Envelope stress response during *Salmonella* infection: role of σ^E

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Statement to my Ph.D. thesis

This work was carried out in the group of Prof. Dr. Dirk Bumann in the Focal Area Infection Biology at the Biozentrum in Basel, Switzerland.

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My Ph. D. thesis is written as a cumulative dissertation. It consists of an abstract, an introduction, a results part composed of a paper manuscript followed by a discussion and a conclusion.

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Abbreviations

CAMP	Cationic Anti Microbial Peptide
IM	Inner Membrane
IMP	Inner Membrane Protein
iNOS	inducible Nitric Oxide Synthase
LPS	LipoPolySaccharide
MDR	Multi-Drug Resistant
NO	Nitric Oxide
OM	Outer Membrane
OMP	Outer Membrane Protein
OMV	Outer Membrane Vesicle
PAMP	Pathogen-Associated Molecular Pattern
PDR	Pan-Drug Resistant
PG	PeptidoGlycan
PL	PhosphoLipid
PRR	Pattern Recognition Receptor
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
SCV	*Salmonella* Containing Vacuole
SPI	*Salmonella* Pathogenicity Island
SS	Secretion System
WT	Wild-Type
XDR	eXtensively-Drug Resistant

1 Abstract

Bacterial infections are a major public health problem worldwide. Over the years, multi-drug resistant (MDR) pathogens emergence combined with approval absence of new antibiotics in clinics has resulted in untreatable infections. Thus, routine medical procedure constitutes a risk of infection for patients. Therefore, it is crucial to identify novel strategies to efficiently and effectively combat bacterial pathogens.

In vitro, it is challenging to reproduce the microenvironment that pathogens encounter *in vivo*. Consequently, important stress conditions are omitted, decreasing the ability to identify relevant inhibitor targets. The host immune response largely focuses on the pathogen envelope that constitutes the first line of defense for pathogens and a strong physical barrier. Pathogens possess several envelope stress response systems that enable an adapted response to host attacks, ensuring survival and growth. Inhibiting these systems may enhance host immunity and provide efficient infection control. The extracytoplasmic stress response factor sigma E (σ^E), encoded by *rpoE*, is crucial for the virulence of several pathogens, including *Salmonella*. σ^E regulates expression of more than 100 genes, including those that encode proteases, chaperones, and sRNAs to maintain envelope homeostasis. However, it remains unclear which σ^E-regulated gene(s) is (are) critical for *in vivo* fitness. Here, we used an unbiased approach to identify target mutations that restore Δ*rpoE* survival by using a transposon library screen. While the parental strain Δ*rpoE* was cleared from infected mice, several transposon mutants with inactivated *ompC* survived, indicating partial fitness rescue. Clean mutations (*i.e.*, Δ*rpoE* Δ*ompC*) reproduced the transposon effect, confirming the involvement of OmpC in Δ*rpoE* survival. OmpC could be the entry pore for a toxic molecule whose damages require σ^E-mediated repair. Or, OmpC itself could be or generate a cargo in the periplasm. Further studies are required to understand the exact underlying molecular mechanisms, but the effect of *ompC* deletion on Δ*rpoE* fitness is remarkable. Another *in vivo* screen of the transposon library identified *pnp* as a target mutation. *pnp* encodes a major regulator involved in mRNAs degradation and cold shock resistance. Truncated *pnp*, in combination with Δ*rpoE* Δ*ompC,* almost reached WT-like fitness. Surprisingly, *in vivo* proteomics data demonstrated few differences between WT and mutant bacteria. This suggests that *Salmonella* can bypass σ^E by a few minor alterations mediated by *ompC* and *pnp* deletions.

Collectively, we demonstrate that a small number of mutations can rescue the *in vivo* fitness of an avirulent regulatory mutant. Synergizing with host studies allows the identification of inhibitor targets that would not be found with our standard *in vitro* mimicking conditions. We therefore invalidated

rpoE as an inhibitor target. Our approach could be more widely used in the future to evaluate other major systems such as the master regulator *phoP* or the general stress response sigma factor *rpoS* before potential inhibitors reach the clinics.

2 Introduction

2.1 Emergence and rise of multi-drug resistant (MDR) pathogens

During his PhD in 1897, French physician Ernest Duchesne paved the way for antibiotic research by discovering that certain mold kill bacteria, but his work eventually was forgotten (1). Sir Alexander Fleming followed similar path in 1928 by identifying and extracting penicillin from *Penicillium notatum* fungus (2). The "Golden Age" of antibiotic discovery lasted from the 1940s to the 1960s (3,4). Doctors thought they had won the battle against bacterial infections. However, emergence of MDR pathogens has occurred in the past decades, posing serious problems during the routine medical interventions. In the meantime, the number of newly discovered and approved antibiotics by the U. S. Food and Drug Administration has decreased rapidly. Even more striking is the absence of new antibiotics class that reached the clinics since 1987 (5).

The emergence of MDR pathogens is due to overuse and misuse of antibiotics by the human population, extensive utilization of antibiotics in livestock food, and use of pesticides in agriculture (6). Pathogens are naturally inclined to mutate their genome, but the mutation rate frequency increases with pressures such as antibiotics. Once exposed to antibiotics, some pathogens pool quickly acquires numerous mechanisms to subvert the effect of many antibiotics. Over the past few decades, the number of MDR pathogens has significantly increased. Additionally, pathogens acquire resistance against antibiotics that have not reached the clinics, such as fluoroquinolones (Figure 1). Extensively drug resistant (XDR) and pan drug resistant (PDR) pathogens have also emerged. MDR refers to the lack of susceptibility to at least one agent in three or more categories, XDR to only one in two categories, and PDR to all agents in all categories (7). The current resistance emergence problem occurs mainly in Gram-negative pathogens, but it is also seen in Gram-positive pathogens. Most nosocomial infections worldwide are caused by *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species, all of them constituting the well-known ESKAPE group (8,9).

ESKAPE pathogens, among others, resist antibiotic effects by inactivating or alterating the drug, modifying the drug binding site, reducing intracellular drug accumulation by decreasing the membrane permeability or increasing efflux, or biofilm formation (10). Extrachromosomal plasmids allow rapid resistance transmission by horizontal gene transfer between unrelated bacteria (11). In developed countries, increasing numbers of infections that were previously controlled now again threaten

hospital patients. In developing countries, problems arise from poor access to diagnosis and medication (12). Antimicrobial resistance is expected to cause 10 million deaths in 2050, which is higher than cancer-related fatalities. Thus, new treatment discovery is crucial for humanity and represents one of the most critical challenges of the century.

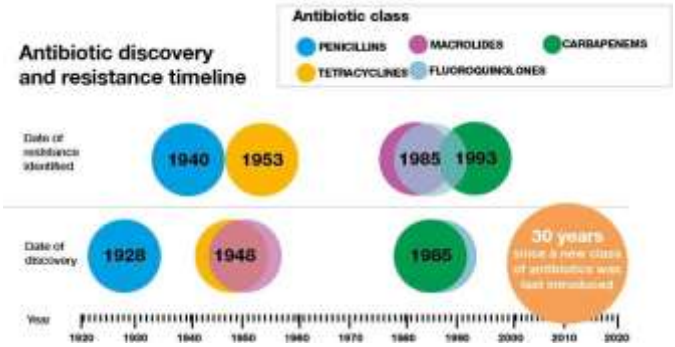

Figure 1. MDR pathogen numbers increased while new antibiotic discoveries number decreased (www.gov.uk).

2.2 *Salmonella* as a pathogen model, and BALB/c mice as an animal model

To discover new strategies to efficiently fight bacterial pathogens, we selected the well-established pathogen *Salmonella* and the BALB/c mice model of infection (13).

2.2.1 *Salmonella* as a pathogen model

Salmonella are Gram-negative, facultatively intracellular pathogens, belonging to the *Enterobacteriaceae* family that includes *Escherichia*, *Shigella*, *Yersinia*, and *Klebsiella*. Due to a common ancestry with *E. coli* (14), *Salmonella* genetic manipulation and laboratory culture is straightforward, making them an excellent pathogen model. Highly reproducible results combined with a wide range of available techniques (Fluorescence-Activated Cell Sorting, microscopy, proteomics, transcriptomics, cell culture, macrophage killing assays, activity reporters) and extensive literature allow a solid understanding of the molecular mechanisms involved in host-*Salmonella* interactions. *Salmonella enterica* serovar Typhi (*S.* Typhi) is the causative agent of typhoid fever, a foodborne disease and life-threatening infection. As *S.* Typhi is a human-restricted pathogen, no practical model exists to study this systemic disease in humans. Indeed, humanized mouse models exist, but exhibit highly variable outcomes (15). *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) is responsible for typhoid-like fever in genetically susceptible mice. Since *S.* Typhi and

S. Typhimurium share 89% of coding sequences (16), *S.* Tyhimurium infection of genetically susceptible BALB/c mice provides a reliable, tractable and widely used model.

2.2.2 The BALB/c mouse model

BALB/c mice lack, among other genes, the NRAMP1 transporter (Natural Resistance Associated Macrophage Protein 1), which is essential for controlling *Salmonella* infection (17). Therefore, BALB/c mice cannot resist *Salmonella* Typhimurium infection as their immune system is unable to clear the pathogen. BALB/c mice are thus a commonly used and well-characterized animal model (18). Therefore, *Salmonella* pathogenesis can be investigated using a mouse model that mimics human infection with *S.* Typhi.

Routes of infection (oral, intraperitoneal or intravenous) depend on the scientific questions and hypotheses formulated. Indeed, *Salmonella* infection results from consumption of contaminated food or water, and most of the bacteria is killed by the stomach acidity. However, the surviving pool generates a gut inflammation, invades gut M cells, and is engulfed by resident macrophages from the submucosa. *Salmonella* then reach the mesenteric lymph nodes and the bloodstream to invade deep organs such as the liver and spleen. There, pathogens can replicate in the so-called *Salmonella*-containing vacuole (SCV) within macrophages at a rate of 0.5 to 1.5 log a day. Therefore, the oral route of infection in the animal model represents the natural course. Unfortunately, variability exists between individual mouse due to a decreased control of the exact number of bacteria that reach the blood. Thus, larger study groups are required.

