducing conditions to trigger a possible evolution process.

To test the hypothesis that ColM producers become resistant to ColM after new acquisition of a ColM plasmid, the pColBM plasmid of the donor Ec^{B1144} was transferred into the colicin sensitive strain Ec^{MG1655} by conjugation. This plasmid harbored a resistance to ampicillin (as tested by a standard antibiotic disc test) and thereby it was possible to select for pColBM plasmid transformants. Since prophages contribute to the release of ColIb (Nedialkova *et al.*, 2015), the adaptation to ColM toxicity might be more pronounced or eventually faster in a strain containing a functional prophage. Thus, pColBM of Ec^{B1144} was also transferred into Ec^{Stx} and $Ec^{Stx\ \Delta SR}$ (**Table 3.1**). These strains are variants of Ec^{MG1655}. Ec^{Stx} is a lysogen for the functional lambdoid temperate phage 933W and was shown to be able to lyse Ec^{Stx} upon induction of the SOS-response and thus release colicin Ib (Nedialkova *et al.*, 2015). $Ec^{Stx\ \Delta SR}$ is a derivate of Ec^{Stx} with disrupted lysis genes of prophage 933W and was shown to be deficient in prophage-dependent cell lysis and thus colicin Ib release (Nedialkova *et al.*, 2015). As described in **section 3.2.3.3**, the *in vitro* evolution assay was conducted as follows. Transconjugant strains were grown in liquid LB media in a flask, and plated the next day in appropriate dilutions on LB agar plates supplemented with 0.25 µg/ml MitC to induce the SOS-response, and therefore induction of colicin production, to direct the adaption process towards a less ColM sensitive strain. Afterwards, single colonies (10 in the first experiment and one in the second experiment) were transferred in a flask containing LB media, grown over night and plated the next day on LB agar plates supplemented with 0.25 µg/µl MitC. This procedure was performed for five times in series. Subsequently, transconjugants were tested for ColB and ColM production and sensitivity (**Figure 4.28** and **Figure 4.29**).

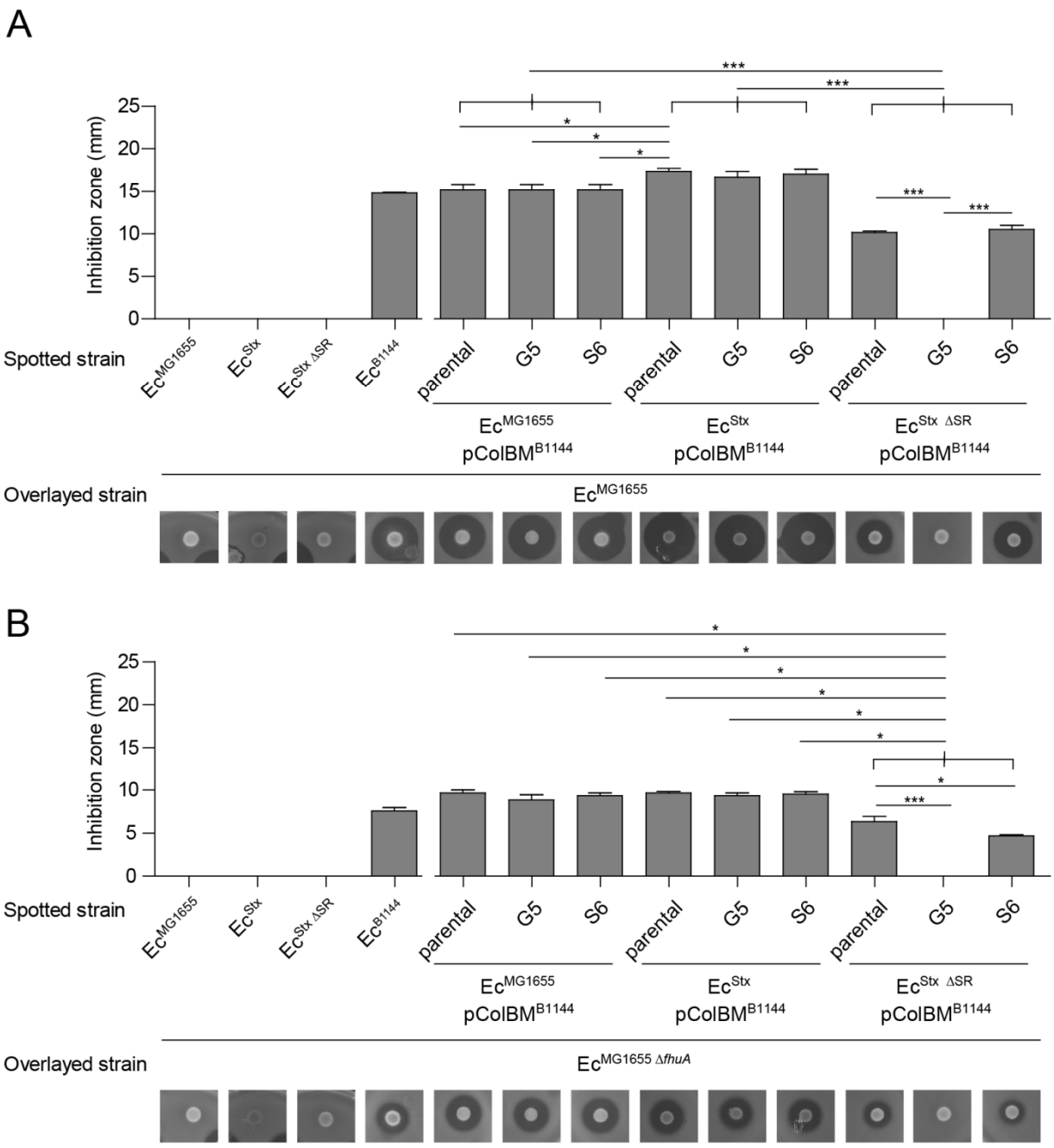

Figure 4.28: Halo-assay to determine ColBM production in newly pColBM transconjugant *E. coli* strains and their mutants. Bacterial strains were cultivated o.n. and spotted on LB agar plates supplemented with 0.25 µg/ml MitC. The next day agar plates were overlayed with LB softagar mixed with o.n. culture of **(A)** Ec^{MG1655} or **(B)** $Ec^{MG1655\ \Delta fhuA}$, respectively. Following an incubation time of 24 h, the halo size was measured. The experiment was performed in triplicates. Statistical analysis was performed using 1-way ANOVA with Tukey's post test (***p<0.001). If not indicated, differences were not statistically significant. Bars represent means and StD.

All transconjugants produced functional ColM and ColB before passaging (**Figure 4.28**). Interestingly, it was shown that prophage-harboring strain Ec^{Stx} $pColBM^{B1144}$ releases more colicin compared to prophage-deficient strain Ec^{MG1655} pColMBM^{B1144}. By the overlay with only ColB-sensitive mutant strain $Ec^{MG1655\ \Delta fhuA}$ it was shown, that this was due to differences in ColM release. The prophage lysis gene deficient $Ec^{Stx\ \Delta SR}$ $pColBM^{B1144}$

transconjugant strain secretes less ColBM than its equivalent strain Ec^{Stx} $pColBM^{B1144}$ with the intact prophage lysis genes, as expected. This is in line with published results on the release of ColIb, where prophage lysis genes are needed to release ColIb (Nedialkova *et al.*, 2015), together by the fact that Ec^{Stx} $pColBM^{B1144}$ releases more ColM than its equivalent Ec^{MG1655} $pColBM^{B1144}$ strain. Notably, ColBM release of $Ec^{Stx\ \Delta SR}$ $pColBM^{B1144}$ is even more decreased compared to the prophage-deficient strain Ec^{MG1655} $pColBM^{B1144}$.

The mutants Ec^{MG1655} $pColMBM^{B1144}$ S6 and Ec^{Stx} $pColBM^{B1144}$ S6 generated during the first set of the *in vitro* evolution did not show a difference in ColBM production or secretion compared to the parental strains, respectively. Although mutant strain $Ec^{Stx\ \Delta SR}$ $pColBM^{B1144}$ S6 shows no differences in total ColBM secretion, when overlayed with the sensitive strain Ec^{MG1655}, it exhibit a decrease of ColB release compared to the parental strain.

The mutants Ec^{MG1655} $pColBM^{B1144}$ G5 and Ec^{Stx} $pColBM^{B1144}$ G5, generated during the second set of the *in vitro* evolution did not exhibit a difference in ColBM production or secretion, respectively, compared to the respective parental strains. Mutant strain $Ec^{Stx\ \Delta SR}$ $pColBM^{B1144}$ G5 showed no colicin production at all (**Figure 4.28**). Further investigations by PCR and halo-assay confirmed the assumption that this mutant lost the pColBM plasmid (data not shown).

All strains were also tested for suicidal properties (**Figure 4.29**). All tested pColBM transconjugant *E. coli* strains were able to kill themselves. The majority of the “evolved” pColBM transconjugant strains did not exhibit a difference in their suicidal phenotype compared to their parental strain. The exception was Ec^{MG1655} $pColBM^{B1144}$ S6, which revealed a slightly reduced sensitivity against its produced colicins. This strain is now under further investigation to decipher the genetic basis for this phenotype. In addition $Ec^{Stx\ \Delta SR}$ $pColBM^{B1144}$ S6 will be further characterized due to its decreased ColB release.

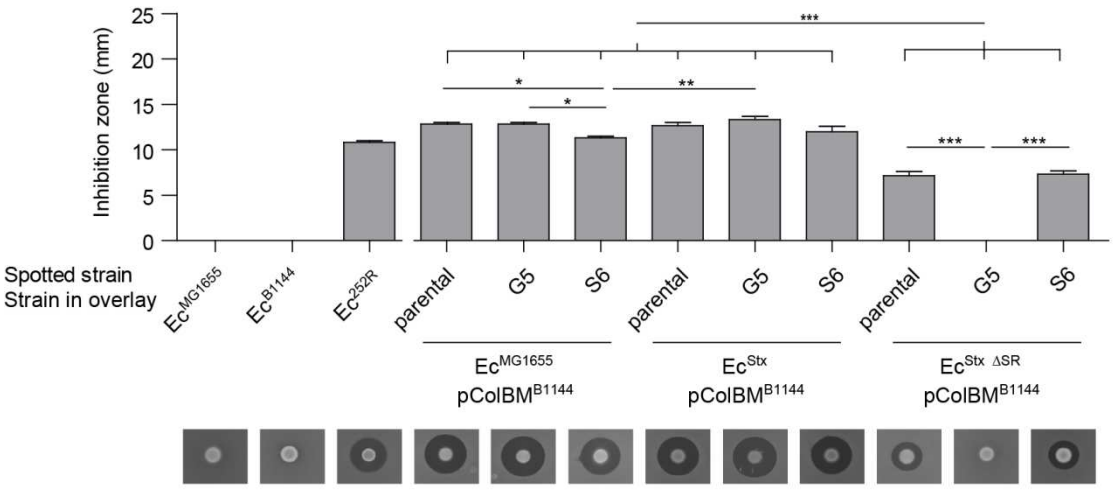

Figure 4.29: Halo-assay to characterize the suicidal phenotypes. pColMB transduced *E. coli* strains, their mutants, as well as the control strains Ec^{MG1655} and Ec^{B1144} (negative controls) and Ec^{252R} (positive control) were analyzed for their self-killing properties. O.n. cultures of these *E. coli* strains were spotted on LB agar plates containing 0.25 µg/ml MitC. The next day, agar plates were overlayed with LB softagar mixed with o.n. cultures of the spotted strain. Afterwards each strain could be overlayed by itself. Halos were measured 24 h later. The experiment was done in triplicates and statistical analysis was performed using 1-way ANOVA with Tukey's post test (***$p<0.001$), if not indicated differences were not statistically significant. Bars represent means and StD.

In conclusion, the hypothesis that new ColBM producers quickly adapt to ColM production could not be verified by the "evolution experiments" under the chosen experimental conditions. Only one "evolved" strain showed a slightly reduced self-killing and another "evolved" strain showed a decrease in ColB release. These "evolved" strains will be characterized further.

5 Discussion

5.1 Single cell analysis of colicin Ib (*cib*) gene expression in *S.* Tm

Colicin Ib production confers a competitive benefit to *S.* Tm^{SL1344} against commensal *E. coli* in the inflamed gut (Nedialkova *et al.*, 2014, Stecher *et al.*, 2012b, Stecher *et al.*, 2013). Notably, colicin-producing bacteria usually lyse, and thus die, in order to release colicins. According to this it was speculated that *S.* Tm gains its fitness benefit over colicin-sensitive *E. coli* by bimodal *cib* expression, thus by division of labor. Colicin production would be taken over, by a sub-fraction of the *S.* Tm population and thus, their lysis served as a "common good" for the entire population. To address this hypothesis, different reporter strains (*S.* Tm $p2^{cib::sfgfp}$, *S.* Tm^{wt} $p^{Pcib\ gfp}$) were generated and comprehensively characterized. In contrast to the hypothesis, results of this thesis revealed that *cib* expression is rather unimodal than bimodal. Thus, other mechanisms must ensure that release only occurs in a subfraction of *S.* Tm population in order that *S.* Tm can benefit from ColIb production.

5.1.1 Analysis of group B colicin gene expression and its characteristics

In contrast to group A colicins, which are mainly encoded by multi-copy (15-20 copies/cell) type I colicinogenic plasmids, group B colicins are encoded by single-copy type II colicinogenic plasmids (Clewell and Helinski, 1970, Hardy *et al.*, 1973). Therefore, the direct exchange of the group B colicin Ib gene against the gene of a fluorescent protein results in rather low fluorescent signal intensities (**Figure 4.1C**). This confirms that pColIB9 (p2) of *S.* Tm^{SL1344} exists in low copy numbers per bacterium, like it was shown before for pColIB9 in *E. coli* (Clewell and Helinski, 1970).