In the laboratory, we use the intravenous route. The advantage of the intravenous infection is bypassing the gut, thus focusing on the systemic part of *Salmonella* infection. The intraperitoneal route is technically easier and should provide the same information as the intravenous route. The main difference is that *Salmonella* must cross the peritoneal barrier before reaching the blood.

2.3 General strategy: synergize with the host defense

Our approach consisted in confronting a mutant lacking an envelope stress response system – which exhibits weak *in vitro* phenotype – with the host immune response. Inhibiting such pathogen stress response might enhance host immunity to provide efficient infection control.

2.3.1 Weak *in vitro* phenotype

Certain gene mutations do not alter a pathogen's ability to grow *in vitro*. For instance, *rpoE*, the gene which encodes the extracytoplasmic stress response factor and also the topic of this thesis, can be deleted with a few effects on *Salmonella* growth *in vitro*, especially at low pH and during stationary phase. Therefore, *rpoE in vitro* does not seem to be a satisfactory inhibitor target. However, *Salmonella*

confront few stresses in different laboratory media (LB, M9, MgMES), while intense and diverse stresses are encountered within a host (13). Thus, we must synergize *rpoE* inhibition with the host defense.

Most of the host attacks are focused on the pathogen envelope which serves as a strong physical barrier.

2.3.2 The pathogen envelope

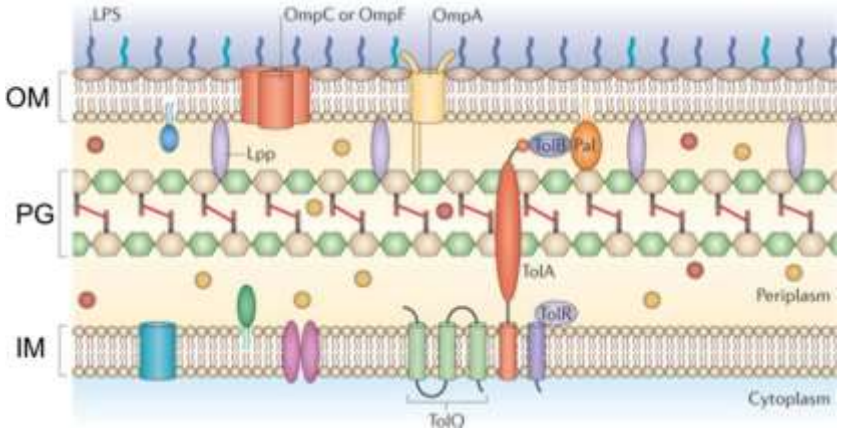

Figure 2. Gram-negative pathogen envelope adapted from (19).

General organization (20)

The envelope of Gram-negative bacteria contains two membranes that separate the aqueous periplasm: the asymmetric outer membrane (OM) and the inner membrane (IM). The OM is composed of a lipopolysaccharide (LPS) layer exposed to the environment, and a phospholipid layer facing the peptidoglycan (PG) layer. The LPS is constituted of lipid A, which serves as an anchor for the core polysaccharide, and a certain number of O-polysaccharide repeats. The PG is composed of a unique polysaccharide with repeating N-acetyl-glucosamine and N-acetyl-muramic acid moieties. The PG possesses vital structural properties. Lipoproteins are inserted into the phospholipid layer, facing the PG. Lipoproteins are crucial for virulence as they allow surface adhesion with the host cells, but also translocation of virulence factors in the cytoplasm. Finally, the IM contains numerous proteins. Highly important systems contain a sensor located in the IM such as PhoQ which is part of the two-component system PhoPQ; or EnvZ-OmpR where EnvZ is the IM sensor that phosphorylates or dephosphorylates the transcription factor OmpR.

Porins (20)

Porins are proteins that insert into the OM and form channels due to their hydrophobic exterior. They form homotrimers and are constituted of 8 or 16 stranded, anti-parallel ß-barrels. The L3 loop folds into the pore and defines the constriction zone or pore diameter. Hydrophilic center allows the passage of water and small water-soluble molecules (less than 600 kDa) and waste excretion. Some porins are specific to the diffusion of small molecules, such as maltose, maltodextrin or phosphate. Host damaging molecules exploit porin function to enter *Salmonella* cells, including hydrogen peroxide (H_2O_2), nitric oxide (NO) and antimicrobial peptides (AMP). *Salmonella* encode several porins with different abundance levels depending on the environment: the major porins are OmpA, OmpC, OmpF, and OmpD.

OmpA is a structural porin that stabilizes bacterial shape, and is highly expressed (21).

OmpC is a small pore strongly cation selective expressed at high osmolarity, such as that found in human tissues. OmpF is a slightly cation selective large pore expressed at low osmolarity. OmpF and OmpC opposing levels are controlled by EnvZ/OmpR. The single mutants were not described as attenuated *in vivo* (22). The double *ompC ompF* mutant was highly attenuated orally – 1,000 fold – but less attenuated intravenously – only 10 fold – in a typhoid fever mouse model (23).

OmpD is another *Salmonella* major porin whose homolog in *E. coli* is NmpC. OmpD participates in paraquat, H_2O_2, and NaOCl entrance (24–26), but its involvement in virulence is under debate due to contradictory studies results. Dorman *et al.* showed that Δ*ompD* is slightly less virulent than WT bacteria after oral infection (22) while Meyer *et al.* claimed there is no difference between WT and mutant fitness (27). More recently, Ipinza *et al.* observed that Δ*ompD* bacteria establish a better systemic infection than WT after oral and intra-peritoneal inoculation (28).

Efflux system (29)

Resistance-Nodulation-Division (RND) superfamily exporters are involved in the active efflux of small molecules, including antibiotics. They are composed of the OMP TolC and a possible combination of IM permease, such as AcrA or AcrB, to form a multidrug efflux complex. *In vitro*, up-regulation of RND leads to increase resistance to antibiotics. Many clinical isolates overexpress RND efflux systems, suggesting a role in antibiotic resistance *in vivo* as well.

By its thickness, the pathogens' envelope constitutes a strong physical barrier. By its content diversity, it represents the first line of defense for pathogens that modulate their envelope surface according to different type of attacks from the host. A few examples of envelope modulation by pathogens is provided below: LPS modification, Bam machinery, and Secretion Systems (SS).

2.3.3 Pathogens' envelope modulation

LPS modification (30)

The LPS is an example of Pathogen-Associated Molecular Pattern (PAMP) recognized by phagocyte Pattern Recognition Receptors (PRR) that are major components of the innate immune system. The LPS is a major virulence factor of Gram-negative bacteria and confers resistance to bile salts, hydrophobic antibiotics, complement-mediated killing and macrophages antibacterial activities. PhoPQ is one of the major regulatory systems that controls the biogenesis and modification of LPS. PhoPQ is a two-component system crucial for virulence. The IM sensor kinase PhoQ senses stresses such as low pH, low magnesium and CAMPs. In response, the transcription factor PhoP activates or represses genes to ensure bacterial survival. LPS modifications focus on the reduction of polymer length of the O-antigen, specific core polysaccharide changes or lipid A structural modifications. PmrAB is another major two-component system that modifies LPS composition after activation by high Fe^{3+} concentration or low pH (31).

Bam machinery (32)

The Bam complex role is to ensure proper folding and insertion of the ß-barrels structure in the OM. It is composed of BamA – the central and essential OMP of the machinery – and four lipoproteins termed BamB, BamC, BamD – which is essential – and BamE. Chaperones support the Bam machinery folding process by avoiding aggregation of unfolded OMPs during their transport across the aqueous periplasm. Thus, the Bam machinery maintains the envelope integrity by regulating and controlling the OM composition, hence its permeability.

Secretion systems (33)

Secretion Systems are used by pathogens to directly secrete effector proteins into the host cytosol. *Salmonella* possess two Type III Secretion Systems (TTSS) encoded on *Salmonella* pathogenicity island-1 (SPI-1) and -2 (SPI-2). TTSS-1 directs invasion of non-phagocytic host cells and promotes inflammation. TTSS-2 permits effector protein translocation across the SCV membrane within macrophages to control intracellular events. SS also allow the secretion of proteins into the surrounding environment to influence host cell physiology. Indeed, secreted proteins can scavenge nutrients or digest peptide complexes.

2.4 Host immune system attacks

During infection, the host innate immune system attacks pathogens to eliminate them using numerous mechanisms such as, among others, Cationic Anti Microbial Peptides (CAMPs), Reactive Oxygen Species (ROS), Reactive Nitrogen Species (RNS) which are further described below.

Cationic Anti Microbial Peptides (34)

CAMPs are produced by innate immune cells to control *Salmonella* infection. Similar to polymyxin B or C18G, CAMPs are positively charged peptides. *Salmonella* have evolved several mechanisms to counter CAMPs' effects. For instance, *Salmonella* regulate LPS O-antigen length, reduce surface negative charges to decrease CAMPs' binding, decrease membrane fluidity to diminish interactions with CAMPs, and increase efflux or proteolytic degradation of CAMPs before they exert their effect.

Reactive Oxygen Species (35)

ROS are produced by host phagocytes. After engulfment, H_2O_2 from macrophages degrades pathogens or damaged cells into smaller compounds for recycling. H_2O_2 can react with Fe^{2+} during the Fenton reaction to generate free radical molecules. As the latter are unstable, they react with other molecules by chain reactions, damaging proteins, lipids, and DNA. *Salmonella* have evolved to produce enzymes that transform ROS into less toxic compounds, including catalases and superoxide dismutases that scavenge H_2O_2 and O^{2-}, respectively.

Reactive Nitrogen Species (36)

Macrophages use inducible Nitric Oxide Synthases (iNOS) to produce copious amount of Nitric Oxide (NO) as part of the innate immune response. The free radical NO is highly reactive and forms intermediates compounds that increase NO damaging activity. *Salmonella* have evolved strategies to detoxify NO thanks to three enzymes that convert NO into less toxic compounds such as ammonium, nitrous oxide and nitrate.