To overcome the limitations of low signal intensities, the multi-copy reporter $p^{Pcib\ gfp}$ was constructed. Notably, multi-copy reporter $p^{Pcib\ gfp}$ is based on plasmid pBAD24 (Guzman *et al.*, 1995). This backbone plasmid contains the *ori* pBR322, thus it is a derivative of pColE1-type plasmid pMB1 and owns the same replication control mechanism of ColE1 and relatives (Bolivar *et al.*, 1992, Guzman *et al.*, 1995). In *E. coli*, pBR322 was shown to exist at about 15-20 copies per bacterium (Lin-Chao and Bremer, 1986). This copy number is negatively regulated by the repressor of primer (ROP) protein (Cesareni *et al.*, 1982). This small RNA-binding protein stabilizes the RNAI/RNAII complex, which inhibits primer formation and consequently plasmid replication (Cesareni *et al.*, 1982, Lacatena *et al.*, 1984). In contrast to plasmid pBR322, plasmids based on pBAD24, such as multi-copy reporter $p^{Pcib\ gfp}$, lack *rop* (Cronan, 2006). Furthermore it was shown, that a deletion of *rop* results in an increase of

copy numbers, similar to that of pUC plasmids (Cronan, 2006, Cesareni *et al.*, 1982, Twigg and Sherratt, 1980). Notably, P_{cib} regulated *gfp* expression by $p^{P_{cib\ gfp}}$ would thus resemble colicin expression of group A colicins rather than of group B colicins.

Colicin expression is generally repressed by LexA which is the global regulator of the SOS-response. Upon DNA damage (e.g. double strand breaks) the protease RecA is activated. This induces auto-cleavage of free LexA dimers and leads to the dissociation of LexA from the SOS-box. Consequently, this is followed by the de-repression of SOS-inducible promoters (Baharoglu and Mazel, 2014). The SOS-response it is responsible for the regulation of more than 50 genes (Zgur-Bertok, 2013). According to this, it essential for bacteria to provide a sufficient quantity of LexA repressor proteins in the cell, to bind all LexA binding sites and secure repression of all SOS-response regulated genes.

In 1983 Anthony P. Pugsley assumed that the introduction of additional LexA binding sites provided by multi copies of a group A colicin plasmid (15-20 copies per cell), could interfere with the SOS-response regulation (Clewell and Helinski, 1970, Hardy *et al.*, 1973, Pugsley, 1983). Consequently, the SOS-response would be induced and this in turn would lead to the expression of the colicin (Pugsley, 1983). However, he could disprove his hypothesis, but only for group A colicins. So far it has never been addressed whether an artificial introduction of multi copies of LexA binding sites has an effect on group B colicin expression. According to this, it was investigated during this thesis, whether an artificial increase of LexA binding sites provided by the multi-copy *gfp*-reporter, interferes with expression of group B colicin *cib*. Construction of different derivatives of $p^{P_{cib\ gfp}}$ (**Figure 4.7**) and their transformation in *S.* Tm $p2^{cib\text{-}HA}$ allowed quantification of intrinsic ColIb-HA-levels as well as multi-copy reporter derived GFP-levels by immunofluorescence microscopy. The results confirmed that the introduction of additional copies of the colicin Ib promoter region (P_{cib}) and thus additional repressor binding sites by $p^{P_{cib\ gfp}}$ does not affect intrinsic *cib*-HA expression (**section 4.1.3**).

Concerning the quantification of *cib* expression at the single cell level, it is very important to ensure that the chosen fluorescent protein does not affect cell viability and reports indeed the expression of the gene of interest. Recent studies support this concern as they showed that fluorescent proteins can additionally stress bacteria leading to an erroneous up-regulation of the reporter gene (Hebisch *et al.*, 2013, Liu *et al.*, 1999). Using *S.* Tm $p2^{cib\text{-}HA}$ $p^{P_{cib\ gfp}}$ I showed that intrinsic ColIb-HA levels correlate well (ρ=0.87) with GFP-levels (**Figure 4.10**). Additionally, analysis of the multi-copy reporter plasmid $p^{P_{cib\ gfp}}$ revealed plasmid stability of >99% after about 24 generations, grown for 5 successive passages in LB medium without selective pressure. This was also true for the copy numbers of $p^{P_{cib\ gfp}}$ in *S.* Tm^{wt}, which stayed stable over the entire time. As expected, $p^{P_{cib\ gfp}}$ exhibited high copy numbers (passage 1: 169±91 StD; passage 2: 84±18 StD; passage 3: 147±57 StD; passage 4: 198±100 StD; passage 5:127±67 StD).

Taken together, this data verifies that $p^{P_{cib\ gfp}}$ is a useful and powerful tool to report and analyse *cib* expression in further experiments (e.g. live cell microscopy).

So far the majority of studies on group A colicin expression was performed under non-inducing conditions in the late logarithmic or stationary growth phase. Bacteria were grown in batch cultures in standard nutrient broth (e.g. Luria Bertani), at 37°C until the stationary growth phase, where colicin expression was determined (Kamensek *et al.*, 2010, Hol *et al.*, 2014). These studies revealed that in early stationary phase colicins are only expressed at low rates, for instance ColA, ColN and ColE1 are expressed in about 0.5%, ColE7 in about 1.5% and ColK in about 3% of the population (Kamensek *et al.*, 2010, Mulec *et al.*, 2003). In a different study, the expression of ColE7 was detected in 2.3% of the population in the exponential growth phase. Moreover this study showed a slight increase in *gfp* expression upon early stationary phase, although the same reporter plasmid was used (as in (Kamensek *et al.*, 2010)) (Majeed *et al.*, 2015). Differences in *gfp* expression might be explained by different media composition or growth conditions or by the sensitivity of the fluorescent protein used as reporter tool. Additionally, the detection limit can vary between different analysis methods in different laboratories. This is supported by the different results of the multi-copy ($p^{P_{cib\ gfp}}$) and single-copy (*S.* Tm $p2^{cib::sfgfp}$) *gfp*-reporter used in this thesis. For instance, using the multi-copy reporter $p^{P_{cib\ gfp}}$ 0.93% of the *S.* Tm population was detected as GFP^+, whereas only 0.01% GFP^+ bacteria were detected using the single-copy reporter strain *S.* Tm $p2^{cib::sfgfp}$ by FACS in the exponential growth phase (**Figure 4.1C-E**). These results revealed that, quantification of *gfp* expression can be biased by reporter sensitivity. As a consequence, comparison on relative expression rates between different reporter tools and analysis methods must be interpreted with care.

5.1.2 Colicin produciton: an extreme form of division of labor?

For the majority of group A colicins it was shown that DNA-damage and, thus induction of the SOS-response is the main trigger of colicin expression which is negatively regulated by two overlapping LexA binding sites located downstream of the -10 box in the promoter region (Gillor *et al.*, 2008, Ghazaryan *et al.*, 2014). When triggered by DNA double strand brakes, the SOS-response can be induced and LexA is removed from its binding site (Pennington and Rosenberg, 2007). This de-represses colicin expression in a small fraction of the bacterial population (Mrak *et al.*, 2007). This bistable gene expression is abrogated in a *recA*-deficient strain or if the LexA binding sites in the colicin promoter region are modified (Mrak *et al.*, 2007). Since bacteria lyse to release intracellular colicin and this results in the eradiation of the entire population of colicin producers. This underlines the strong need for a tight regulation of colicin expression to ensure the survival of a sufficiently high fraction so that colicin production remains beneficial for the bacterial fitness.

To ensure tight control of colicin expression, several colicins are under negative control by other regulators besides LexA. For instance, colicin K expression is growth-phase dependent, as it is only induced upon nutrient starvation by the stringent response alarmone (p)ppGpp (guanosine pentaphosphate) (Kuhar and Žgur-Bertok, 1999). Furthermore, colicin K synthesis can also be regulated by the transcription regulator IscR (iron-sulfur cluster regulator), which is suggested to stabilize LexA at the promoter and is de-repressed upon nutrient starvation (Butala *et al.*, 2012). Other colicins are also found to be regulated by transcription regulator IscR e.g. ColE1 and ColN (Butala *et al.*, 2012) or by transcription regulator AsnC, e.g. ColE2, ColE6, ColE8 (Kamenšek *et al.*, 2015). Interestingly, colicin Ib as well as colicin Ia, harbour only one single LexA binding site located downstream of the -10 box (Gillor *et al.*, 2008). Additionally, colicin Ib is negatively regulated by Fur which binds upstream of the -35 region and mediates Fe^{2+}-dependent repression of P_{cib} (**Figure 4.1A**) (Nedialkova *et al.*, 2014). Thus, it is likely that Fur- and LexA-mediated repression together fulfil a "double-locking" function, to reduce suicidal colicin production (Butala *et al.*, 2012).

In this thesis, *cib* expression in *S.* Tm^{SL1344} was analysed and quantified in individual bacteria in response to SOS-induction (by MitC) and Fe^{2+}-limitation (by DTPA). If applied individually, both supplements de-repress P_{cib}, which in turn leads to a significant increase in *gfp* expression. Compared to conditions were both inducers are supplemented at the same time, single application leads to rather small increase in fluorescence signal intensity. This could also be confirmed at the level of intrabacterial ColIb-HA protein (**Figure 4.8**). These results demonstrated that, upon full induction, *cib* is unimodally expressed rather than bimodally. Inducer titration experiments confirmed these expression patterns (**Figure 4.2**).

Recently it has been shown that, although the colicin Ib locus does not encode a cognate lysis gene, bacteria lyse to release colicin by prophage-mediated cell lysis (Nedialkova *et al.*, 2015). Assuming that all colicin producing bacteria lyse, under inducing conditions unimodal *cib* expression observed in *S.* Tm would lead to the death of almost the entire population. Similar to this observation, single cell analysis of colicin E2 (*cea*) expression revealed that only upon non-inducing conditions, *cea* is expressed in a small fraction of the population. In contrast to this, upon SOS-induction *cea* expression was observed in almost each bacterium (Mader *et al.*, 2015).

As colicin producers lyse to release colicin, the question arises: how could colicin Ib expression confer a growth benefit to *S.* Tm over *E. coli* in the inflamed gut, if >90% of the population produced ColIb and thus eventually died to release colicin?

Using the intrabacterial staining protocol for ColIb-HA it was observed that without lysozyme treatment, ColIb-HA was detectable in a small fraction of bacteria grown under inducing conditions (LB+MitC+DTPA) (**Figure 4.5C**). This suggests that bacteria of this small fraction are undergoing lysis. Based on this data it was assumed that upon induction of *cib*

expression only a small fraction would lyse and thus die to release colicin. Therefore, under colicin and lysis gene inducing conditions, division of labor might not be the case at the level of colicin expression but rather at the level of colicin release by cell lysis. This forms a new scenario of how colicin Ib production could increase the fitness of a bacterial population.

5.2 Analysis of group B colicin release at the single cell level

It is known, that the release mechanisms of group A and B colicin are different. The operons of group A colicins in general encode for small cognate lysis protein, which is, like the colicin, expressed upon prolonged and severe DNA damage (Cavard, 1997). Anchored within the periplasmic leaflet of the IM the lysis protein confers perturbation of the IM and activates the outer membrane phospholipase A (OmpLA). Activated OmpLA leads to the permeabilization of the OM and thus results in the release of colicins (Pugsley and Schwartz, 1984, Snijder and Dijkstra, 2000). The operons of group B colicins do not encode for a cognate lysis gene, and it has been unclear, how group B colicins are released (Cascales *et al.*, 2007). Recently, it has been discovered that group B colicin Ib is released by prophage-mediated lysis in *S.* Tm and *E. coli* (Nedialkova *et al.*, 2015). Furthermore, this study showed that the major contributors for phage-mediated cell lysis in *S.* Tm^{SL1344}, and thus release of ColIb, are the lysis genes of the lambdoid prophage ST64B (*SL1344_1955-SL1344_1957*). Thus, colicin Ib does not only hijack host receptor proteins for their entry into the target cell, but also phage lysis proteins to be released by the producer cell.

In the second part of this thesis, interaction of ColIb and prophage was investigated at the single cell level (**section 4.2**). Comparative single cell analysis of *S.* Tm^{WT} $p^{P\mathit{cib\ gfp}}$ and *S.* $Tm^{\Delta Ph}$ $p^{P\mathit{cib\ gfp}}$ using live cell microscopy and FACS revealed that induction of the SOS-response resulted only in case of *S.* Tm^{WT} $p^{P\mathit{cib\ gfp}}$ in bacterial lysis and, thus, in colicin release. Under the same conditions, bacteria of *S.* $Tm^{\Delta Ph}$ $p^{P\mathit{cib\ gfp}}$ did not lyse and showed higher GFP signal intensity levels and extreme long cell shapes compared to *S.* Tm^{WT} $p^{P\mathit{cib\ gfp}}$ bacteria. Importantly, the differences in GFP signal intensity levels are not only caused by differences in the cell size. Therefore, as shown in bulk assays (Nedialkova *et al.*, 2015), single cell analysis revealed that only in the presence of prophages, *S.* Tm lyses and thus release ColIb. Prophage-mediated lysis starts after sufficient amounts of ColIb have been synthesized intracellularly. Furthermore this indicates that, upon SOS-induction, the amounts of produced colicin released into the environment is not dependent on intracellular colicin concentrations but is regulated by the prophage-lysis genes. Similar observations were recently published for group A colicin E2 (ColE2) and its cognate lysis gene (Mader *et al.*,

2015). This study showed that upon SOS-induction, ColE2 is only produced until a certain saturation level caused by ColE2 release through cell lysis. In contrast to this, in a lysis gene-deficient strain, ColE2 production is significantly increased above this saturation level (Mader *et al.*, 2015).