2.5 *Salmonella* envelope stress response systems

During their life cycle, *Salmonella* encounter a wide variety of environments. *Salmonella* use cues to activate appropriate stress systems and ensure survival. Six partially overlapping systems overcome envelope stress (Figures 3-8): Outer Membrane Vesicles (OMVs), Conjugative plasmid expression (CpxAR), Bacterial adaptive response (BaeSR), Phage Shock Protein (PSP), Regulator of capsule synthesis (Rcs), and the extracytoplasmic stress response factor σ^E that are further detailed in this part

(37,38). Conservation of these systems among Gram-negative pathogens suggests a high relevance for *in vivo* fitness (39).

2.5.1 OMVs

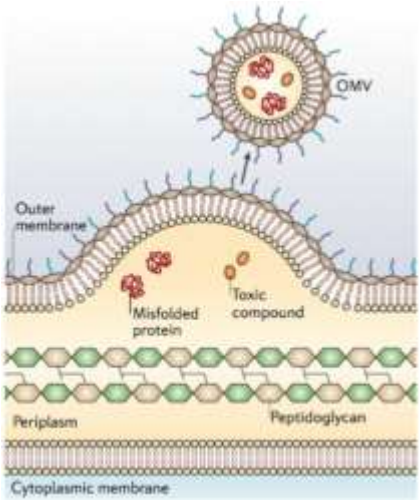

Figure 3. OMVs allow excretion of overproduced components (19).

OMVs are produced by cells from all domains of life, including Eukarya, Archaea and Bacteria. OMVs are spherical structures with a diameter of 30-300 nm. Pathogens use these structures to eliminate overproduced or damaged components whose accumulation can be toxic, to promote infection spread to different loci, and to transfer genetic information or nutrient. Interestingly, a *degP* mutant produces higher levels of OMVs, DegP being an essential protease regulated by two other envelope stress systems, σ^E and CpxAR.

2.5.2 CpxAR

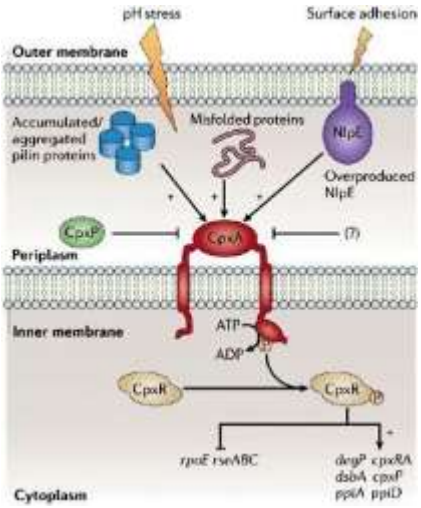

Figure 4. The CpxAR system (37).

CpxAR is a two-component system that responds to aggregated pili proteins, high pH, misfolded proteins or surface adhesion (40–43). CpxAR consists of CpxA, an IM kinase and phosphatase sensor, CpxR, a cytoplasmic response regulator, and CpxP, a negative regulator (44). Upon stress, CpxA autophosphorylates a conserved histidine residue. The phosphate is then transferred to an aspartate residue of CpxR. CpxR~P regulates the expression of more 100 genes, including *cpxP*, by binding to a specific recognition site (45,46). Interestingly, Cpx and σ^E share overlapping functions as, for example, CpxR~P activates *degP* transcription (47,48). Another example is that σ^E senses increased expression of the P subunit (49). Interestingly, CpxR is dispensable for *Salmonella* virulence (50).

2.5.3 BaeSR

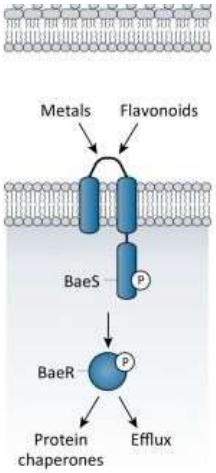

Figure 5. The BaeSR system (51).

BaeSR is a two-component system which, after activation by a plethora of compounds, enhances efflux system expression to expel toxic compounds. BaeSR is composed of BaeS, an IM kinase sensor, and BaeR, a cytoplasmic response regulator (52). The BaeSR system is activated by spheroplast, misfolded pilus subunits, tannins, bile salts, flavonoid, stationary phase growth, some metals and some antibiotics (53–62). BaeS autophosphorylates a conserved histidine residue before funneling the phosphate to a conserved aspartate residue on BaeR. BaeR~P binds to the promoter region of the multidrug efflux systems *acrD* and *mdtABC* (58,63,64). Interestingly, the CpxAR and BaeSR systems regulate overlapping genes such as *acrD*, *mdtA*, and *spy* (65–67). Involvement in *Salmonella* pathogenesis has not been described yet.

2.5.4 PSP

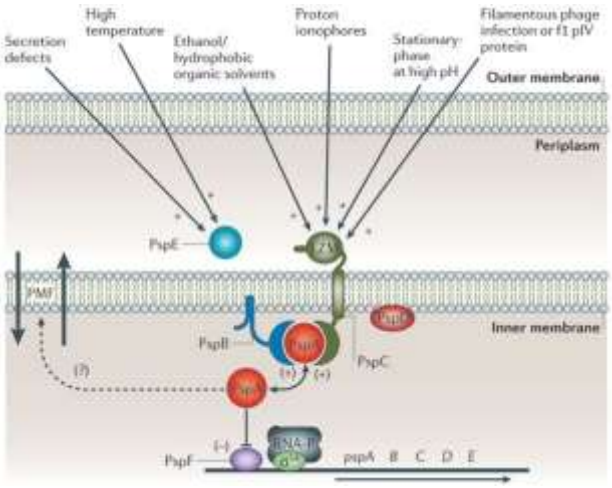

Figure 6. The PSP system (37).

The PSP system is activated by changes in the IM proton motive force due to filamentous bacteriophage infection, secretion defects, ethanol, lipid biosynthesis disruption, extreme temperature, or osmolarity (68–70). The system is composed of 6 proteins. Under non-inducing conditions, PspA, the cytoplasmic negative regulator, binds free cytoplasmic activator PspF (71,72). Under inducing conditions, PspA associates with the IMP PspB and PspC. *pspD* encodes an IMP of unknown function, while *pspE* encodes a periplasmic enzyme (73). Interestingly, the PSP and σ^E systems share overlapping activities in *Salmonella* as *rpoE* and *pspA* mutants exhibit lower proton motive force compared to WT cells (74).

2.5.5 Rcs

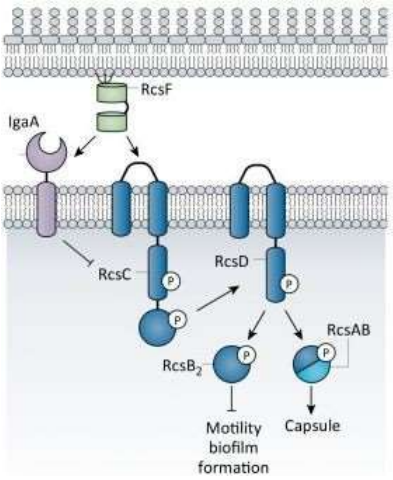

Figure 7. The Rcs system (51).

The Rcs system is an atypical phosphorelay system activated by any defect in the OM or LPS. It is composed of the IM sensor histidine kinase RcsC, the intermediate IM phosphorelay protein RcsD, and the DNA-binding proteins RcsA and RcsB, the latter of which is also the cytoplasmic response regulator (75,76). Under non-stressed conditions, the inhibitor IgaA (Inhibitor of growth activity A) maintains low system activity (77). IgaA is essential unless the Rcs system is also inactivated (78). The lipoprotein RcsF acts as a sensor (79,80). Recently, it was suggested that RcsF directly interacts with RcsC (81). RcsC autophosphorylates a conserved histidine residue, transfers the phosphate to its conserved aspartate residue, then to a RcsD conserved histidine residue (76). The cytoplasmic transcriptional factor RcsB, either preferentially by itself, or in combination with RcsA, receives the phosphate and induces expression of genes involved in biofilm formation, motility, periplasmic quality control, osmotic homeostasis, and surface-antigen production (77). The Rcs system is activated by Penicillin-Binding Protein loss (82), RcsF overexpression (83), AMPs (84), and some antibiotics (85). Maximal activation of the Rcs system attenuates *Salmonella* virulence (86).

2.5.6 The extracytoplasmic envelope stress response

σ^{32} is the sigma factor involved in heat shock and is encoded by *rpoH*. In 1987, *Erickson et al.* noted that σ^{32} does not recognize its own promoter upstream of *rpoH* (87). Later, the sigma factor that transcribes promoter 3 of *rpoH* was discovered and termed extracytoplasmic stress response factor sigma E, σ^{E}, or σ^{24} (88,89). The *rpoE* gene encodes sigma E (90). In *E. coli*, *rpoE* is essential for viability (91,92), but suppressor mutations often arise to allow growth. Mutations in *ptsN*, which encodes an enzyme involved in carbohydrate metabolism, *yhbW*, encoding a gene of unknown function, and *ydcQ*, encoding a putative DNA-binding protein, abolish σ^{E} essentiality (93,94). A nucleotide change in the *rpoE* second codon's third base creates a faster and more robust σ^{E} envelope stress response (95). Interestingly, unlike *E. coli*, σ^{E} is not essential for *Salmonella* viability (96). Reasons proposed for this observation are the over-reaction of *E. coli* to σ^{E} absence (94), or the requirement of σ^{E} to transcribe genes that are not properly regulated, leading to loss of envelope homeostasis (93). However, σ^{E} represents the only envelope stress system that is crucial for *Salmonella* virulence as an *rpoE* mutant is completely attenuated in the mouse typhoid fever models (96).