5.2.1 Co-regulation of colicin gene expression and phage-mediated lysis

It has been previously shown, that the majority of colicins are negatively regulated by LexA and additionally co-regulated by other transcription regulators such as IscR (ColE1, ColK, ColN) (Butala *et al.*, 2012), Fur (ColIb) (Nedialkova *et al.*, 2014) or AsnC (ColE2, ColE6, ColE8) (Kamenšek *et al.*, 2015). Such "double locking" mechanisms might protect the bacterium against a premature colicin production, under conditions where DNA damage could be overcome by cellular repair mechanisms (Butala *et al.*, 2012). Lysis genes of group A colicins are located downstream of their cognate colicin gene and thus are regulated by the same "double locking" mechanism (Cascales *et al.*, 2007). In addition it has been shown that some group A colicin lysis genes (ColE2 and ColE7) are also post-transcriptional regulated by the RNA binding protein CsrA (Hol *et al.*, 2014). This suggests, at least for group A colicins, a co-regulation of colicin and cognate lysis gene expression. Indeed, single cell analysis tools revealed that group A colicin E2 lysis gene (*cel*) is co-regulated with colicin E2 (*cea*) and both genes showed similar, if not identical, gene expression patterns, with the difference that *cea* is expressed at higher intensity levels compared to *cel* (Mader *et al.*, 2015).

Is such co-regulation also possible for group B colicins and prophage lysis genes? In particular, is ColIb production co-regulated with prophage-mediated cell lysis? And furthermore, do all colicin producing bacteria lyse and release colicins?

Single cell analysis revealed that upon non-induced conditions (only LB medium), during the exponential growth phase, prophage lysis genes are expressed only in a small fraction of the population (0.1% or 0.8%, respectively) (**section 4.2.2**). Interestingly, this fraction did not co-expressed colicin Ib. This might be due to spontaneous induction of the ST64B prophage in *S.* Tm as shown for CGP3 prophage of *Corynebacterium glutamicum*. Under similar conditions (as in this work) CGP3 prophage was spontaneously induced in about 0.01% to 0.08% of the bacterial population measured by FACS (Nanda *et al.*, 2014). It was also shown for CGP3 in *Corynebacterium glutamicum*, that spontaneous prophage induction is not solely caused by spontaneous SOS-induction but also by other so far unknown factors (Helfrich *et al.*, 2015). This would explain the small fraction of $LysST^+$ but $ColIb^-$ bacteria in non-inducing conditions (**Figure 4.15**). Additionally this would also explain small fraction of $LysST^+$ but

ColIb$^-$ bacteria observed in induced conditions (**Figure 4.15**). Interestingly, the fraction of *lysST*-expressing bacteria increased significantly by induction of the SOS-response using MitC only, or MitC and DTPA. However, as before, under these conditions only a fraction of the population induced the prophage lysis genes (6.5% to 33.7%), whereas almost the entire population responded with *cib* expression (91.4% to 99.1%) (**Table 4.1**). This reflects a bimodality in the prophage-decision between lysogenic and lytic state.

However, upon Fe^{2+}-limiting condition only a slight induction of *cib* expression was observed. This was expected since, in contrast to prophage lysis genes, ColIb is also negatively regulated by Fur. According to this, Fur-mediated repression of *cib* expression can protect the cell against premature colicin production under conditions where DNA damage could be overcome by cellular repair mechanisms. In this scenario prophage-mediated cell lysis and thus colicin release is prevented and *cib* expression would not be required.

Interestingly, it was previously shown in bulk assays that induction of prophage-lysis genes is delayed compared to *cib* expression (Nedialkova *et al.*, 2015). This was also supported by live cell microscopy (done in this work). Results showed that *cib* expressing bacteria start to lyse ~2 h after induction of the SOS-response (**Figure 4.11B** and **Movie S1**). In conclusion, results showed that production of ColIb is co-regulated with prophage-mediated cell lysis ~2 h after induction of the SOS-response.

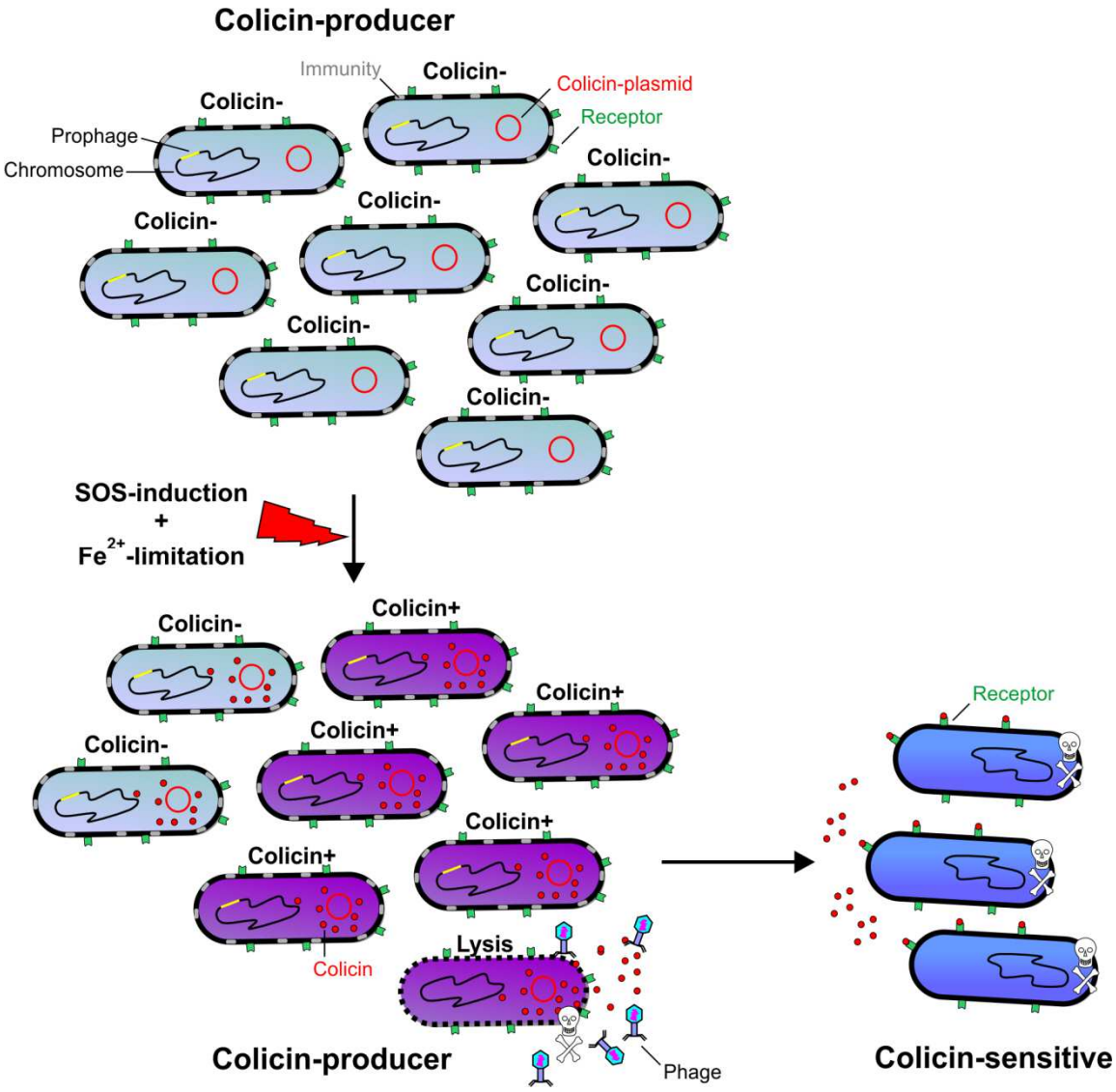

Figure 5.1:Colicin warfare: an extreme form of "division of labor". Upon conditions which trigger the bacterial SOS-response and Fe^{2+}-limitation, colicinogenic bacteria (light blue) start to express colicin Ib (purple). Depending on the environmental conditions colicins are expressed in a small fraction or the majority of the population. Upon prolonged and severe DNA-damage colicins are then only released by a small fraction of the population by prophage-mediated cell lysis. This leads to colicin-depending killing of colicin sensitive competitors (blue). Sacrificing only a small fraction of the population the remaining (majority) of the population can benefit from colicin-mediated killing and gain a fitness increase over colicin sensitive competitors.

Taken together, it can be assumed that under colicin and lysis gene-inducing conditions, division of labor is a strategy of *S.* Tm for colicin release by cell lysis rather than for *cib* expression (see **section 5.1.2**). Furthermore, growth in the inflamed intestine provides *S.* Tm an excellent environment for ColIb-dependent competition against ColIb-sensitive *E. coli*. As under these conditions not only production of ColIb but also the production of its receptor and translocator CirA is maximally induced. In addition, the delayed ColIb release, due to the delayed induction of prophage lysis gene expression would provide the possibility to accumulate sufficient amounts of intracellular ColIb before the start of prophage-mediated cell lysis.

5.2.2 Benefits conferred by prophage-mediated release of ColIb

The fact that, in contrast to group A, group B colicins are released by prophage-mediated lysis raised the question, what is the benefit of the genetic un-coupling of release process from colicin production and immunity?

With respect to protection against premature cell lysis, it might be favorable to induce cell lysis only, if sufficient amounts of colicin have been produced to guarantee the killing of the surrounding competing bacteria by the toxin. That means, if cell lysis, and thus the colicin release mechanism, was under the control of a different system (e.g. prophages) this would confer a “double locking” mechanism which secures premature lysis eventually better than the double locking system of lysis gene regulation of group A colicins (e.g. E2 and E7) or at least to similar extent. However, in case of ColE2 (*cea*) its cognate lysis gene (*cel*) is post-transcriptionally regulated by CsrA, thus CsrA prevents premature lysis. This is important as it has been recently shown, that induction of *cel* expression is similar, if not identical to *cea* expression (Mader *et al.*, 2015). Thus if post-transcriptional regulation by CsrA is not sufficient it would lead to cell lysis even if only little amounts of colicin are produced. Furthermore, based on the results from the same study, it is likely that upon induction, all group A colicin producing bacteria also produce lysis proteins and thus die to release colicin, especially if lysis gene regulation failed. Thus colicinogenic bacteria would suffer from the cost of colicin and immunity production as well as cell lysis, therefore being unable to compete with other bacteria. Notably, MitC concentrations used before (Nedialkova *et al.*, 2014), in this study but also in other studies e.g. (Mader *et al.*, 2015) are rather high and thus might be un-physiological. In the gut, DNA damaging agents might be present at concentrations which only locally trigger the SOS-response in a small part of the population. Therefore, it can not be excluded, that colicin gene expression is bimodal under *in vivo* conditions. This will be addressed in the future.

In contrast to lysis genes of group A colicins, lysis genes of group B colicin Ib are un-coupled from the colicin plasmid and under the control of *S.* Tm prophages, in particular prophage ST64B. In *S.* Tm^{SL1344} it has been recently shown that expression of prophage ST64B (*lysST*) lysis genes is delayed (~60 min) compared to *cib* expression (Nedialkova *et al.*, 2015). The difference in timing of expression was also supported by single cell analysis in this study. It was shown that 2 h after SOS-induction, the majority of bacteria were already $ColIb^{+}$ (MitC only: 91.4%) but out of these bacteria only a very small fraction was $LysST^{+}$ (MitC only: 5.5%), with rather weak signal intensity compared to induction for 4 h (**Figure 4.15 and Table 4.1**). Furthermore, it was shown that full induction of *cib* expression was achieved by MitC and DTPA with the highest signal intensities at 2 h post induction, whereas 4 h post induction the ColIb signal intensity levels were significantly decreased, due to cell lysis. In

contrast to this, lysis genes were slightly expressed after 2 h of induction, but were significantly increased after 4 h, which supports the delay in induction of expression. With this delay bacteria might have time enough to produce sufficient amounts of ColIb before lysis genes are also expressed, followed by the lysis of the cell.

Recently, it was also shown for *Corynebacterium glutamicum* that the induction of the SOS-response can be transient and those bacteria which are switched to the “ON” state can also switch back to the “OFF” state, if DNA-damage can be repaired by the bacterium (Helfrich *et al.*, 2015). This study also demonstrated that only upon severe DNA damage SOS-response stays in the “ON” state and results in CPG3 prophage induction always leading to cell lysis.

Therefore, upon SOS-induction *S.* Tm^{SL1344} is switched to the “ON” state and start to express *cib*. If the SOS-inducer is gone and it was possible to repair the DNA-damage, before induction of ST64B prophage lysis genes, bacteria might switch back to the “OFF” state (regarding the SOS-response). If ColIb production was only induced by the SOS-response induction ColIb production would be inhibited. Intracellular ColIb might be degraded by produced proteases, as it is known that expression of proteases is also induced upon cell stress. It might also be the case that colicins are just out-diluted over the next generations due to cell division. This behavior might be more likely if bacteria show a delay in lysis gene induction due to prophage induction, as this confers the bacterium enough time to repair the DNA-damage and to outlast a short period of cellular stress. In this scenario Fur-mediated repression would prevent the cell against high concentrations of intracellular ColIb. This simplifies elimination of small amounts of intracellular ColIb.

Furthermore, as the exploitation of prophage lysis genes for colicin release is not restricted to a specific colicin it can also release all types of colicins. This is of advantage as colicinogenic plasmids can harbor more than one colicin, as e.g. pColBM encodes genes for colicin B and M. Prophage-mediated release of both colicins would save costs as instead of two lysis genes only prophage lysis genes would have to be expressed. This leads also to the question, if other group B colicins without cognate lysis genes are also released by prophage-mediated cell lysis as for instance ColB and ColM (**section 5.3.3**). Generation of bacterial mutants with and without prophages as well as generation of reporter tools would be required to address this question.

Group B colicin plasmids can be easily transferred into colicin sensitive strains by conjugation. These strains would pay the cost for colicin production and its cognate immunity protein but would not gain ColIb dependent growth benefit as the colicin remains intracellular. Thus only if the plasmid acceptor strain carries prophages, it can benefit from colicin production. This circumstance would serve as selective pressure on maintenance of intact prophages or cryptic prophages with intact lysis function. Therefore, outsourcing colicin lysis

genes to prophages would also enable the use of a colicin plasmid directly as “weapon”. For instance, it was shown that upon gut inflammation colicin Ib plasmid p2 of *S.* Tm^{SL1344} can be transferred by conjugation with an efficiency of about 100% (Stecher *et al.*, 2012b). Under such conditions, plasmid p2 could be conjugated into ColIb-sensitive *E. coli* lacking functional prophages. This *E. coli* strain would suffer from the cost of ColIb production (i.e metabolic load of protein synthesis) but would not benefit from ColIb production as this strain lacks the ColIb release mechanism (Nedialkova *et al.*, 2015). Furthermore, the requirement of prophages for colicin release secures the co-occurrence of colicin plasmids and prophages and thus provides the simultaneous release of two different weapons: the toxin colicin and infectious phage progenies. According to this, outsourcing colicin lysis genes from the colicin plasmid would broaden the range of targeted competitors. Competitors might be killed either by the bactericidal activity of colicins, by the increased metabolic load of colicin production provided by the colicin plasmid, or by phage-dependent lysis.

5.2.2.1 Does prophage-mediated release of ColIb provide an evolutionary stable strategy to prevent the emergence of cheaters?

Furthermore, uncoupling of colicin production and immunity from cell lysis may lead to evolutionary stabilization of the ColIb production phenotype and may prevent the emergence of cheaters (e.g. lysis-deficient mutants). Therefore it may form an evolutionary stable strategy (ESS) even in a well-mixed environment. Colicin production is highly costly, since the producer lyses to release colicin. Thus, the emergence of cheaters, e.g. defective in cell lysis, is very likely. As a consequence, cheaters do not contribute to the provision of the public good (=colicin) but benefit from its production by the cooperators. In case, the genes for colicin, immunity and lysis are encoded on the same location (plasmid or chromosome) cheaters usually have a fitness benefit over the cooperators. In well-mixed environments (e.g. in batch cultures) cheaters can arise and outcompete cooperators, which bear the burden of colicin release. The emergence and spread of cheaters can only be prevented if counteracting mechanisms exist such as bottlenecks, which promote group selection (Ackermann *et al.*, 2008). In contrast to this, in spatially structured environments (e.g. growth on agar plates or in biofilms), cooperators which produce and release the common good (colicin) will have a benefit over cheaters, as only those can invade other territories of colicin sensitive populations (Chao and Levin, 1981, Kerr, 2007).

Temperate phages only have a fitness benefit if their lysis genes are functional, to secure the release of infectious phage particles and the infection of a new host to propagate. This fitness benefit would be lost, if phage lysis genes were disrupted. Furthermore, this would lead to extinction of the phage. Thus, the selective pressure for phages to maintain functional

lysis genes is very high. According to this outsourcing costly lysis from colicin production and immunity would evolutionary stabilize the colicin production phenotype. Moreover, a single bacterium can harbor several prophages. Thus, if a bacterium harbored a prophage with disrupted lysis genes, infection with a new functional phage could restore prophage-mediated cell lysis. This presumes that not a particular prophage is required for colicin release. Interestingly, it was shown, ColIb is released by prophage ST64B-mediated cell lysis in *S.* Tm but also by prophage 933W-mediated lysis in *E. coli* (Nedialkova *et al.*, 2015). This suggests that not a particular prophage is required for ColIb release. Additionally, a future work objective will be, to investigate if prophage-mediated colicin release provides an ESS to stabilize the ColIb production phenotype and prevent the emergence of cheaters. This will be analyzed by mathematical modeling (e.g. by adaptive dynamics) as well as suitable *in vitro* studies.

5.2.3 Colicin production and release by prophage-mediated cell lysis *in vivo*

It was shown that *S.* Tm can colonize the mammalian intestine and induce inflammation (Stecher *et al.*, 2007). This inflammation is primarily characterized by transmigration of neutrophils into the gut lumen, which produce antibacterial proteins like lipocalin-2, elastase, calprotectin and antibacterial effectors like reactive oxygen and nitrogen species (ROS, RNS). These neutrophil effectors induce iron-starvation or DNA damage in bacteria and thus lead to induction of the SOS-response (Winter *et al.*, 2010b, Loetscher *et al.*, 2012). Furthermore, it was shown that inflammation leads to a bloom of Enterobacteriaceae, which are usually found at low densities ($<10^8$ cfu/g) in a healthy gut (Stecher *et al.*, 2012a). Under these stressful environmental conditions *S.* Tm upregulates expression of the colicin Ib gene which, in turn, enables *S.* Tm to outcompete commensal *E. coli* (Nedialkova *et al.*, 2014). Furthermore, a recent *in vitro* study from our lab elucidated the release mechanism of ColIb (Nedialkova *et al.*, 2015). Like the majority of colicins, prophages are activated by the SOS-response to induce the lytic cycle (Oppenheim *et al.*, 2005). Interestingly, it has been reported recently that IBD (inflammatory bowel disease) patients exhibit increased fecal phage titer compared to healthy controls (Norman *et al.*, 2015). Therefore, it is likely that gut inflammation leads to induction of prophages, which in turn mediate cell lysis and release of phage particles and colicin Ib, explaining the ColIb-dependent fitness benefit of *S.* Tm over *E. coli*. After all, the relative importance of iron-starvation and induction of the SOS-response for *cib* expression and thus prophage mediated lysis was not elucidated in detail *in vivo*. Due to the high costs of colicin production (death of the producer) it is important to keep a balance between the colicin-mediated fitness loss and the fitness gain. Therefore colicin production and its release should be tightly regulated and restricted to environmental niches

with high frequencies of potential competitors such as in Enterobacterial blooms. Further studies will be directed to define the optimal rate for *cib* expression and prophage-mediated lysis *in vivo* to explore the conditions how ColIb production and the release by prophage-mediated lysis has evolved as cooperative phenotype in Enterobacteriaceae.

5.3 How do colicin producers become immune to their own colicin?

Ec^{252R} shows a self-killing phenotype under SOS-response conditions. By performing transposon library screens the Ec^{252R} mutant strains Ec^{P10A10} (no self-killing) and Ec^{P13C5} (reduced self-killing) could be isolated. Further analysis revealed that Ec^{252R} harbors a pColBM plasmid and in case of Ec^{P10A10} the transposon Tn*10* was inserted at the beginning of the gene encoded for colicin M (*cma*). Generation and analysis of mutant strains with deletion either of *cma* or *cba* (colicin B) revealed that *cma* expression is responsible for the self-killing phenotype of Ec^{252R} (**Figure 4.20**). Colicin M-mediated self-killing of Ec^{252R} was surprising, as generally, colicinogenic bacteria are protected against their own colicins by simultaneous production of a cognate immunity protein, which is usually encoded on the same locus (Cascales *et al.*, 2007). Further investigations showed that pColBM of Ec^{252R} also encodes a ColM immunity protein (*cmi*), which is expressed constitutively at low levels and increased by MitC (Stayrook *et al.*, 2008) and Fe^{2+}-limitation (DTPA) (**Figure 4.21B**). Furthermore, although *cmi* of Ec^{252R} exhibits a point mutation (G233T), which results in an amino acid exchange (D26Y) in the hinge region of CMI (**Figure 4.22**), it is as functional as other ColM immunity proteins of immune ColM producers (**Figure 4.23E**). Interestingly, the same experiment revealed that, in general, the ColM immunity protein does not fully protect bacteria against ColM, suggesting that full ColM-immunity is not only mediated by ColM immunity protein. Nuclease colicins are directly active after synthesis and thus have to be inactivated by their cognate immunity protein already in the cytosol of the producing strain. In contrast, the immunity protein of ColM is integrated in the periplasmic leaflet of the IM as it is the case for pore-forming colicins (Usón *et al.*, 2012). According to the literature, this should sufficiently protect bacteria from ColM toxicity. In the course of this thesis two other *E. coli* strains which harbor the pColBM plasmid (Ec^{CI139} and Ec^{B195}) were also found to be only partially immune against their own colicins. Thus this seems to be a property of ColM producers that can be encountered occasionally.

Taken all data together I hypothesized that bacteria which have just taken up a pColBM plasmid by conjugation need to adapt to ColM production and its cytotoxicity. Thus Ec^{252R}

might be a candidate for an evolutionary "early" ColBM producer that needs to adapt to become fully ColM resistant.

5.3.1 Adaptation to colicin M production

Is there evidence that colicin M producers, in general, have to adapt to become fully ColM resistant? Interestingly, immunity proteins of nuclease-type colicins form tight complexes with their cognate colicins. In fact, the interaction of Im9 to its ColE9 has been shown to be a very strong protein-protein interaction (Wallis *et al.*, 1995). In contrast, the interaction between ColM immunity protein and the enzymatically active ColM is very weak (Usón *et al.*, 2012). Additionally, it was shown that immunity can be overcome by high colicin concentrations (Levisohn *et al.*, 1968, Šmajs *et al.*, 2006). Considering this and with the finding, that *cmi* expression alone is not sufficient to protect bacteria against ColM (**Figure 4.23E**) additional resistance mechanisms besides CMI-mediated immunity may have evolved.

To test the hypothesis, an *in vitro* evolution experiment was carried out. As ColM is usually encoded together with ColB on the same conjugative plasmid (Cascales *et al.*, 2007) a pColBM plasmid was transferred in a ColBM sensitive *E. coli* strain. Indeed, this "new" ColBM producer was not completely immune to ColM and exhibited a "self-killing" phenotype in the presence of MitC. This strain was passaged several times on LB agar plates containing MitC. Results however showed that only one strain (Ec^{MG1655} $pColBM^{B1144}$ S6) showed a slightly decreased self-killing phenotype compared to its parental strain (Ec^{MG1655} $pColBM^{B1144}$) (**Figure 4.29**). All other strains exhibited the same ColM sensitivity after five rounds of passaging.

It was shown that prophage-mediated cell lysis contributes to ColIb release in *S.* Tm (Nedialkova *et al.*, 2015). This was not only restricted to a *S.* Tm strain but also shown for ColIb release by *E. coli* strains harboring a ColIb plasmid and a functional prophage 933W (Ec^{Stx}). In contrast, ColIb release was not observed in a derivative of Ec^{Stx} with disrupted 933W prophage lysis genes ($Ec^{Stx\Delta S\ R}$). Thus only Ec^{Stx} with intact lysis genes released ColIb in the supernatant (Nedialkova *et al.*, 2015). According to this, we assumed that prophage-mediated cell lysis may also contribute to ColM release and the prophage may thus be required for the adaptation process. Therefore, pColBM was also transferred in Ec^{Stx} and in the derivative $Ec^{Stx\Delta S\ R}$. These strains were also passaged several times. As expected, results confirmed that the prophage-harboring strain (Ec^{Stx} $pColBM^{B1144}$) released higher amounts of ColBM compared to its derivative lacking the prophage lysis genes ($Ec^{Stx\Delta S\ R}$ $pColBM^{B1144}$) and compared to *E. coli* lacking the entire prophage (Ec^{MG1655} $pColBM^{B1144}$) (**Figure 4.28**). Interestingly, it seems that only release of ColM is increased in the presence of a functional

prophage compared to a strain lacking the entire prophage, whereas the release of ColB was similar in these strains (**Figure 4.28**). Comparing colicin release of the strain harboring the intact prophage and its derivative lacking the prophage lysis genes, the results showed expression of 933W prophage lysis genes increased ColM and also slightly ColB release (**Figure 4.28**). Therefore, prophage-mediated lysis mainly contributes to the release of ColM and this might explain the slightly decreased self-killing phenotype of a ColBM producer strain lacking the 933W prophage lysis genes compared to the strain harboring the intact prophage (**Figure 4.29**). However, 933W prophage-mediated ColM release did not alter the adaption process to a fully ColM resistant strain under the tested conditions.

In the course of this experiment passaging of the strain lysogenic for temperate 933W but lacking the prophage lysis genes, resulted in the mutant strain $Ec^{Stx\Delta S\ R}$ $pColBM^{B1144}$ S6. This mutant showed a decrease in ColB-dependent killing compared to its parental strain (**Figure 4.28B**).

In conclusion, only two mutant strains were identified showing either a slight decreased self-killing phenotype (Ec^{MG1655} $pColBM^{B1144}$ S6) or a phenotype with decreased ColB-dependent killing ($Ec^{Stx\Delta S\ R}$ $pColBM^{B1144}$ S6). This suggests that the *in vitro* evolution experiment was insufficient to select for mutants which become fully immune to ColM production. A possible explanation for this could be that the selective pressure by growth on LB agar plates containing the antibiotic MitC was too low. Future *in vitro* evolution experiments should be performed for instance encompassing more rounds of passaging. Or passaging of the strains could be done in liquid medium containing MitC. This may create increased selective pressure for ColM resistant mutants, as bacteria are all exposed to the same concentration of MitC. The use of recombinant ColM as selective pressure could also be a useful tool to select for ColM resistant strains, especially because purified ColM can be applied in defined concentrations.