2.6 Role of σ^E

2.6.1 Sigma factors role

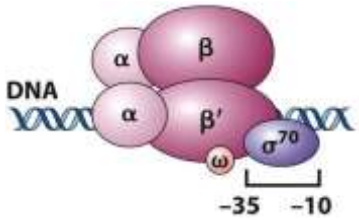

Figure 8. RNA polymerase subunits composition.

Four subunits compose the core RNA polymerase: p, p', two copies of α, and m (Figure 8). When the last subunit sigma (σ) binds to the core RNA polymerase, the complex is referred to as the RNA polymerase holoenzyme. σ recognizes DNA promoters to initiate transcription start. The promoter sequence is composed of two consensus sequences located at the -35 bp and -10 bp sites from the transcriptional start site. In *E. coli*, *rpoD* encodes σ^{70}, the general sigma factor, that allows normal growth. *rpoN*, *rpoS*, *rpoH* and *rpoE* encode alternative sigma factors. Alternative sigma factors use environmental cues to activate gene regulons and promote survival. *rpoN* encodes σ^{54} and is involved in nitrogen assimilation while *rpoS* encodes σ^{38} and is implicated in stationary phase, oxidative and osmotic stresses. σ^{32} is encoded by *rpoH* and is involved in the heat shock response.

rpoE encodes σ^E, or σ^{24}, and is involved in the extracytoplasmic stress response.

2.6.2 σ^E expression under non-stressed conditions

rpoE is part of an operon containing *rseD*, *rseA*, *rseB*, and *rseC*. *rse* refers to "regulator of sigma E". Expression of the *rpoErseArseBrseC* operon is driven by several promoters (90) and σ^E activates its own transcription (97,98). Under non-stressed conditions, σ^E is sequestered at the IM by the cytoplasmic domain of the anti-sigma factor RseA (99,100). RseA sterically occludes σ^E binding sites to the core RNA polymerase (101). *rseB* encodes a periplasmic protein that assumes the role of a second anti-sigma factor as it inhibits DegS proteolysis (99,100,102). RseB stimulates RseA binding to σ^E as RseB increases σ^E:RseA association (103) and is necessary for efficient binding to RseA (104). RseC has a slight positive effect on σ^E activity as deleting *rseC* in *rseB* mutant abolishes the slight induction of σ^E activity in an *rseB* mutant (99). The σ^E:RseA:RseB ratio is 2:5:1 (103), indicating that the amount of free σ^E is very low. Recently, a gene encoded upstream of *rpoE* has been described: *rseD* is predicted to encode the *rpoE* leader peptide that aids *rpoE* translation (105).

2.6.3 σ^E expression under certain stressed conditions

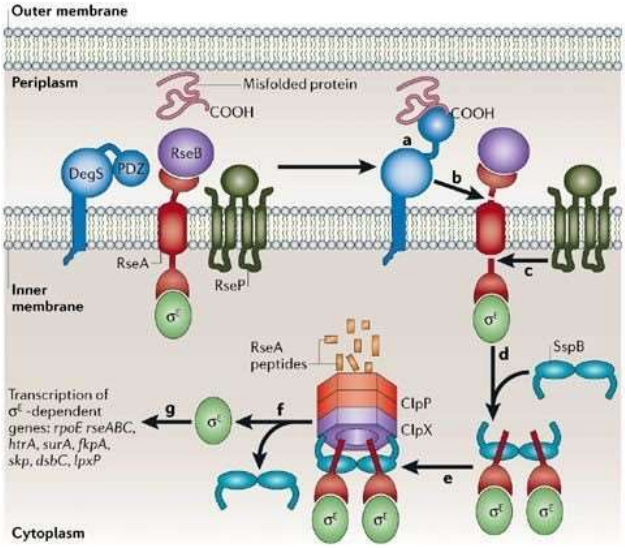

Figure 9. Molecular mechanisms of σ^E activation (37).

In *E. coli*, some stress conditions activate σ^E, including heat shock (106), entry into stationary phase (107), metal exposure (108,109), hyperosmotic shock (110), altered LPS structure (111), OMPs overproduction, and unfolded or misfolded OMPs (112). In *Salmonella*, AMP (113) and acid exposure (114) are additional σ^E-activity inducers.

YQF constitutes the C terminal part of most *Salmonella* OMPs (115). This motif from unfolded and misfolded OMPs binds to the PDZ domain of the essential IM protease, DegS (116,117). This binding results in conformational changes that activate DegS protease activity (118) by relieving inhibitory contacts between the PDZ domain and protease domain of DegS (119). The periplasmic domain of RseA is subject to a first cleavage by DegS (120). Another essential IM protease termed RseP further cleaves RseA (121–124). RseB and DegS independently inhibit RseP cleavage of intact RseA. Recognition of the cleaved amino acid by the RseP PDZ domain is not essential for sequential cleavage of RseA and the σ^E stress response *in vivo* (125). As opposed to substrate recognition, the PDZ domain of RseP likely acts as an inhibitor of proteolytic activity (126). The serine protease complex ClpXP degrades the remaining part of RseA freeing σ^E in the cytoplasm (127). σ^E then recruits the core RNA polymerase and activates transcription of over 100 genes in *E. coli*.

Acid activation of σ^E is independent of the unfolded OMP signal or degradation by DegS, but requires RseA degradation by RseP. An atypical stimulus for σ^E upon entry into stationary phase is the alarmone guanosine 3',5'-bispyrophosphate (ppGpp), which is a general signal of starvation-induced stress.

Interestingly, ppGpp is the only cytoplasmic activator and does not use the classical unfolded OMP pathway as seen in acid activation (107). Additionally, by directing cell lysis in late stationary phase, σ^E provides nutrients from dead cells for the next generation (128). Together, these data imply that σ^E has a broader role than previously recognized.

2.6.4 σ^E-regulated genes

σ^E maintains envelope homeostasis by (i) ensuring assembly, proper folding, and insertion of OMPs (chaperone), (ii) clearing the periplasm of debris (protease), (iii) providing membrane with LPS and PL (biosynthesis enzyme), (iv) rapidly balancing disorder in the amount of OMPs produced (sRNAs), and (v) likely other unknown tasks. Not all the σ^E-regulated genes contain a consensus sequence, allowing a direct and indirect - thus broader - regulation.

Chaperones (32)

Chaperones have two functions: the maturation of proteins located in the OM (OMPs and Lipoproteins), and the protection of periplasmic proteins under stress conditions. σ^E activates periplasmic chaperones expression such as FkpA or SurA, two peptidyl-prolyl isomerases (PPIase). FkpA plays a role in the folding of some OMPs. SurA is necessary for the transport of unfolded OMPs to the Bam machinery. SurA recognizes their surface exposed hydrophobic areas and protects them from aggregation in the aqueous periplasm. Skp functions during OMP assembly (129,130). The general model proposes SurA as the primary pathway which transport the bulk mass of OMPs across the periplasm. The combination DegP/Skp constitutes the secondary pathway which rescues the proteins that fall out of the primary pathway. At least one of these pathways has to be functional as double mutants *skp surA* or *degP surA* are not viable in *E. coli*.

Proteases

Proteases role is to degrade unfolded or misfolded OMPs in the periplasm. σ^E triggers likewise proteases expression like the essential proteins DegP, a serine endoprotease (90,91,131,132), or RseP, a zinc metalloprotease (121). DegP possesses the specificity to act as a chaperone below 28°C and as a protease at higher temperature. It degrades abnormal proteins such as mutant proteins and oxidatively damaged proteins. RseP degrades remnant signal peptides left in the IM, such as RseA.

LPS and PL biosynthesis (97,133)

Other σ^E-regulated genes are involved in lipid A biosynthesis such as *lpxD* and *lpxP. plsB* is involved in PL biosynthesis, and *tolR* plays a role in OM invagination during cell division. The two essential components of the Bam machinery, *bamA* and *bamD,* are under σ^E-control.

sRNAs

σ^E also leads to the expression of 3 non-coding small regulatory RNAs (sRNAs) conserved among *Enterobacteriacea* namely MicL (134), MicA (135), and RybB (136). MicA, MicL and RybB require the RNA binding protein Hfq, a chaperone that brings the sRNA in the vicinity of the mRNA and modifies its conformation to expose the binding site for base-pairing (137). The sRNAs bind imperfectly to the mRNA targets close to the ribosome binding site thus inhibiting translation (138). MicA downregulates OmpA expression, a highly abundant structural porin (139) as well as OmpX, an outer membrane protein (140). RybB facilitates rapid decay of OmpC, OmpD, OmpF, OmpN and OmpS porins (141,142). MicL targets the very abundant lipoprotein Lpp. Therefore, sRNAs allow σ^E to rapidly downregulate the amount of major OMPs in case of accumulation of misfolded or unfolded proteins, thus preserving envelope homeostasis (143).

Primary metabolism and others

Other genes regulated by σ^E are involved in primary metabolism such as *fusA*, *eno, tufA, recR and ppA*. FusA is an elongation factor that facilitates ribosome translocation along the mRNA molecule while Eno is a component of the degradosome complex that degrades RNAs. TufA is an elongation factor that validates codon/anticodon match during translation, and RecR is a DNA repair protein. PpA catalyzes a reaction that provides energy for protein, RNA and DNA synthesis.

The general sigma factor *rpoD* and the alternative sigma factor *rpoH* also belong to the σ^E regulon (97). Finally, some σ^E-dependent genes are involved in unknown mechanism such as *sbmA*, *ygiM*, *yggN and bacA* (144,133).

In *Salmonella*, most of the target genes are identical (145–149). σ^E represents a central component of a successful infection as it seems to up-regulate SPI-2 genes both in the early and late stages of infection (150). In *Salmonella Typhi*, it was suggested that σ^E upregulates sPI-1 and sPI-2 expression. Evidences suggest that σ^Eregulation also occurs post-transcriptionally (151).

By regulating directly and indirectly more than 100 genes in *E. coli* and *Salmonella*, σ^E is crucial for viability and virulence, respectively. Interestingly, σ^E expression is also relevant for other pathogens.