Further future tasks will be to analyze the mutant strains Ec^{MG1655} $pColBM^{B1144}$ S6 (decreased self-killing phenotype) and $Ec^{Stx\Delta S\ R}$ $pColBM^{B1144}$ S6 (decreased ColB release). Therefore, genomes of both mutant strains will be sequenced to identify localization of the mutations. Western blot analysis of Ec^{MG1655} $pColBM^{B1144}$ S6 using ColM specific antiserum will give insights whether colicin production is decreased or only ColM-release. The generation of a specific ColB antiserum would be of advantage to study ColB production as well. This would allow us to investigate if the observed phenotype of $Ec^{Stx\Delta S\ R}$ $pColBM^{B1144}$ S6 is due to decreased c*ba* expression, or if ColB release is defective. The *in vitro* evolution experiment could also be repeated with other ColM sensitive *E. coli* strains.

5.3.2 Is the self-killing phenotype also found in other "new" colicin producers?

Mutations in the *cba* gene appear to be frequent (Christenson and Gordon, 2009). Christenson and Gordon showed that the majority (~90%) of *E. coli* strains harboring the pColBM plasmid encode, instead of full length *cba*, only a truncated version (*cba*-remnant). Interestingly, these strains exhibit only a rather low *cma* expression (Christenson and Gordon, 2009). Such a "polar" effect on *cma* expression has also been shown before (Ölschläger *et al.*, 1984). It was demonstrated that a transposon insertion in *cba* reduces *cma* expression (Ölschläger *et al.*, 1984). This is reasonable, since *cba* is encoded upstream of *cma* and both are regulated by the same promoter (**Figure 4.19A**).

In the course of this study, a collection of 35 *E. coli* strains identified as ColM-producers was characterized. Our results revealed that the majority of these strains (63%) encode a *cba*-remnant version instead of a full length *cba* (**Table 4.2**). As noted above, this mutation leads to downregulation of ColM production in these strains (**Table 4.2** and **Figure 4.25**). In this case a derivative of a ColBM plasmid might have become positively selected and spread in the *E. coli* population, on which both, *cba* and *cma* expression is non-functional. Truncation of *cba* also leads to deletion of the common promoter, and thus to massive reduction of *cma* expression. It can be speculated that the ancestor ColBM producer strain experienced a benefit from this mutation as it alleviated self-killing. Why has this plasmid spread so successfully in the *E. coli* population? It can be assumed that the plasmid harbors other fitness factors besides *cba* and *cma* that lead to positive selection of this plasmid(s), such as virulence factors like siderophore receptors, iron transport systems, antibiotic resistances or pili (Christenson and Gordon, 2009, Kröger *et al.*, 2012). Interestingly a different study observed that the majority of *E. coli* isolates from wild mice (50-98%) are resistant against different colicins (Riley and Wertz, 2002a). This supports the idea that, besides exhibiting immunity to self-produced colicins, colicin producers, in general, may benefit from mutations rendering them colicin resistant. This is actually in contrast to the concept "rock-paper-scissors", where a colicin sensitive strain would beat a resistant strain as it does not suffer from costs arising by mutations in the import pathway (Hibbing *et al.*, 2010). This suggests that immunity to colicins alone is generally insufficient to protect colicin producers against self-killing. As a consequence all strains which just have become colicin-producers would show a (more or less pronounced) self-killing phenotype and hence may accumulate mutations and become fully resistant to their produced colicins.

It was shown that the ColIb-producer S.Tm^{SL1344} is resistant to its own colicin even after the strain was cured from the colicin plasmid p2 (Stecher, unpublished data). To test if ColIb immunity protein does sufficiently protect the strain against self-killing, the ColIb plasmid p2 was transferred in a colicin-sensitive strain (Ec^{MG1655}) by conjugation. However, this strain

was highly immune to ColIb. After curing the strain of p2 it became sensitive to ColIb as before (data not shown). In conclusion, production of the ColIb immunity protein is apparently sufficient to protect *E. coli* against self-killing. Based on these first results, other colicin producers also should be tested to further support our hypothesis. However, the self-killing phenotype may also only be restricted to ColM producers. This might be explained by the fact that ColM has a unique mode of action among all colicins (El Ghachi *et al.*, 2006). In addition, its immunity protein is (in contrast to other enzymatic colicins) inserted in the inner membrane of the bacterium (similar to immunity proteins pore-forming colicins) and forms a dimer to protect against ColM toxicity (Olschläger and Braun, 1987, Cascales *et al.*, 2007, Usón *et al.*, 2012).

5.3.3 Is prophage-mediated release also true for group B colicins ColB and ColM?

ColB and ColM are classified as group B colicins, thus they do not encode cognate lysis genes and their release mechanism is still unclear. Recently, it has been shown that the pore-forming group B colicin Ib (ColIb) is released by prophage-mediated cell lysis (Nedialkova *et al.*, 2015). Interestingly, the transposon library screen conducted in this thesis yielded a mutant strain (Ec^{P13C5}) which showed a reduced self-killing phenotype (**Figure 4.18**). The insertion of Tn*10* in Ec^{P13C5} was located in a gene for a putative prophage protein of Ec^{252R}. However, this prophage gene was excluded to be a lysis gene. Interestingly, the self-killing phenotype was almost lost but this mutant was still able to kill sensitive Ec^{MG1655} as efficiently as ColM-deficient Ec^{P10A10}. This suggests, that mainly ColM is released by prophages, or at least, by this prophage. This hypothesis was also supported by the observation that increased ColM release was observed in Ec^{MG1655} when transduced with intact prophage 933W. Further analysis is required, for instance identification of all Ec^{252R} prophages followed by generation and analysis of Ec^{252R} mutant strains lacking prophage lysis genes or cured from all prophages, and additionally carrying deletions in either *cba* or *cma*. Furthermore, generation of a *fepA* mutant strain, the gene encoding the ColB receptor (FepA), would be necessary. Using this *fepA* mutant as test strain in the overlay of a halo-assay, ColM-dependent killing and thus ColM-release could be investigate.

In fact, ColB and ColM might be released by different mechanisms. It was shown that although ColM is detected as clear band in the cell pellet by western blot analysis, it is released only in small amounts by a fraction of the population (1%) (Ölschläger, 1991). Surprisingly, this small amount is sufficient to inhibit growth of sensitive bacteria (Ölschläger, 1991). Interestingly, in the course of this work, western blot analysis for ColM production and release by 13 ColM-producing *E. coli* strains (including Ec^{252R}) revealed that in half of the

strains, ColM was only detected in the cell pellet but not or in rather low amounts, in the supernatant. This suggests that ColM concentration in the supernatant is rather low and under the detection limit of a western blot analysis. Ölschläger (1991) assumed that according to the low amounts of released ColM, ColM might be secreted without cell lysis (Ölschläger, 1991). Intriguingly, it was shown that ColM (one of the smallest colicins (~30 kDa) is able to enter the cell from the outside by osmotic shock in a strain lacking the ColM-receptor (Harkness and Braun, 1990). This suggests that ColM is small enough to enter the cell and possibly to exit the cell without an active transport machinery. Whether ColM-producing (of an immune ColM producer) bacteria do not lyse during colicin production and colicin release could be tested by single cell analysis using the CellASIC® ONIX Microfluidic Platform (Millipore).

Although ColB has a higher molecular mass (~55 kDa) compared to ColM, it is assumed that ColB is released by cell leakage, since it was also only detected in low amounts in the supernatant similar to ColM (Mende and Braun, 1990, Ölschläger, 1991, Ölschläger *et al.*, 1984). Mende *et al.* could also show in their study that ColB can enter the cell from the outside by osmotic shock in a strain lacking the ColB receptor FepA (Mende and Braun, 1990). This suggests that ColB might also exit the cell without an active transport machinery or cell lysis. Secretion of ColB might also explain the phenotype of Ec^{P13C5} which was observed to kill sensitive Ec^{MG1655} as efficiently as the ColM-deficient strain Ec^{P10A10}. Furthermore, our results showed that ColB release was not increased in Ec^{MG1655} when transduced with intact prophage 933W.

Taken together, our data supports the hypothesis that ColB is secreted by its producer by a so far unknown mechanism not involving phage-mediated cell lysis. Whether ColB-producing (of an immune ColB producer) bacteria do not lyse during colicin production and colicin release could be tested by single cell analysis using the CellASIC® ONIX Microfluidic Platform (Millipore).

Supplementary Data

Movie S1: Live cell microscopy of *S.* Tm^{WT} p$^{P cib\ gfp}$. First, microcolonies of *S.* Tm^{WT} p$^{P cib\ gfp}$ were grown for 3 h in LB, in the Onix microfluidic system (Millipore). Afterwards, media was switched first to LB medium supplemented with 100 µM DTPA (Fe^{2+}-limiting conditions). After 20 min the SOS-response was induced by applying LB supplemented with 100 µM DTPA and 0.5 µg/ml MitC. Again after 20 min, media was switched back to LB supplemented with 100 µM DTPA only, for the remaining time. **(A)** Merge of brightfield and GFP-channel and **(B)** GFP-channel only.

Movie S2: Live cell microscopy of *S.* Tm$^{\Delta Ph}$ p$^{P cib\ gfp}$. First, microcolonies of *S.* Tm$^{\Delta Ph}$ p$^{P cib\ gfp}$ were grown for 3 h in LB, in the Onix microfluidic system (Millipore). Afterwards, media was switched first to LB medium supplemented with 100 µM DTPA (Fe^{2+}-limiting conditions). After 20 min the SOS-response was induced by applying LB supplemented with 100 µM DTPA and 0.5 µg/ml MitC. Again after 20 min, media was switched back to LB supplemented with 100 µM DTPA only, for the remaining time. **(A)** Merge of brightfield and GFP-channel and **(B)** GFP-channel only.

Literature

ACKERMANN, M. 2015. A functional perspective on phenotypic heterogeneity in microorganisms. *Nature Reviews Microbiology,* 13**,** 497-508.

ACKERMANN, M., STECHER, B., FREED, N. E., SONGHET, P., HARDT, W.-D. & DOEBELI, M. 2008. Self-destructive cooperation mediated by phenotypic noise. *Nature,* 454**,** 987-990.

ALEXEYEV, M. F. & SHOKOLENKO, I. N. 1995. Mini-Tn10 transposon derivatives for insertion mutagenesis and gene delivery into the chromosome of gram-negative bacteria. *Gene,* 160**,** 59-62.

ALONSO, A., PUCCIARELLI, M. G., FIGUEROA-BOSSI, N. & GARCIA-DEL PORTILLO, F. 2005. Increased excision of the Salmonella prophage ST64B caused by a deficiency in Dam methylase. *J Bacteriol,* 187**,** 7901-11.

ARKIN, A., ROSS, J. & MCADAMS, H. H. 1998. Stochastic kinetic analysis of developmental pathway bifurcation in phage λ-infected Escherichia coli cells. *Genetics,* 149**,** 1633-1648.

BADARINARAYANA, V., ESTEP, P. W., 3RD, SHENDURE, J., EDWARDS, J., TAVAZOIE, S., LAM, F. & CHURCH, G. M. 2001. Selection analyses of insertional mutants using subgenic-resolution arrays. *Nat Biotechnol,* 19**,** 1060-5.

BAGGESEN, D. L., SORENSEN, G., NIELSEN, E. & WEGENER, H. C. 2010. Phage typing of Salmonella Typhimurium-is it still a useful tool for surveillance and outbreak investigation. *Euro Surveill,* 15**,** 19471.

BAHAROGLU, Z. & MAZEL, D. 2014. SOS, the formidable strategy of bacteria against aggressions. *FEMS microbiology reviews,* 38**,** 1126-1145.

BALABAN, N. Q., MERRIN, J., CHAIT, R., KOWALIK, L. & LEIBLER, S. 2004. Bacterial persistence as a phenotypic switch. *Science,* 305**,** 1622-1625.

BEBEACUA, C., FAJARDO, L., CARLOS, J., BLANGY, S., SPINELLI, S., BOLLMANN, S., NEVE, H., CAMBILLAU, C. & HELLER, K. J. 2013. X-ray structure of a superinfection exclusion lipoprotein from phage TP-J34 and identification of the tape measure protein as its target. *Molecular microbiology,* 89**,** 152-165.

BERNHARDT, T. G., STRUCK, D. K. & YOUNG, R. 2001a. The lysis protein E of φX174 is a specific inhibitor of the MraY-catalyzed step in peptidoglycan synthesis. *Journal of Biological Chemistry,* 276**,** 6093-6097.

BERNHARDT, T. G., WANG, N., STRUCK, D. K. & YOUNG, R. 2001b. A protein antibiotic in the phage Qβ virion: diversity in lysis targets. *Science,* 292**,** 2326-2329.

BERNHARDT, T. G., WANG, N., STRUCK, D. K. & YOUNG, R. 2002. Breaking free:"protein antibiotics" and phage lysis. *Research in microbiology,* 153**,** 493-501.

BERRY, J., SUMMER, E. J., STRUCK, D. K. & YOUNG, R. 2008. The final step in the phage infection cycle: the Rz and Rz1 lysis proteins link the inner and outer membranes. *Molecular microbiology,* 70**,** 341-351.

BOLIVAR, F., RODRIGUEZ, R. L., GREENE, P. J., BETLACH, M. C., HEYNKER, H. L., BOYER, H. W., CROSA, J. H. & FALKOW, S. 1992. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. 1977. *Biotechnology,* 24**,** 153-71.

BOSSI, L., FUENTES, J. A., MORA, G. & FIGUEROA-BOSSI, N. 2003. Prophage contribution to bacterial population dynamics. *Journal of bacteriology,* 185**,** 6467-6471.

BRÜSSOW, H., CANCHAYA, C. & HARDT, W.-D. 2004. Phages and the evolution of bacterial pathogens: from genomic rearrangements to lysogenic conversion. *Microbiology and Molecular Biology Reviews,* 68**,** 560-602.

BUTALA, M., SONJAK, S., KAMENSEK, S., HODOSCEK, M., BROWNING, D. F., ZGUR-BERTOK, D. & BUSBY, S. J. 2012. Double locking of an Escherichia coli promoter by

two repressors prevents premature colicin expression and cell lysis. *Mol Microbiol,* 86**,** 129-39.
BUTALA, M., ŽGUR-BERTOK, D. & BUSBY, S. J. 2009. The bacterial LexA transcriptional repressor. *Cellular and Molecular Life Sciences,* 66**,** 82-93.
CAMPBELL, A. 2003. The future of bacteriophage biology. *Nature Reviews Genetics,* 4**,** 471-477.
CASCALES, E., BUCHANAN, S. K., DUCHE, D., KLEANTHOUS, C., LLOUBES, R., POSTLE, K., RILEY, M., SLATIN, S. & CAVARD, D. 2007. Colicin biology. *Microbiol Mol Biol Rev,* 71**,** 158-229.
CAVARD, D. 1997. Role of the colicin A lysis protein in the expression of the colicin A operon. *Microbiology,* 143**,** 2295-2303.
CESARENI, G., MUESING, M. A. & POLISKY, B. 1982. Control of ColE1 DNA replication: the rop gene product negatively affects transcription from the replication primer promoter. *Proceedings of the National Academy of Sciences,* 79**,** 6313-6317.
CHAO, L. & LEVIN, B. R. 1981. Structured habitats and the evolution of anticompetitor toxins in bacteria. *Proceedings of the National Academy of Sciences,* 78**,** 6324-6328.
CHEN, Y.-R., YANG, T.-Y., LEI, G.-S., LIN, L.-J. & CHAK, K.-F. 2011. Delineation of the translocation of colicin E7 across the inner membrane of Escherichia coli. *Archives of microbiology,* 193**,** 419-428.
CHEREPANOV, P. P. & WACKERNAGEL, W. 1995. Gene disruption in Escherichia coli: TcR and KmR cassettes with the option of Flp-catalyzed excision of the antibiotic-resistance determinant. *Gene,* 158**,** 9-14.
CHRISTENSON, J. K. & GORDON, D. M. 2009. Evolution of colicin BM plasmids: the loss of the colicin B activity gene. *Microbiology,* 155**,** 1645-55.
CLEWELL, D. B. & HELINSKI, D. R. 1970. Existence of the colicinogenic factor-sex factor coli-P9 as a supercoiled circular DNA-protein relaxation complex. *Biochemical and biophysical research communications,* 41**,** 150-156.
COBURN, B., GRASSL, G. A. & FINLAY, B. 2007. Salmonella, the host and disease: a brief review. *Immunology and cell biology,* 85**,** 112-118.
COLEMAN, D. C., SULLIVAN, D. J., RUSSEL, R. J., P, A. J., CAREY, B. F. & POMEROY, H. M. 1989. Staphylococcus aureus bacteriophages mediating the simultaneous lysogenic conversion of β-lysin, staphylokinase and enterotoxin A: molecular mechanism of triple conversion. *Journal of general microbiology,* 135**,** 1679-1697.
COMEAU, A. M., HATFULL, G. F., KRISCH, H. M., LINDELL, D., MANN, N. H. & PRANGISHVILI, D. 2008. Exploring the prokaryotic virosphere. *Research in microbiology,* 159**,** 306-313.
CORNFORTH, D. M. & FOSTER, K. R. 2013. Competition sensing: the social side of bacterial stress responses. *Nature Reviews Microbiology,* 11**,** 285-293.
COURT, D. L., OPPENHEIM, A. B. & ADHYA, S. L. 2007. A new look at bacteriophage lambda genetic networks. *J Bacteriol,* 189**,** 298-304.
CRONAN, J. E. 2006. A family of arabinose-inducible Escherichia coli expression vectors having pBR322 copy control. *Plasmid,* 55**,** 152-157.
CZÁRÁN, T. L., HOEKSTRA, R. F. & PAGIE, L. 2002. Chemical warfare between microbes promotes biodiversity. *Proceedings of the National Academy of Sciences,* 99**,** 786-790.
D'HERELLE, F. 1917. Sur un microbe invisible antagoniste des bacilles dysentériques. *CR Acad. Sci. Paris,* 165**,** 373-375.
DATSENKO, K. A. & WANNER, B. L. 2000. One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. *Proc Natl Acad Sci U S A,* 97**,** 6640-5.
DAVIES, J. K. & REEVES, P. 1975a. Genetics of resistance to colicins in Escherichia coli K-12: cross-resistance among colicins of group A. *Journal of Bacteriology,* 123**,** 102-117.
DAVIES, J. K. & REEVES, P. 1975b. Genetics of resistance to colicins in Escherichia coli K-12: cross-resistance among colicins of group B. *Journal of Bacteriology,* 123**,** 96-101.
DONNENBERG, M. S. 2002. *Escherichia coli: virulence mechanisms of a versatile pathogen*, Elsevier.

DOZOIS, C. M. & CURTISS, R. 1999. Pathogenic diversity of Escherichia coli and the emergence of'exotic'islands in the gene stream. *Veterinary research,* 30**,** 157-179.

ECKBURG, P. B., BIK, E. M., BERNSTEIN, C. N., PURDOM, E., DETHLEFSEN, L., SARGENT, M., GILL, S. R., NELSON, K. E. & RELMAN, D. A. 2005. Diversity of the human intestinal microbial flora. *science,* 308**,** 1635-1638.

EL GHACHI, M., BOUHSS, A., BARRETEAU, H., TOUZÉ, T., AUGER, G., BLANOT, D. & MENGIN-LECREULX, D. 2006. Colicin M exerts its bacteriolytic effect via enzymatic degradation of undecaprenyl phosphate-linked peptidoglycan precursors. *Journal of Biological Chemistry,* 281**,** 22761-22772.

ELOWITZ, M. B., LEVINE, A. J., SIGGIA, E. D. & SWAIN, P. S. 2002. Stochastic gene expression in a single cell. *Science,* 297**,** 1183-1186.

ESPESSET, D., DUCHE, D., BATY, D. & GELI, V. 1996. The channel domain of colicin A is inhibited by its immunity protein through direct interaction in the Escherichia coli inner membrane. *The EMBO journal,* 15**,** 2356.

FÀBREGA, A. & VILA, J. 2013. Salmonella enterica serovar Typhimurium skills to succeed in the host: virulence and regulation. *Clinical microbiology reviews,* 26**,** 308-341.

FIALKOW, L., WANG, Y. & DOWNEY, G. P. 2007. Reactive oxygen and nitrogen species as signaling molecules regulating neutrophil function. *Free Radical Biology and Medicine,* 42**,** 153-164.

FIGUEROA-BOSSI, N. & BOSSI, L. 2004. Resuscitation of a defective prophage in Salmonella cocultures. *Journal of bacteriology,* 186**,** 4038-4041.

FIGUEROA-BOSSI, N., UZZAU, S., MALORIOL, D. & BOSSI, L. 2001. Variable assortment of prophages provides a transferable repertoire of pathogenic determinants in Salmonella. *Mol Microbiol,* 39**,** 260-71.

FIGUEROA-BOSSI, N., COISSAC, E., NETTER, P. & BOSSI, L. 1997. Unsuspected prophage-like elements in Salmonella typhimurium. *Molecular microbiology,* 25**,** 161-173.

FOSTER, K. R. & BELL, T. 2012. Competition, not cooperation, dominates interactions among culturable microbial species. *Current biology,* 22**,** 1845-1850.

FRIEBEL, A., ILCHMANN, H., AEPFELBACHER, M., EHRBAR, K., MACHLEIDT, W. & HARDT, W.-D. 2001. SopE and SopE2 from Salmonella typhimurium activate different sets of RhoGTPases of the host cell. *Journal of Biological Chemistry,* 276**,** 34035-34040.

FRYE, J. G., PORWOLLIK, S., BLACKMER, F., CHENG, P. & MCCLELLAND, M. 2005. Host gene expression changes and DNA amplification during temperate phage induction. *Journal of bacteriology,* 187**,** 1485-1492.

GHAZARYAN, L., TONOYAN, L., ASHHAB, A. A., SOARES, M. I. & GILLOR, O. 2014. The role of stress in colicin regulation. *Arch Microbiol,* 196**,** 753-64.

GILLOR, O., VRIEZEN, J. A. & RILEY, M. A. 2008. The role of SOS boxes in enteric bacteriocin regulation. *Microbiology,* 154**,** 1783-92.

GORDON, D. M. & O'BRIEN, C. L. 2006. Bacteriocin diversity and the frequency of multiple bacteriocin production in Escherichia coli. *Microbiology,* 152**,** 3239-3244.

GORDON, D. M., OLIVER, E. & LITTLEFIELD-WYER, J. 2007. The diversity of bacteriocins in Gram-negative bacteria. *Bacteriocins.* Springer.

GRATIA, A. 1925. Sur un remarquable exemple d'antagonisme entre deux souches de colibacille. *CR Soc Biol,* 93**,** 1040-1041.

GUZMAN, L. M., BELIN, D., CARSON, M. J. & BECKWITH, J. 1995. Tight regulation, modulation, and high-level expression by vectors containing the arabinose PBAD promoter. *J Bacteriol,* 177**,** 4121-30.

HARDY, K., MEYNELL, G., DOWMAN, J. & SPRATT, B. 1973. Two major groups of colicin factors: their evolutionary significance. *Molecular and General Genetics MGG,* 125**,** 217-230.

HARKNESS, R. E. & BRAUN, V. 1990. Colicin M is only bactericidal when provided from outside the cell. *Molecular and General Genetics MGG,* 222**,** 37-40.

HARKNESS, R. E. & ÖLSCHLÄGER, T. 1991. The biology of colicin M. *FEMS microbiology reviews,* 88**,** 27-42.

HARTL, D. L. & DYKHUIZEN, D. E. 1984. The population genetics of Escherichia coli. *Annual review of genetics,* 18**,** 31-68.

HEBISCH, E., KNEBEL, J., LANDSBERG, J., FREY, E. & LEISNER, M. 2013. High variation of fluorescence protein maturation times in closely related Escherichia coli strains. *PLoS One,* 8**,** e75991.

HELBIG, S., PATZER, S. I., SCHIENE-FISCHER, C., ZETH, K. & BRAUN, V. 2011. Activation of colicin M by the FkpA prolyl cis-trans isomerase/chaperone. *Journal of Biological Chemistry,* 286**,** 6280-6290.

HELFRICH, S., PFEIFER, E., KRÄMER, C., SACHS, C. C., WIECHERT, W., KOHLHEYER, D., NÖH, K. & FRUNZKE, J. 2015. Live cell imaging of SOS and prophage dynamics in isogenic bacterial populations. *Molecular microbiology,* 98**,** 636-650.

HELLER, K. J. 1992. Molecular interaction between bacteriophage and the gram-negative cell envelope. *Archives of microbiology,* 158**,** 235-248.

HERRERO-FRESNO, A., LEEKITCHAROENPHON, P., HENDRIKSEN, R. S., OLSEN, J. E. & AARESTRUP, F. M. 2014. Analysis of the contribution of bacteriophage ST64B to in vitro virulence traits of Salmonella enterica serovar Typhimurium. *Journal of medical microbiology,* 63**,** 331-342.

HERSCHMAN, H. R. & HELINSKI, D. R. 1967. Comparative study of the events associated with colicin induction. *Journal of bacteriology,* 94**,** 691-699.

HIBBING, M. E., FUQUA, C., PARSEK, M. R. & PETERSON, S. B. 2010. Bacterial competition: surviving and thriving in the microbial jungle. *Nature Reviews Microbiology,* 8**,** 15-25.

HOISETH, S. K. & STOCKER, B. A. 1981. Aromatic-dependent Salmonella typhimurium are non-virulent and effective as live vaccines. *Nature,* 291**,** 238-9.

HOL, F. J., VOGES, M. J., DEKKER, C. & KEYMER, J. E. 2014. Nutrient-responsive regulation determines biodiversity in a colicin-mediated bacterial community. *BMC biology,* 12**,** 68.

HUDDLESTON, J. R. 2014. Horizontal gene transfer in the human gastrointestinal tract: Potential spread of antibiotic resistance genes. *Infection and drug resistance,* 7**,** 167.

HUSSEINY, M. I. & HENSEL, M. 2005. Rapid method for the construction of Salmonella enterica Serovar Typhimurium vaccine carrier strains. *Infect Immun,* 73**,** 1598-605.

JACOB, F., LWOFF, A., SIMINOVITCH, A. & WOLLMAN, E. Définition de quelques termes relatifs à la lysogénie. ANNALES DE L INSTITUT PASTEUR, 1953. MASSON EDITEUR 120 BLVD SAINT-GERMAIN, 75280 PARIS 06, FRANCE, 222-224.

JAKES, K. S. & FINKELSTEIN, A. 2010. The colicin Ia receptor, Cir, is also the translocator for colicin Ia. *Molecular microbiology,* 75**,** 567-578.

JOHNSON, C. L., RIDLEY, H., MARCHETTI, R., SILIPO, A., GRIFFIN, D. C., CRAWFORD, L., BONEV, B., MOLINARO, A. & LAKEY, J. H. 2014. The antibacterial toxin colicin N binds to the inner core of lipopolysaccharide and close to its translocator protein. *Molecular microbiology,* 92**,** 440-452.

JOHNSON, T. J., JOHNSON, S. J. & NOLAN, L. K. 2006. Complete DNA sequence of a ColBM plasmid from avian pathogenic Escherichia coli suggests that it evolved from closely related ColV virulence plasmids. *Journal of bacteriology,* 188**,** 5975-5983.

JUSTICE, S. S., HUNSTAD, D. A., CEGELSKI, L. & HULTGREN, S. J. 2008. Morphological plasticity as a bacterial survival strategy. *Nature Reviews Microbiology,* 6**,** 162-168.