2.6.5 σ^E relevance in other pathogens

In *Vibrio cholerae, rpoE* significantly contributes to virulence (152). *rpoE* is essential for *Yersinia enterocolita* growth (153) while *rpoE* is required for intracellular survival of *Haemophilus influenzae* in macrophages (154). In *Mycobacterium tuberculosis*, Δ*rpoE* is more susceptible to the activated murine macrophages killing (155). *E. coli rpoE* restores the mucoidy of a *Pseudomonas aeruginosa algU* mutant

(156) showing functional equivalence of these genes. AlgU is responsible for alginate overproduction, leading to mucoidy and chronic infections of cystic fibrosis patients (157).
Rhodius *et al.* demonstrated that some genes regulated by σ^E are conserved among different genomes: *E. coli*, *S. typhi*, *S. typhimurium*, *S. flexneri*, *Y. pestis*, the insect pathogen *P. luminescens* and the plant pathogen *E. carotovora* highlighting the importance of *rpoE* (158).

2.7 Goal of the Ph.D. project

Even though σ^E regulates numerous genes, their impact on virulence is subtle. Some genes are involved in virulence and exhibit an attenuated phenotype *in vivo* such as *degS*, *bamE*, *skp* or *surA*, but their phenotype is not as strong as an *rpoE* mutant, which is completely cleared by the host immune system in typhoid fever mouse model (159).

Therefore, the goal of my Ph.D. project was to:

- seek inhibitor targets of σ^E essentiality *in vivo*;
- understand the molecular mechanisms of σ^E activation *in vivo*;
- assess the ability of *rpoE* and the efflux system as potential inhibitor targets.

To address this topic, our specific aims were to:

- use transposon screenings to identify target genes that could restore *rpoE* virulence *in vivo*;
- investigate the target genes' molecular mechanisms involved in the virulence rescue strain;
- analyze the relevance of our *in vivo*-mimicking condition

3 Publications

3.1 A two-step bypass rescues virulence of *Salmonella* lacking σ^E

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State of the paper: manuscript in preparation

A two-step bypass rescues virulence of *Salmonella* lacking σ^E

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Abstract

Salmonella enterica lacking the extracytoplasmic stress factor σ^E (encoded by *rpoE*), is avirulent and gets completely cleared from infected mice within a few days, suggesting that σ^E may represent an attractive target for urgently needed novel antimicrobials. However, we show here that just two mutations can bypass the requirement for σ^E and restore almost wild-type levels of virulence. As an essential first step, inactivation of the major porin OmpC boosted in vivo fitness about 1,000fold, in part by reducing periplasmic misfolded proteins, (probably including OmpC itself as a major component). Under mildly acidic in vitro conditions, inactivation of OmpC rescued the poor viability of *Salmonella* Δ*rpoE* during

stationary phase, and reversed most proteome alterations during exponential growth. σ^E-dependent gene regulation thus seemed to be a largely indirect consequence of preventing OmpC-mediated damage. N-terminal truncation of polynucleotide phosphorylase boosted in vivo fitness another 1,000fold and caused mostly subtle differences in protein abundance. The final virulence-restored *Salmonella* bypass mutant had also highly similar in vitro and in vivo proteome profiles compared to wild-type. Instead of switching to a distinct envelope maintenance program in absence of σ^E, the two-step bypass thus restored *Salmonella* virulence by removing a major trouble-maker in the cell envelope (OmpC), as well as fine- tuning of many other components. Minor fitness costs of both mutational steps in wild-type *Salmonella*, and the clinical occurrence of OmpC mutations in various pathogenic bacteria, suggest a substantial risk for resistance emergence against σ^E inhibitors. These data demonstrate the importance of early in vivo assessment of potential resistance pathways to avoid costly late-stage failures. Our methods provide a generally applicable strategy for such experiments.

Bacterial infections are a major threat to human health worldwide [1]. All major pathogens show rapidly increasing resistance to available drugs. Without effective novel antimicrobials, infections become untreatable, and medical procedures such as organ transplantation, cancer chemotherapy, or major surgery, are at risk. Unfortunately, antimicrobial discovery and development are slow for Gram-negative bacterial pathogens, with no approval of new antibiotic classes in the past decades. Traditional pipelines for antimicrobial discovery employ rich in vitro conditions that miss important opportunities that may exist in host microenvironment. In particular, antibacterial attacks of the host innate immune system can damage pathogen cells, and pathogens need to limit and repair this damage for survival and growth. Inhibiting such pathogen stress responses might enforce host immunity to provide efficient infection control [2].

The bacterial cell envelope constitutes the host/pathogen interface and is a hotspot of host attacks and counteracting bacterial defenses. A key regulator of the bacterial cell envelope, the RNA polymerase extracytoplasmic stress subunit σ^E (also called σ^{24}, encoded by *rpoE*) is crucial for virulence of diverse pathogens [3,4]. σ^E may thus represent an attractive target whose inhibition could render pathogens more vulnerable to hostile conditions in host tissues [3] (Figure 1a). The role of σ^E in virulence has been most extensively studied in *Salmonella enterica* serovar Typhimurium. σ^E is essential for systemic *Salmonella* virulence in a mouse model of typhoid fever [5], in contrast to only minor roles for the two other major cell envelope regulators Rcs [6] and Cpx [7]. σ^E is induced by unfolded outer membrane proteins, accumulating lipopolysaccharides in the periplasm, or by low pH [8,9]. σ^E regulates hundreds of proteins at transcriptional and post-transcriptional levels, including proteins associated with the type-3 secretion system (T3SS) encoded on pathogenicity island 2 (SPI-2), but most affected proteins show only modest changes in abundance [10-15]. In general, σ^E facilitates delivery of LPS and porins to the outer membrane, repairs/degrades misfolded porins in the

periplasm, and limits the synthesis of outer membrane constituents. These and other σ^E-dependent processes protect *Salmonella* during extended stationary phase [16] as well as during exposure to oxidative stress [16], antimicrobial peptides [17], or severe acid shock [9]. However, the relative importance of these and other stresses in vivo, and the underlying molecular mechanisms remain largely unclear. This knowledge gap impairs thorough assessment of σ^E as a potential target, in particular in terms of potential pathways to resistance against σ^E inhibitors. The possibility of such resistance pathways is supported by (i) several suppressor mutations that bypass the otherwise essential role of σ^E in closely related *Escherichia coli* [18-20] and *Vibrio cholerae* [21]; and (ii) the fact that σ^E can also be bypassed by exposure to low levels of various antibiotics [18] or overexpression of a single small RNA [22].

OmpC causes in vivo essentiality of σ^E

σ^E regulates directly and indirectly the expression of several hundreds of genes including MicA, MicL and RybB. These three noncoding sRNAs have broad impact on cell envelope functions and might mediate a large part of σ^E-dependent processes [4,23]. However, a *Salmonella enterica* serovar Typhimurium SL1344 Δ*micA* Δ*micL* Δ*rybB* mutant lacking all three small RNAs retained wild-type levels of in vivo fitness (in marked contrast to *Salmonella* Δ*rpoE*, which could not be recovered from infected mice at day 5 post-infection) (Figure 1d), indicating that other σ^E-dependent genes are required for *Salmonella* in vivo fitness.

To identify such genes, we generated a transposon library in *Salmonella* Δ*rpoE*. We employed TN*5pp*, a Tn5-derived transposon in which a kanamycin-resistance cassette is flanked by two outward facing σ^{70} promoters with high (P_{tac}) or moderate (P_{bla}) activity, respectively (Figure 1b). Chromosomal insertion of this transposon insertion might drive elevated transcription of adjacent genes, thereby rescuing otherwise σ^E-dependent expression.

At day 5 post-infection of a single mouse with this transposon library, we recovered a few hundred CFU from spleen and liver, indicating partial rescue of in vivo fitness. Sequencing of transposon insertion sites (Figure 1c) revealed three dominant clones (29, 16, and 3 out of a total of 57) with insertions in, or upstream, of *ompC*, which encodes a major outer membrane channel (porin) (Figure 1b). Single strain infections with the most identified transposon mutant *Salmonella* Δ*rpoE ompC1*::Tn*5pp* revealed at least 1,000fold improved in vivo fitness compared to *Salmonella* Δ*rpoE* (Figure 1d).

*ompC*3::Tn*5pp* interrupted the *ompC* signal peptide sequence (Figure 1b), which should render OmpC dysfunctional. *ompC*1::Tn*5pp* preserved the entire *ompC* gene but diminished its expression almost 100fold (Figure 1e), without affecting the abundance of two other major porins, OmpD and OmpF (Supplementary Figure 1a). Together, these data suggested that loss of OmpC might increase the in vivo fitness of *Salmonella* in absence of σ^E. Indeed, a clean *ompC* deletion rescued survival of *Salmonella* Δ*rpoE* to the same extent as *ompC*1::Tn*5pp*, while *ompC* complementation in trans again abolished spleen colonization (Figure 1d, pOmpC). By contrast, deletion of *ompC* in wild-type *Salmonella* caused a slight but highly significant fitness defect (Supplementary Figure 2a), that was undetectable in less sensitive single-strain infection assays (Figure 1d), as reported before [24]. Together, these data show that the dominant porin OmpC contributes to *Salmonella* in vivo fitness, presumably by permitting nutrient uptake. On the other hand, OmpC causes major problems that require mitigation by σ^E-dependent processes to maintain fitness. A similar link between essentiality of σ^E and the dominant porin OmpU has previously been observed in vitro for *Vibrio cholerae* [21], while deletion of *ompC* together with *ompF* and *ompA* only partially bypasses the requirement for σ^E in more closely related *E. coli* [25].