KAMENŠEK, S., BROWNING, D. F., PODLESEK, Z., BUSBY, S. J., ŽGUR-BERTOK, D. & BUTALA, M. 2015. Silencing of DNase Colicin E8 Gene Expression by a Complex Nucleoprotein Assembly Ensures Timely Colicin Induction. *PLoS Genet,* 11**,** e1005354.

KAMENSEK, S., PODLESEK, Z., GILLOR, O. & ZGUR-BERTOK, D. 2010. Genes regulated by the Escherichia coli SOS repressor LexA exhibit heterogeneous expression. *BMC Microbiol,* 10**,** 283.

KARLYSHEV, A. V., PALLEN, M. J. & WREN, B. W. 2000. Single-primer PCR procedure for rapid identification of transposon insertion sites. *Biotechniques,* 28**,** 1078, 1080, 1082.

KERR, B. 2007. The ecological and evolutionary dynamics of model bacteriocin communities. *Bacteriocins.* Springer.

KERR, B., RILEY, M. A., FELDMAN, M. W. & BOHANNAN, B. J. 2002. Local dispersal promotes biodiversity in a real-life game of rock–paper–scissors. *Nature,* 418**,** 171-174.

KIM, Y. C., TARR, A. W. & PENFOLD, C. N. 2014. Colicin import into E. coli cells: a model system for insights into the import mechanisms of bacteriocins. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research,* 1843**,** 1717-1731.

KIRKUP, B. C. & RILEY, M. A. 2004. Antibiotic-mediated antagonism leads to a bacterial game of rock–paper–scissors in vivo. *Nature,* 428**,** 412-414.

KLEANTHOUS, C. 2010. Swimming against the tide: progress and challenges in our understanding of colicin translocation. *Nat Rev Microbiol,* 8**,** 843-8.

KLEANTHOUS, C., KÜHLMANN, U. C., POMMER, A. J., FERGUSON, N., RADFORD, S. E., MOORE, G. R., JAMES, R. & HEMMINGS, A. M. 1999. Structural and mechanistic basis of immunity toward endonuclease colicins. *Nature Structural & Molecular Biology,* 6**,** 243-252.

KLEANTHOUS, C. & WALKER, D. 2001. Immunity proteins: enzyme inhibitors that avoid the active site. *Trends in biochemical sciences,* 26**,** 624-631.

KLOOS, D.-U., STRÄTZ, M., GÜTTLER, A., STEFFAN, R. J. & TIMMIS, K. N. 1994. Inducible cell lysis system for the study of natural transformation and environmental fate of DNA released by cell death. *Journal of bacteriology,* 176**,** 7352-7361.

KÖCK, J., OLSCHLÄGER, T., KAMP, R. & BRAUN, V. 1987. Primary structure of colicin M, an inhibitor of murein biosynthesis. *Journal of bacteriology,* 169**,** 3358-3361.

KRÖGER, C., DILLON, S. C., CAMERON, A. D., PAPENFORT, K., SIVASANKARAN, S. K., HOKAMP, K., CHAO, Y., SITTKA, A., HÉBRARD, M. & HÄNDLER, K. 2012. The transcriptional landscape and small RNAs of Salmonella enterica serovar Typhimurium. *Proceedings of the National Academy of Sciences,* 109**,** E1277-E1286.

KUHAR, I. & ŽGUR-BERTOK, D. 1999. Transcription Regulation of the Colicin Kcka Gene Reveals Induction of Colicin Synthesis by Differential Responses to Environmental Signals. *Journal of bacteriology,* 181**,** 7373-7380.

LACATENA, R., BANNER, D., CASTAGNOLI, L. & CESARENI, G. 1984. Control of initiation of pMB1 replication: purified Rop protein and RNA I affect primer formation in vitro. *Cell,* 37**,** 1009-1014.

LEE, C. Y. & IANDOLO, J. J. 1985. Mechanism of bacteriophage conversion of lipase activity in Staphylococcus aureus. *Journal of bacteriology,* 164**,** 288-293.

LEVISOHN, R., KONISKY, J. & NOMURA, M. 1968. Interaction of colicins with bacterial cells IV. Immunity breakdown studied with colicins Ia and Ib. *Journal of bacteriology,* 96**,** 811-821.

LIN-CHAO, S. & BREMER, H. 1986. Effect of the bacterial growth rate on replication control of plasmid pBR322 in Escherichia coli. *Molecular and General Genetics MGG,* 203**,** 143-149.

LITTLE, A. E., ROBINSON, C. J., PETERSON, S. B., RAFFA, K. F. & HANDELSMAN, J. 2008. Rules of engagement: interspecies interactions that regulate microbial communities. *Annu Rev Microbiol,* 62**,** 375-401.

LIU, H. S., JAN, M. S., CHOU, C. K., CHEN, P. H. & KE, N. J. 1999. Is green fluorescent protein toxic to the living cells? *Biochem Biophys Res Commun,* 260**,** 712-7.

LIU, X., JIANG, H., GU, Z. & ROBERTS, J. W. 2013. High-resolution view of bacteriophage lambda gene expression by ribosome profiling. *Proceedings of the National Academy of Sciences,* 110**,** 11928-11933.

LOETSCHER, Y., WIESER, A., LENGEFELD, J., KAISER, P., SCHUBERT, S., HEIKENWALDER, M., HARDT, W.-D. & STECHER, B. 2012. Salmonella transiently reside in luminal neutrophils in the inflamed gut. *PLoS One,* 7**,** e34812.

LOPEZ, C. A., WINTER, S. E., RIVERA-CHÁVEZ, F., XAVIER, M. N., POON, V., NUCCIO, S.-P., TSOLIS, R. M. & BÄUMLER, A. J. 2012. Phage-mediated acquisition of a type III secreted effector protein boosts growth of salmonella by nitrate respiration. *MBio,* 3**,** e00143-12.

LUNA-CHÁVEZ, C., LIN, Y.-L. & HUANG, R. H. 2006. Molecular basis of inhibition of the ribonuclease activity in colicin E5 by its cognate immunity protein. *Journal of molecular biology,* 358**,** 571-579.

MADER, A., VON BRONK, B., EWALD, B., KESEL, S., SCHNETZ, K., FREY, E. & OPITZ, M. 2015. Amount of colicin release in Escherichia coli is regulated by lysis gene expression of the colicin E2 operon. *PLoS One,* 10**,** e0119124.

MADIGAN, M. T., MARTINKO, J. M. & BROCK, T. D. 2009. *Brock Mikrobiologie*, Pearson Deutschland GmbH.

MAJEED, H., GHAZARYAN, L., HERZBERG, M. & GILLOR, O. 2015. Bacteriocin expression in sessile and planktonic populations of Escherichia coli. *The Journal of antibiotics,* 68**,** 52-55.

MAJEED, H., GILLOR, O., KERR, B. & RILEY, M. A. 2011. Competitive interactions in Escherichia coli populations: the role of bacteriocins. *ISME J,* 5**,** 71-81.

MCGRATH, S., FITZGERALD, G. F. & SINDEREN, D. V. 2002. Identification and characterization of phage-resistance genes in temperate lactococcal bacteriophages. *Molecular microbiology,* 43**,** 509-520.

MCLAUGHLIN, L. M., GOVONI, G. R., GERKE, C., GOPINATH, S., PENG, K., LAIDLAW, G., CHIEN, Y.-H., JEONG, H.-W., LI, Z. & BROWN, M. D. 2009. The Salmonella SPI2 effector SseI mediates long-term systemic infection by modulating host cell migration. *PLoS Pathog,* 5**,** e1000671.

MENDE, J. & BRAUN, V. 1990. Import-defective colicin B derivatives mutated in the TonB box. *Molecular microbiology,* 4**,** 1523-1533.

MIAO, E. A. & MILLER, S. I. 2000. A conserved amino acid sequence directing intracellular type III secretion by Salmonella typhimurium. *Proceedings of the National Academy of Sciences,* 97**,** 7539-7544.

MIROLD, S., RABSCH, W., ROHDE, M., STENDER, S., TSCHÄPE, H., RÜSSMANN, H., IGWE, E. & HARDT, W.-D. 1999. Isolation of a temperate bacteriophage encoding the type III effector protein SopE from an epidemic Salmonella typhimurium strain. *Proceedings of the National Academy of Sciences,* 96**,** 9845-9850.

MMOLAWA, P. T., SCHMIEGER, H. & HEUZENROEDER, M. W. 2003. Bacteriophage ST64B, a genetic mosaic of genes from diverse sources isolated from Salmonella enterica serovar typhimurium DT 64. *Journal of bacteriology,* 185**,** 6481-6485.

MODI, S. R., LEE, H. H., SPINA, C. S. & COLLINS, J. J. 2013. Antibiotic treatment expands the resistance reservoir and ecological network of the phage metagenome. *Nature,* 499**,** 219-222.

MOLLER, A. K., LEATHAM, M. P., CONWAY, T., NUIJTEN, P. J., DE HAAN, L. A., KROGFELT, K. A. & COHEN, P. S. 2003. An Escherichia coli MG1655 lipopolysaccharide deep-rough core mutant grows and survives in mouse cecal mucus but fails to colonize the mouse large intestine. *Infect Immun,* 71**,** 2142-52.

MRAK, P., PODLESEK, Z., VAN PUTTEN, J. P. & ZGUR-BERTOK, D. 2007. Heterogeneity in expression of the Escherichia coli colicin K activity gene cka is controlled by the SOS system and stochastic factors. *Mol Genet Genomics,* 277**,** 391-401.

MULEC, J., PODLESEK, Z., MRAK, P., KOPITAR, A., IHAN, A. & ZGUR-BERTOK, D. 2003. A cka-gfp transcriptional fusion reveals that the colicin K activity gene is induced in only 3 percent of the population. *J Bacteriol,* 185**,** 654-9.

MÜLLER, A. J., HOFFMANN, C., GALLE, M., VAN DEN BROEKE, A., HEIKENWALDER, M., FALTER, L., MISSELWITZ, B., KREMER, M., BEYAERT, R. & HARDT, W.-D. 2009. The S. Typhimurium effector SopE induces caspase-1 activation in stromal cells to initiate gut inflammation. *Cell host & microbe,* 6**,** 125-136.

NANDA, A. M., HEYER, A., KRAMER, C., GRUNBERGER, A., KOHLHEYER, D. & FRUNZKE, J. 2014. Analysis of SOS-induced spontaneous prophage induction in Corynebacterium glutamicum at the single-cell level. *J Bacteriol,* 196**,** 180-8.

NARDI, A., CORDA, Y., BATY, D. & DUCHÉ, D. 2001. Colicin A Immunity Protein Interacts with the Hydrophobic Helical Hairpin of the Colicin A Channel Domain in theEscherichia coli Inner Membrane. *Journal of bacteriology,* 183**,** 6721-6725.

NEDIALKOVA, L. P., DENZLER, R., KOEPPEL, M. B., DIEHL, M., RING, D., WILLE, T., GERLACH, R. G. & STECHER, B. 2014. Inflammation fuels colicin Ib-dependent competition of Salmonella serovar Typhimurium and E. coli in enterobacterial blooms. *PLoS Pathog,* 10**,** e1003844.

NEDIALKOVA, L. P., SIDSTEDT, M., KOEPPEL, M. B., SPRIEWALD, S., RING, D., GERLACH, R. G., BOSSI, L. & STECHER, B. 2015. Temperate phages promote colicin-dependent fitness of Salmonella enterica serovar Typhimurium. *Environ Microbiol*.

NORMAN, J. M., HANDLEY, S. A., BALDRIDGE, M. T., DROIT, L., LIU, C. Y., KELLER, B. C., KAMBAL, A., MONACO, C. L., ZHAO, G. & FLESHNER, P. 2015. Disease-specific alterations in the enteric virome in inflammatory bowel disease. *Cell,* 160**,** 447-460.

O'BRIEN, A. D., MARQUES, L. R., KERRY, C. F., NEWLAND, J. W. & HOLMES, R. K. 1989. Shiga-like toxin converting phage of enterohemorrhagic Escherichia coli strain 933. *Microbial pathogenesis,* 6**,** 381-390.

O'BRIEN, A. D., NEWLAND, J. W., MILLER, S. F., HOLMES, R. K., SMITH, H. W. & FORMAL, S. B. 1984. Shiga-like toxin-converting phages from Escherichia coli strains that cause hemorrhagic colitis or infantile diarrhea. *Science,* 226**,** 694-696.

ÖLSCHLÄGER, T. 1991. A colicin M derivative containing the lipoprotein signal sequence is secreted and renders the colicin M target accessible from inside the cells. *Archives of microbiology,* 156**,** 449-454.

OLSCHLÄGER, T. & BRAUN, V. 1987. Sequence, expression, and localization of the immunity protein for colicin M. *Journal of bacteriology,* 169**,** 4765-4769.

ÖLSCHLÄGER, T., SCHRAMM, E. & BRAUN, V. 1984. Cloning and expression of the activity and immunity genes of colicins B and M on ColBM plasmids. *Molecular and General Genetics MGG,* 196**,** 482-487.

OPPENHEIM, A. B., KOBILER, O., STAVANS, J., COURT, D. L. & ADHYA, S. 2005. Switches in bacteriophage lambda development. *Annu. Rev. Genet.,* 39**,** 409-429.

OZBUDAK, E. M., THATTAI, M., LIM, H. N., SHRAIMAN, B. I. & VAN OUDENAARDEN, A. 2004. Multistability in the lactose utilization network of Escherichia coli. *Nature,* 427**,** 737-740.

PEDELACQ, J. D., CABANTOUS, S., TRAN, T., TERWILLIGER, T. C. & WALDO, G. S. 2006. Engineering and characterization of a superfolder green fluorescent protein. *Nat Biotechnol,* 24**,** 79-88.