We used SWATH [26] to compare the proteomes of the various *Salmonella* strains grown under in vitro conditions that mimic conditions that *Salmonella* may encounter during

systemic infection including mildly acidic pH (5.5) and magnesium limitation. Numerous proteins showed lower abundance in *Salmonella* Δ*rpoE* compared to wild-type (Figure 1f). This included many SPI-2 associated proteins as expected [10,12,15], but also members of the PhoPQ regulon, which was surprising given previous reports on PhoP-repression of σ^E through MicA [4]. This suboptimal abundance of these crucial virulence proteins could contribute to poor in vivo fitness of *Salmonella* Δ*rpoE*. By contrast, outer membrane proteins such as porins, the Bam complex for outer membrane protein insertion, the Lpt machinery for LPS delivery to the outer leaflet of the outer membrane, or LolAB that facilitate lipoprotein delivery to the outer membrane, all showed no significant alterations as observed previously for *E. coli* [19] and *Salmonella* [10-15] (Supplementary Figure 1b). There was thus a disconnect under our experimental conditions between the proposed main function of σ^E in orchestrating proper delivery of outer membrane components, and lacking changes in respective cell envelope proteins in its absence.

Strikingly, additional mutation of *ompC* annihilated most proteome alterations (Supplementary Figure 1c), and no significant differences remained between the *Salmonella* Δ*rpoE* Δ*ompC* double mutant and wild-type *Salmonella* (Figure 1g), despite the fact that all direct σ^E regulation was absent in *Salmonella* Δ*rpoE* Δ*ompC*. These surprising data suggested that under our experimental conditions, largely indirect mechanisms mediate the σ^E impact on the *Salmonella* proteome, while direct transcriptional and post-transcriptional control by σ^E and its target genes played an apparently minor role, at least in absence of OmpC-mediated perturbations of *Salmonella* physiology.

Mildly acidic stationary cultures phenocopy in vivo σ^E requirements

The porin OmpC could cause cell envelope perturbations by (i) its channel function facilitating translocation of toxic environmental compounds ("channel hypothesis"); (ii) as a

major cargo that could overwhelm folding and delivery pathways for outer membrane proteins, resulting in toxic protein aggregates in the periplasm ("cargo hypothesis") [27]; or combinations of these and other unknown mechanisms [25] (Figure 2a).

OmpC has been shown to permit entry of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) [28]. ROS are key components of anti-*Salmonella* immunity [29-31], and ROS are more active against *Salmonella* lacking σ^E [16]. Absence of OmpC could thus diminish ROS entry into the *Salmonella* cell, and this would diminish requirements for σ^E-dependent defense/damage repair. To test this model, we used a sensitive P_{katG}-*gfp-ova* reporter fusion that specifically detects host-generated ROS that reach the *Salmonella* cytosol [30,31], to determine *Salmonella* exposure to ROS (Figure 2b). In vitro, wild-type *Salmonella* showed higher GFP fluorescence upon exposure to 10 µM H_2O_2 compared to *Salmonella* Δ*ompC* (Supplementary Figure 3a), confirming previous in vitro data [28]. However, in vivo *Salmonella* Δ*ompC* and wild-type *Salmonella* had indistinguishable GFP levels with about 10-15% cells with high GFP content (GFP^{Hi}) (Figure 2d), indicating that OmpC was dispensable for ROS entry in vivo. A ROS-related mechanism was also unlikely to explain the OmpC-σ^E link since *Salmonella* Δ*rpoE* shows enhanced sensitivity only at H_2O_2 exposure levels that are several orders of magnitude higher compared to physiological levels (4 mM [16] vs. less than 20 µM [31]). Together, these data suggested that factors other than ROS might cause the OmpC-dependent requirement of σ^E in vivo.

To further test the OmpC "channel hypothesis", we occluded the OmpC restriction zone by a glycin132 to aspartate in the L3 loop. An analogous Gly119Asp mutation in OmpF diminishes translocation through OmpF with minimal impact on the overall porin structure [32] (Figure 2c). *Salmonella* Δ*rpoE ompC* G132D carrying this "occluded" variant had in vivo fitness comparable to *Salmonella* Δ*rpoE* Δ*ompC* totally lacking OmpC (Figure 1d), but OmpC G132D had also diminished abundance (Figure 1e), suggesting that OmpC channel

closure, lower OmpC cargo, or both might have contributed to the fitness gain from this variant, rendering these data non-informative for discriminate the "channel" and "cargo" hypotheses.

Salmonella expressed closely related porins, OmpD and OmpF, at moderate and very low levels, respectively (Supplementary Figure 1a). Deletion of *ompD* did not improve in vivo fitness of *Salmonella* Δ*rpoE* (Figure 1d), consistent with our inability to identify *ompD* mutants in our transposon screen. Moreover, replacing the *ompC* gene by *ompD* (*ompC*::*ompD*), rescued in vivo fitness as well as a simple *ompC* deletion (Figure 1d). These data demonstrate that OmpD did not contribute significantly to perturbations that require σ^E for maintaining in vivo fitness. Likewise, inactivation of the small RNA MicF that represses OmpF [33], had no impact on in vivo fitness of *Salmonella* Δ*rpoE* Δ*ompC*. Together, these data suggest that in contrast to OmpC, OmpD and OmpF do not cause detectable perturbations that need mitigation by σ^E. Again, this information was not conclusive with respect to the "channel" and " cargo" hypotheses, as these porins differ both in channel properties and abundance from OmpC.

Some contribution of misfolded periplasmic proteins to the poor fitness of *Salmonella* Δ*rpoE* was indicated by the moderate fitness rescue that we obtained by overexpressing of the periplasmic protease DegP [34,35] (Figure 1d, pDegP). By contrast, DegP overexpression did not further enhance in vivo fitness of *Salmonella* Δ*rpoE* Δ*ompC* (Figure 1d) suggesting that once OmpC is inactive, periplasmic protein aggregates might not be a major problem affecting in vivo fitness. Again, this could be either a consequence of less translocation of toxic molecules ("channel" hypothesis), or less cargo due to the absence of OmpC itself.

A prerequisite for the channel hypothesis would be actual presence of toxic molecules that enter exclusively through OmpC. Although such compounds might exist in the *Salmonella* microenvironment in infected tissues, two reporters for envelope stress, the

strictly σ^E-dependent fusion P_{micA}-*gfp-ova* (Supplementary Figure 3b) and the broadly responsive fusion P_{degP}-*gfp-ova*, had both non-impressive in vivo activities (Figure 2d, red lines) similar to baseline activities of these fusion in our in vivo-mimicking in vitro culture conditions with no toxic molecules, and clearly lower than in *Salmonella* exposed to 5 μg ml^{-1} polymyxin B, a widely used inducer of envelope stress (Figure 2d, black lines). These data could suggest that the detectable live *Salmonella* have to deal mostly with internally generated envelope stress during infection, with limited impact of external stress. However, some *Salmonella* could still be exposed to overwhelming envelope attacks that cause lethal damage, rendering this *Salmonella* subset undetectable for flow cytometry (which depends on fluorescent protein retention by an intact envelope) [31,36] and plating. We have previously observed an analogous situation for host ROS attacks that killed *Salmonella* in neutrophils and inflammatory monocytes, whereas *Salmonella* in red pulp macrophages experienced only weak non-lethal ROS stress [31].

The hypothesis that toxic external molecules entering through OmpC may not explain the in vivo phenotypes, was supported by data for in vitro stationary cultures in mildly acidic minimal medium with no toxic compounds (Figure 2e,f). Live/dead staining revealed extensive loss of viability in *Salmonella* Δ*rpoE* and *Salmonella* Δ*rpoE* Δ*ompD*, but not *Salmonella* Δ*rpoE* Δ*ompC* or *Salmonella* Δ*rpoE ompC* G132D, thus phenocopying respective in vivo fitness data (Figure 1d). *Salmonella* Δ*rpoE* had few dead cells in exponential cultures, or exponential or overnight cultures at pH 7.4. A specific role of σ^E at mildly acidic conditions in host microenvironments was compatible with previous data for cell culture infections that showed less severe *Salmonella* Δ*rpoE* fitness defects in presence of ammonium chloride that diminishes acidification of *Salmonella*-containing vacuoles [9]. Acidic pH can increase protein misfolding and aggregation in the periplasm, especially during stationary phase [35] (that resembles very slow *Salmonella* in vivo growth [37]). Coping with such

protein aggregates requires increased chaperone and protease activity as induced by σ^E, to minimize envelope damage [38]. Together, these data show that (i) slow growth under mildly acidic conditions with no external toxic compounds, phenocopies the in vivo OmpC- σ^E link; and (ii) that OmpC can cause a requirement for σ^E most likely because of its ability to overwhelm folding and delivery pathways under adverse conditions ("cargo" hypothesis). How distinct properties of OmpC such as abundance and/or propensity to misfold and aggregate at acidic conditions, might cause its dominant role for σ^E requires further studies.

Restoration of high in vivo fitness by a truncated polynucleotide phosphorylase

OmpC inactivation rescued spleen colonization of *Salmonella* Δ*rpoE* at least 1,000fold, but bacterial loads at day 4 post-infection were still 10,000fold lower compared to wild-type *Salmonella* (Figure 1d), and mice showed no detectable disease signs. Poor in vivo *Salmonella* colonization could reflect reduced proliferation, enhanced killing, or both. *Salmonella* Δ*rpoE* Δ*ompC* carrying the growth rate reporter TIMER^{bac} [37] showed almost wild-type TIMER^{bac} signals indicative of substantial proliferation, suggesting that the overall low fitness of this strain was mostly due to rapid killing.

Envelope stress due to dysfunctional σ^E can result in increased induction of another cell envelope stress response system, Rcs [19], which could lead to poor *Salmonella* in vivo fitness [6]. *Salmonella* Δ*rpoE* Δ*ompC* Δ*rcsD* lacking phosphotransferase RcsD, which is required for activating the response regulator RcsB, showed strong down-regulation of RcsB but no other significant proteome differences (Figure 2h), and slightly increased in vivo fitness (Figure 3b). We used this mutant as a basis for further investigations.

To identify other factors involved in rapid killing, we performed another round of transposon mutagenesis with TN*5pp*. Surprisingly, after two rounds of enrichment, mice infected with the transposon mutant pool showed clear disease signs at days four to five post-

infection. Sequencing of transposon insertion sites revealed a dominant clone with an insertion in *pnp* encoding polynucleotide phosphorylase (PNPase), which is involved in RNA metabolism [39], and three minor clones with insertions in *cadA*, *ftsI2*, and *yicH* (Figure 3a). Single strain infections confirmed at least 1,000fold increased spleen loads (compared to *Salmonella* Δ*rpoE* Δ*ompC* Δ*rcsD*) for *Salmonella* Δ*rpoE* Δ*ompC* Δ*rcsD pnp*::Tn*5pp* achieving almost wild-type levels, and more than 100fold increased levels for the other three clones (Figure 3b). A clean deletion of *cadA* failed to boost in vivo fitness and we did not further investigate the three minor clones.

A clean deletion of *pnp* in *Salmonella* Δ*rpoE* Δ*ompC* Δ*rcsD* resulted in elevated, but highly variable in vivo fitness (Figure 3b). As a potential explanation for this discrepancy, the transposon insertion might not fully inactivated *pnp*, but rather generate truncated N-terminal and C-terminal (from an internal ATG codon) fragments of this multi-domain protein [40] (Figure 3c), that could have conferred the fitness rescue. To test this hypothesis, we deleted the *pnp* N-terminal sequence upstream of the transposon insertion site (amino acids 2-199). It is possible that the N-terminal fragment of polynucleotide phosphorylase in the transposon mutant contributed to in vivo fitness but for sake of simplicity, we focused on the strain encoding the truncated C-terminus. This strain *Salmonella* Δ*rpoE* Δ*ompC* Δ*rcsD pnp*Δ2-199 (abbreviated as Δ4) caused clear disease signs such as rough hair coat and reduced surface temperature, and had rather consistent 1,000fold increased spleen loads. A more accurate analysis using competitive infections together with wild-type *Salmonella*, revealed a competitive index 0.16 ± 0.09 (Supplementary Figure 2b). This was equivalent to a six-fold difference in spleen loads at day 4 post-infection between wild-type and mutant, a minor difference compared to the over 10,000fold in vivo expansion of wild-type *Salmonella* during this time interval. These data suggested an almost complete rescue of in vivo fitness and virulence in the mutant.

Despite the marked impact of the N-terminal truncation of *pnp* in the *Salmonella* Δ*rpoE* Δ*ompC* Δ*rcsD* background, the same *pnp* truncation had only small impact on fitness of *Salmonella* Δ*rpoE* (Figure 3b), consistent with our inability to recover *pnp* mutants in our first screen. This indicated an indispensable role of *ompC* inactivation as a first step in fitness rescue in absence of σ^E.

PNPase contains two RNase PH-like domains PH1 and PH2, which are connected by an all α-helical domain AAHD [39]. The C-terminus is composed of two RNA binding domains KH and S1 (Figure 3c). The active center is located in PH2, but PH1 (which is largely missing in our Δ2-199 mutant) also affects PNPase activity, in part by stabilizing the trimeric structure. PNPase has both 3'->5' RNA exonuclease and RNA polymerase (adding untemplated nucleotides) activities [39]. PNPase is part of the RNase E degradosome and the poly(A) polymerase I polyadenylation complexes, and regulates a wide range of complex phenotypes. PNPase has a broad impact on various RNA species including sRNAs, rRNAs, and tRNAs. In addition, PNPase can regulate virulence factor activities by its RNA binding activity independent of RNA metabolism. A *Salmonella* mutant with dysfunctional PNPase due to truncation of RNA binding domain S1, increases *Salmonella* expression of virulence genes associated with SPI-1 and SPI-2 and abolishes formation of Agf fibers [41] in rich media. These alterations do not affect *Salmonella* systemic virulence but may enable the mutant to cause persistent infections after oral inoculation [42].

Proteome analysis of *Salmonella* Δ*rpoE* Δ*ompC* Δ*rcsD pnp* Δ2-199 (Δ4) showed a threefold decrease in PNPase levels as well as many subtle and a few more pronounced differences to *Salmonella* Δ*rpoE* Δ*ompC* Δ*rcsD* (Figure 3d). Similar to the effects of dysfunctional PNPase [41], N-terminal truncation stimulated expression of SPI-2 associated factors (red). We did not detect upregulation of SPI-1 associated factors, probably as SPI-1 is strongly repressed under in vitro-mimicking conditions. The cold-shock associated regulator

CspA had increased abundance consistent with a role of PNPase in degradation of *cspA* transcripts [39]. Together, these data could suggest decreased activity of the N-terminally truncated PNPase variant. On the other hand, the marked difference of this variant to a full *pnp* deletion indicated that the N-truncated variant had still some residual functions.

We also detected alterations in three envelope proteins (green), the glutamate transporter GltS, porin OmpD, and the lipoprotein NlpI. The 3.5fold increase in OmpD levels might partially compensate the diminished nutrient uptake across the outer membrane due to the absence of OmpC, but how this would contribute to reduced *Salmonella* killing is unclear. Decreased NlpI levels can cause hyper-production of outer membrane vesicles (OMV), which increases resistance to membrane-damaging agents such as antimicrobial peptides [43], and helps to get rid of damaged periplasmic components [35]. Diminished NlpI levels also stimulate increased peptidoglycan synthesis [44]. Comparison of *Salmonella* Δ*rpoE* Δ*ompC* Δ*rcsD pnp* Δ2-199 (Δ4) with wild-type *Salmonella* revealed the combined effects of the different steps in fitness rescue and a few additional proteins with unclear relevance to *Salmonella* virulence including colicin Ib (Cib), YggN, YccU and the flavin transferase Ftp (Figure 3e).

Although the relevance of these individual alterations for fitness rescue remains to be investigated, the low number of differentially proteins associated with *Salmonella* outer membrane maintenance was striking given the major role of σ^E for mitigating envelope stress. In vitro cultures might imperfectly reflect the in vivo conditions that *Salmonella* encounter in infected host tissues, but our (less comprehensive) data for *Salmonella* purified ex vivo from spleen also revealed highly similar proteome profiles for wild-type *Salmonella* and *Salmonella* Δ*rpoE* Δ*ompC* Δ*rcsD pnp* Δ2-199 (Δ4) (Supplementary Figure 4). The only clear difference was the tenfold lower abundance of the outer membrane porin for fatty acids FadL in the Δ4 mutant in vivo, in contrast to in vitro cultures where FadL abundance remained in 1.3fold range for all strains. As the intermediate *Salmonella* strains were poor colonizers we

were unable to purify enough material for assigning this in vivo FadL modulation to a specific step (Δ*rpoE*, Δ*ompC*, Δ*rcsD*, or *pnp* Δ2-199). OmpD that showed fivefold higher abundance in vitro in Δ4, had only twofold (and non-significantly) elevated levels in vivo. Taken together, these data suggest that *Salmonella* can bypass σ^E, an absolutely crucial contributor to in vivo fitness and virulence, by a number of minor alterations, instead of using a completely distinct envelope maintenance program, under the precondition that the major "trouble-maker" OmpC is absent. Such mostly subtle differences might have been expected since (i) σ^E itself modulates expression of many genes in only moderate and apparently largely indirect ways, and (ii) *Salmonella* in vivo properties are already selected for high fitness and might be incompatible with major alterations associated with a distinct envelope maintenance program.

Conclusion

The extracytoplasmic stress factor σ^E plays a crucial role in *Salmonella* virulence in a mouse typhoid fever model, providing in vivo validation for σ^E as an attractive target for antimicrobial chemotherapy. However, we show here that just two mutations were sufficient to rescue spleen colonization by 100,000,000 fold resulting in almost wild-type levels of virulence.

The specific mutations and their physiological consequences provide new insights into key issues for *Salmonella* envelope maintenance. In particular, σ^E was primarily needed to mitigate lethal damage caused by the major porin OmpC in vivo and during stationary phase in mildly acidic in vitro cultures. Inactivation of OmpC also reversed most distortions in protein abundances that were caused in absence of σ^E, further supporting a crucial OmpC - σ^E link. Truncation of PNPase had mostly subtle consequences on gene expression and was not required under our in vitro conditions, but dramatically boosted in vivo fitness. The final

virulence-restored *Salmonella* bypass mutant had highly similar in vitro and in vivo proteome profiles compared to wild-type. Instead of switching to an alternative envelope maintenance program in absence of σ^E, the two-step bypass thus restored *Salmonella* virulence by removing a major trouble-maker in the cell envelope as well as fine-tuning of many other components. σ^E-dependent gene regulation seemed to be a largely indirect consequence of preventing OmpC-mediated damage, at least under our conditions.

These data demonstrate an effective two-step bypass pathway for developing in vivo resistance against potential σ^E inhibitors. Both steps have only minimal fitness costs in vitro and in vivo. The requirement for two consecutive steps could diminish resistance rates to acceptable levels. However, the first step, *ompC* inactivation, is also associated with resistance to clinically relevant antibiotics [45,46], and clinical strains with low OmpC levels are repeatedly isolated [45,47-49]. At least in some clinical infections, only a single inactivating mutation in PNPase (or maybe other genes, Figure 3a) would thus be needed to render σ^E inhibitors ineffective. Together, these findings might devaluate σ^E as an antimicrobial target.

In general, in vivo validation of potential targets relies mostly on quantifying attenuation levels. We propose that this should be augmented by a detailed in vivo risk assessment for potential pathways to resistance. It is important to perform such experiments in a relevant in vivo setting, since gene essentiality and bypass pathways are highly condition-dependent. It is generally unclear how well particular in vitro cultures mimic relevant in vivo environments, and in vivo/in vitro discrepancies in resistance rates and pathways (as also seen in this study) are common [50]. This can lead to a major waste of resources when compounds fail late in clinical trials because of inacceptable resistance emergence in patients, in spite of low resistance in vitro (e.g., [51]). Our approach provides an effective, broadly applicable strategy to mitigate such risks.