PENNINGTON, J. M. & ROSENBERG, S. M. 2007. Spontaneous DNA breakage in single living Escherichia coli cells. *Nat Genet,* 39**,** 797-802.

PORWOLLIK, S. & MCCLELLAND, M. 2003. Lateral gene transfer in Salmonella. *Microbes and infection,* 5**,** 977-989.

PUGSLEY, A. P. 1983. Autoinduced synthesis of colicin E2. *Mol Gen Genet,* 190**,** 379-83.

PUGSLEY, A. P. & SCHWARTZ, M. 1984. Colicin E2 release: lysis, leakage or secretion? Possible role of a phospholipase. *The EMBO journal,* 3**,** 2393.

QUIRÓS, P., COLOMER-LLUCH, M., MARTÍNEZ-CASTILLO, A., MIRÓ, E., ARGENTE, M., JOFRE, J., NAVARRO, F. & MUNIESA, M. 2014. Antibiotic resistance genes in the bacteriophage DNA fraction of human fecal samples. *Antimicrobial agents and chemotherapy,* 58**,** 606-609.

RAFFATELLU, M., GEORGE, M. D., AKIYAMA, Y., HORNSBY, M. J., NUCCIO, S.-P., PAIXAO, T. A., BUTLER, B. P., CHU, H., SANTOS, R. L. & BERGER, T. 2009. Lipocalin-2 resistance confers an advantage to Salmonella enterica serotype Typhimurium for growth and survival in the inflamed intestine. *Cell host & microbe,* 5**,** 476-486.

RAJ, A. & VAN OUDENAARDEN, A. 2008. Nature, nurture, or chance: stochastic gene expression and its consequences. *Cell,* 135**,** 216-226.

RAJAGOPALA, S. V., CASJENS, S. & UETZ, P. 2011. The protein interaction map of bacteriophage lambda. *BMC microbiology,* 11**,** 1.

RAJAURE, M., BERRY, J., KONGARI, R., CAHILL, J. & YOUNG, R. 2015. Membrane fusion during phage lysis. *Proceedings of the National Academy of Sciences,* 112**,** 5497-5502.

RILEY, M. A. & GORDON, D. M. 1999. The ecological role of bacteriocins in bacterial competition. *Trends in microbiology,* 7**,** 129-133.

RILEY, M. A. & WERTZ, J. E. 2002a. Bacteriocin diversity: ecological and evolutionary perspectives. *Biochimie,* 84**,** 357-64.

RILEY, M. A. & WERTZ, J. E. 2002b. Bacteriocins: evolution, ecology, and application. *Annual Reviews in Microbiology,* 56**,** 117-137.

RUSSEL, M. 1995. Moving through the membrane with filamentous phages. *Trends in microbiology,* 3**,** 223-228.

SALLES, B., WEISEMANN, J. & WEINSTOCK, G. M. 1987. Temporal control of colicin E1 induction. *Journal of bacteriology,* 169**,** 5028-5034.

SALMOND, G. P. & FINERAN, P. C. 2015. A century of the phage: past, present and future. *Nature Reviews Microbiology,* 13**,** 777-786.

SAUER, R., ROSS, M. & PTASHNE, M. 1982. Cleavage of the lambda and P22 repressors by recA protein. *Journal of Biological Chemistry,* 257**,** 4458-4462.

SCHLUMBERGER, M. C., MULLER, A. J., EHRBAR, K., WINNEN, B., DUSS, I., STECHER, B. & HARDT, W. D. 2005. Real-time imaging of type III secretion: Salmonella SipA injection into host cells. *Proc Natl Acad Sci U S A,* 102**,** 12548-53.

SCHMIEGER, H. 1972. Phage P22-mutants with increased or decreased transduction abilities. *Mol Gen Genet,* 119**,** 75-88.

SCHNEIDER, C. A., RASBAND, W. S. & ELICEIRI, K. W. 2012. NIH Image to ImageJ: 25 years of image analysis. *Nat Methods,* 9**,** 671-5.

SIMON, R., PRIEFER, U. & PÜHLER, A. 1983. A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in gram negative bacteria. *Nature Biotechnology,* 1**,** 784-791.

SINGH, J. & GHOSH, C. 2012. Ribosomal encoded bacteriocins: their functional insight and applications. *J. Microbiol. Res,* 2**,** 19-25.

SLILATY, S. N., RUPLEY, J. A. & LITTLE, J. W. 1986. Intramolecular cleavage of lexA and phage. lambda. repressors: dependence of kinetics on repressor concentration, pH, temperature, and solvent. *Biochemistry,* 25**,** 6866-6875.

ŠMAJS, D., MATĚJKOVÁ, P. & WEINSTOCK, G. M. 2006. Recognition of pore-forming colicin Y by its cognate immunity protein. *FEMS microbiology letters,* 258**,** 108-113.

ŠMAJS, D. & WEINSTOCK, G. M. 2001. Genetic organization of plasmid ColJs, encoding colicin Js activity, immunity, and release genes. *Journal of bacteriology,* 183**,** 3949-3957.

ŠMARDA, J. & ŠMAJS, D. 1998. Colicins—exocellular lethal proteins ofEscherichia coli. *Folia microbiologica,* 43**,** 563-582.

SMITS, W. K., KUIPERS, O. P. & VEENING, J.-W. 2006. Phenotypic variation in bacteria: the role of feedback regulation. *Nature Reviews Microbiology,* 4**,** 259-271.

SNIJDER, H. & DIJKSTRA, B. 2000. Bacterial phospholipase A: structure and function of an integral membrane phospholipase. *Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids,* 1488**,** 91-101.

SPRIEWALD, S., GLASER, J., BEUTLER, M., KOEPPEL, M. B. & STECHER, B. 2015. Reporters for Single-Cell Analysis of Colicin Ib Expression in Salmonella enterica Serovar Typhimurium. *PLoS One,* 10**,** e0144647.

STANLEY, T. L., ELLERMEIER, C. D. & SLAUCH, J. M. 2000. Tissue-specific gene expression identifies a gene in the lysogenic phage Gifsy-1 that affects Salmonella enterica serovar Typhimurium survival in Peyer's patches. *Journal of bacteriology,* 182**,** 4406-4413.

STAYROOK, S., JARU-AMPORNPAN, P., NI, J., HOCHSCHILD, A. & LEWIS, M. 2008. Crystal structure of the &lgr; repressor and a model for pairwise cooperative operator binding. *Nature,* 452**,** 1022-1025.

STECHER, B., DENZLER, R., MAIER, L., BERNET, F., SANDERS, M. J., PICKARD, D. J., BARTHEL, M., WESTENDORF, A. M., KROGFELT, K. A. & WALKER, A. W. 2012a.

Gut inflammation can boost horizontal gene transfer between pathogenic and commensal Enterobacteriaceae. *Proceedings of the National Academy of Sciences,* 109**,** 1269-1274.

STECHER, B., DENZLER, R., MAIER, L., BERNET, F., SANDERS, M. J., PICKARD, D. J., BARTHEL, M., WESTENDORF, A. M., KROGFELT, K. A., WALKER, A. W., ACKERMANN, M., DOBRINDT, U., THOMSON, N. R. & HARDT, W. D. 2012b. Gut inflammation can boost horizontal gene transfer between pathogenic and commensal Enterobacteriaceae. *Proc Natl Acad Sci U S A,* 109**,** 1269-74.

STECHER, B., HAPFELMEIER, S., MULLER, C., KREMER, M., STALLMACH, T. & HARDT, W. D. 2004. Flagella and chemotaxis are required for efficient induction of Salmonella enterica serovar Typhimurium colitis in streptomycin-pretreated mice. *Infect Immun,* 72**,** 4138-50.

STECHER, B., MAIER, L. & HARDT, W. D. 2013. 'Blooming' in the gut: how dysbiosis might contribute to pathogen evolution. *Nat Rev Microbiol,* 11**,** 277-84.

STECHER, B., ROBBIANI, R., WALKER, A. W., WESTENDORF, A. M., BARTHEL, M., KREMER, M., CHAFFRON, S., MACPHERSON, A. J., BUER, J., PARKHILL, J., DOUGAN, G., VON MERING, C. & HARDT, W. D. 2007. Salmonella enterica serovar typhimurium exploits inflammation to compete with the intestinal microbiota. *PLoS Biol,* 5**,** 2177-89.

TOMAN, Z., DAMBLY-CHAUDIÈRE, C., TENENBAUM, L. & RADMAN, M. 1985. A system for detection of genetic and epigenetic alterations in Escherichia coli induced by DNA-damaging agents. *Journal of molecular biology,* 186**,** 97-105.

TWIGG, A. J. & SHERRATT, D. 1980. Trans-complementable copy-number mutants of plasmid ColE1. *Nature,* 283**,** 216-218.

TWORT, F. W. 1915. An investigation on the nature of ultra-microscopic viruses. *The Lancet,* 186**,** 1241-1243.

USÓN, I., PATZER, S. I., RODRÍGUEZ, D. D., BRAUN, V. & ZETH, K. 2012. The crystal structure of the dimeric colicin M immunity protein displays a 3D domain swap. *Journal of structural biology,* 178**,** 45-53.

UZZAU, S., FIGUEROA-BOSSI, N., RUBINO, S. & BOSSI, L. 2001. Epitope tagging of chromosomal genes in Salmonella. *Proc Natl Acad Sci U S A,* 98**,** 15264-9.

VAN GESTEL, J., WEISSING, F. J., KUIPERS, O. P. & KOVÁCS, Á. T. 2014. Density of founder cells affects spatial pattern formation and cooperation in Bacillus subtilis biofilms. *The ISME journal,* 8**,** 2069-2079.

WALDOR, M. K. & MEKALANOS, J. J. 1996. Lysogenic conversion by a filamentous phage encoding cholera toxin. *Science,* 272**,** 1910-1914.

WALLIS, R., MOORE, G. R., JAMES, R. & KLEANTHOUS, C. 1995. Protein-protein interactions in colicin E9 DNase-immunity protein complexes. 1. Diffusion-controlled association and femtomolar binding for the cognate complex. *Biochemistry,* 34**,** 13743-13750.

WANG, R. F. & KUSHNER, S. R. 1991. Construction of versatile low-copy-number vectors for cloning, sequencing and gene expression in Escherichia coli. *Gene,* 100**,** 195-9.

WEAVER, C. A., KAGAN, B. L., FINKELSTEIN, A. & KONISKY, J. 1981a. Mode of action of colicin Ib formation of ion-permeable membrane channels. *Biochimica et Biophysica Acta (BBA)-Biomembranes,* 645**,** 137-142.

WEAVER, C. A., KAGAN, B. L., FINKELSTEIN, A. & KONISKY, J. 1981b. Mode of action of colicin ib: formation of ion-permeable membrane channels. *Biochim Biophys Acta,* 645**,** 137-42.

WEGRZYN, G., LICZNERSKA, K. & WEGRZYN, A. 2012. Phage-new insights into regulatory circuits. *Adv. Virus Res,* 82**,** 155-178.

WINTER, S. E., LOPEZ, C. A. & BÄUMLER, A. J. 2013a. The dynamics of gut-associated microbial communities during inflammation. *EMBO reports,* 14**,** 319-327.

WINTER, S. E., THIENNIMITR, P., WINTER, M. G., BUTLER, B. P., HUSEBY, D. L., CRAWFORD, R. W., RUSSELL, J. M., BEVINS, C. L., ADAMS, L. G. & TSOLIS, R. M. 2010a. Gut inflammation provides a respiratory electron acceptor for Salmonella. *Nature,* 467**,** 426-429.

WINTER, S. E., THIENNIMITR, P., WINTER, M. G., BUTLER, B. P., HUSEBY, D. L., CRAWFORD, R. W., RUSSELL, J. M., BEVINS, C. L., ADAMS, L. G., TSOLIS, R. M., ROTH, J. R. & BAUMLER, A. J. 2010b. Gut inflammation provides a respiratory electron acceptor for Salmonella. *Nature,* 467**,** 426-9.

WINTER, S. E., WINTER, M. G., XAVIER, M. N., THIENNIMITR, P., POON, V., KEESTRA, A. M., LAUGHLIN, R. C., GOMEZ, G., WU, J. & LAWHON, S. D. 2013b. Host-derived nitrate boosts growth of E. coli in the inflamed gut. *Science,* 339**,** 708-711.

YANG, T.-Y., SUNG, Y.-M., LEI, G.-S., ROMEO, T. & CHAK, K.-F. 2010. Posttranscriptional repression of the cel gene of the ColE7 operon by the RNA-binding protein CsrA of Escherichia coli. *Nucleic acids research,* 38**,** 3936-3951.

YOUNG, R. 2013. Phage lysis: do we have the hole story yet? *Current opinion in microbiology,* 16**,** 790-797.

YOUNG, R. 2014. Phage lysis: Three steps, three choices, one outcome. *Journal of Microbiology,* 52**,** 243-258.

ZETH, K., RÖMER, C., PATZER, S. I. & BRAUN, V. 2008. Crystal Structure of Colicin M, a Novel Phosphatase Specifically Imported by Escherichia coli>. *Journal of Biological Chemistry,* 283**,** 25324-25331.

ZGUR-BERTOK, D. 2012. Regulating colicin synthesis to cope with stress and lethality of colicin production. *Biochem Soc Trans,* 40**,** 1507-11.

ZGUR-BERTOK, D. 2013. DNA damage repair and bacterial pathogens. *PLoS Pathog,* 9**,** e1003711.

ZHANG, X. Y.-Z., LLOUBÈS, R. & DUCHÉ, D. 2010. Channel domain of colicin A modifies the dimeric organization of its immunity protein. *Journal of Biological Chemistry,* 285**,** 38053-38061.

ZINDER, N. D. & LEDERBERG, J. 1952. Genetic exchange in Salmonella. *Journal of bacteriology,* 64**,** 679.

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