Methods

Salmonella cultures

Salmonella strains used in this study were derived from *Salmonella enterica* serovar Typhimurium *SL1344 hisG rpsL xyl* [52,53]. The H_2O_2 biosensor construct P_{katG}-*gfp-ova* was described previously[31]. Envelope stress reporter constructs P_{degP}-*gfp-ova* and P_{micA}-*gfp-ova* were obtained by exchanging the P_{katG} promoter for SL1344 bp 244,513 – 244,323 (P_{degP}) or 2,989,124 - 2,989,445 (P_{micA}). *Salmonella* chromosomal mutants were generated using red recombinase-mediated allelic followed by P22 phage transduction and FLP recombinase-mediated removal of the resistance cassette [54], or using two consecutive single-crossovers with positive and negative selection markers (*aphT*; *sacB or i-sceI*). Tn*5pp* was synthesized by GENEART. Transposon libraries were generated by electroporation of a transposome complex formed by mixing PCR-amplified Tn*5pp* with EZ-Tn5™ Transposase (Epicentre). Plasmids pOmpC and pDegP carried *ompC* or *degP*, respectively, under control of the constitutive P_{ybaJ} promoter on pBR322 backbones. *Salmonella* were cultured at 37°C with aeration (200 rpm) in Lennox LB with addition of 90 μg ml^{-1} streptomycin with or without 100 μg ml^{-1} ampicillin or in MgMES medium [55] adjusted to pH 5.5.

Mouse Infections and Tissue Collection

All animal experiments were approved (license 2239, Kantonales Veterinäramt Basel) and performed according to local guidelines (Tierschutz-Verordnung, Basel) and the Swiss animal protection law (Tierschutz-Gesetz). Female 8-12 weeks old BALB/cAnNCr were infected by tail vein injection of 800-2800 CFU (virulent *Salmonella* strains), or up to 100,000 CFU (strongly attenuated strains, transposon libraries) in 100 μl PBS and euthanized at 4-5 days post infection. Spleen tissue was collected from each mouse and dissected into several pieces. CFU counts were determined by plating. Competitive indices were determined using mixed

infections by dividing output ratios (mutant/wild-type) by the inoculum ratios (mutant/wild-type). We estimated sample size by a sequential statistical design. We first infected three mice each based on effect sizes and variation observed in our previous studies [56], and used the results to estimate group sizes for obtaining statistical significance with sufficient power. We did neither randomize nor blind the experiments. However, flow cytometry analysis was carried out using an automated unbiased approach (see Flow Cytometry section).

Flow Cytometry

Spleen homogenates were prepared for flow cytometry as described [31]. Relevant spectral parameters of 10,000 to 50,000 *Salmonella* were recorded in a FACS Fortessa II equipped with 488 nm and 561 nm lasers (Becton Dickinson), using thresholds on SSC and FSC to exclude electronic noise (channels: tagBFP2, excitation 405 nm, emission 460-480 nm; GFP, excitation 488 nm, emission 502-525; mCherry, excitation 561 nm, emission 595-664 nm; yellow autofluorescence channel, excitation 488 nm, emission 573-613 nm; infrared autofluorescence channel, excitation 561 nm, emission 750-810 nm). *Salmonella* viability was determined using the LIVE/DEAD™ BacLight™ Bacterial Viability Kit (InVitrogen) (channels: Syto 9, excitation 488 nm, emission 502-525 nm; Propidium iodide, excitation 561 nm, emission 595-664 nm). Data processing was done with FlowJo and FCS Express. $TIMER^{bac}$ fluorescence was measured with green (excitation 488 nm, emission 502-525) and orange (excitation 561 nm, emission 604-628 nm) channels and displayed as log ratio.

For purification of *Salmonella* from infected spleen, samples were sorted using an Aria IIIu cell sorter (BD Biosciences) with scatter and fluorescence channels (green, excitation 488 nm, emission 499-529 nm; orange, excitation 561 nm, emission 595-664 nm).

Proteomics

Triplicate or quadruplicate cell pellets from independent experiments were prepared for proteomics with the sample preparation kit from PreOmics (PreOmics GmbH, Germany), according to the protocol version 2.2. The cell pellets were lysed in 50 µl of the provided lysis buffer and heated for 5 min at 95 °C. The samples were sonicated with a Bioruptor (Diagenode) applying the standard 10 min method (30 s on, 30 s off, 10 cycles). The protein concentration was determined by BCA assay (Thermo-Pierce, 23252). A maximum of 100 µg protein was digested at 37 °C for four hours. After clean-up according to the manufacturers procedure, the peptides were dried in a vacuum centrifuge and frozen at -20 °C until further use.

Data independent analysis (DIA) was performed using a HRM DIA method as previously described [57]. Liquid chromatography and mass spectrometer setup was identical to the PRM analysis. Peptide separation was performed over 90 min. The HRM DIA method contained a MS1 scan (target setting: 5e6 ions, maximum accumulation time: 100 ms, scan range: 400-1220 m/z, resolution: 120,000 FWHM) followed by acquisition of 38 DIA windows (target setting: 3e6 ions, maximum accumulation time: auto, resolution: 30,000 FWHM). Stepped collision energy was 22.5%, 25%, 27.5%. Spectra were recorded in profile mode.

SWATH data files were converted to htrms file format using HTRMS Converter (Biognosys AG, Switzerland). Converted files were imported into Spectronaut 11 (Biognosys AG, Switzerland) using default parameters with the following exceptions for the quantification settings: no cross run normalization and only proteotypic peptides allowed. An in-house *Salmonella enterica* serovar Typhimurium SL1344 spectral library was generate from *Salmonella* grown under different standard media conditions and from *Salmonella* purified from mouse spleen. This spectral library was merged with an in-house generated mouse spectral library. Peptide and protein identification was performed allowing a q-value of

0.01. Protein abundance was taken from the protein group quantity calculation (PG.quantity) as provided by the software. Samples were normalized using the top 100 most abundant proteins.

Statistical analysis

Colonization data were log-transformed, which results in normally distributed data [58], and analyzed by one-way ANOVA followed by comparison of selected columns to a control column taking into account the multiple comparison problem using GraphPad Prism 7.03. Proteome data were log-transformed and analyzed by two-tailed t-tests followed by determining the Benjamini-Hochberg false-discovery rate q for multiple comparisons. A q-value of 0.05 is shown as a dashed line in the respective volcano plots.

Data availability

The data that support the findings of this study are available from the corresponding author upon request.

Correspondence and requests for materials should be addressed to D.B.

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Author contributions

P.M. performed experiments; P.M. and D.B analyzed the data; D.B. wrote the paper with major input from P.M.

Additional information

Supplementary information is available online.

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Figure legends

Figure 1: OmpC causes in vivo essentiality of σ^E.

a, Schematic model for host attacks targeting the *Salmonella* envelope and *Salmonella* defenses that mitigate the damage. **b**, Upper panel: Structure of transposon Tn5pp with two outward facing promoters; lower panel, map of identified transposon insertions sites at the *ompC* locus. **c**, Frequency of transposon insertions sites in *Salmonella* Δ*rpoE* after mouse passage. **d**, In vivo growth of various *Salmonella* strains. Spleen loads at day 4 (virulent strains) or day 5 (attenuated strains) post intravenous infection, normalized by inoculum size are shown. Statistical significance of differences to the control strain *Salmonella* Δ*rpoE* as tested by one-way ANOVA is shown in color (red, $P< 0.0001$; orange, $P< 0.001$; black, $P> 0.05$; det.thr., detection threshold). **e**, Abundance of OmpC protein relative to the most abundant outer membrane protein OmpA in various *Salmonella* strains. Statistical significance of differences to wild-type strain *Salmonella* Δ*rpoE* as tested by one-way ANOVA (****, $P< 0.0001$). **f**, Protein abundance differences between *Salmonella* Δ*rpoE* and wild-type. The dashed line represents a Benjamini-Hochberg false-disovery rate q = 0.05. *Salmonella* pathogenicity island 2 (SPI-2) - associated proteins are shown in red, PhoP-associated proteins are shown in blue. **g**, Protein abundance differences between *Salmonella* Δ*rpoE* Δ*ompC* and wild-type. No protein passed the q = 0.05 criterion.

Figure 2: Mildly acidic stationary cultures phenocopy in vivo σ^E requirements.

a, Schematic model for OmpC-dependent envelope damage. Toxic molecules might translocate across the outer membrane through OmpC (i; "channel" hypothesis). OmpC might provide a major contribution to misfolded protein aggregates overwhelming folding and delivery pathways to the outer membrane (ii, "cargo" hypothesis). **b**, Left panel:

Simultaneous detection of two different *Salmonella* strains containing either blue fluorescent protein (BFP, Δ*ompC*) or mCherry (WT, wild-type) in the same spleen using flow cytometry. GFP reporter as a read-out for P_{katG} promoter activity can then be detected for both strains as an independent third fluorescence color (right panel). The dashed line was used as a threshold to determine the fraction of *Salmonella* cells with high GFP content (GFP^{Hi}). **c**, Structures of OmpF (PDB entry 2omf) and OmpF G119D (1mpf). **d**, Activities of cell envelope stress reporter fusions to *gfp* in MgMES medium (grey), MgMES medium containing 5 mg l^{-1} poylmyxin B, or in mouse spleen (red). The insets show the median GFP fluorescence. **e**, Viability of various *Salmonella* strains in MgMES medium at pH 5.5. **f**, quantification of independent replicates of data shown in e. Differences in viability compared to wild-type (WT) was tested by one-way ANOVA (****, $P< 0.0001$; n.s., not significant). **g**, $TIMER^{bac}$ log(green/orange) ratios for *Salmonella* Δ*rpoE* Δ*ompC* and wild-type in spleen as detected by flow cytometry. **h**, Protein abundance differences between *Salmonella* Δ*rpoE* Δ*ompC* Δ*rcsD* and *Salmonella* Δ*rpoE* Δ*ompC* wild-type. Only RcsB passed the q = 0.05 criterion.

Figure 3: Restoration of high in vivo fitness by a truncated polynucleotide phosphorylase.

a, Frequency of transposon insertions sites in *Salmonella* Δ*rpoE* Δ*ompC* Δ*rcsD* after mouse passage. **b**, In vivo growth of various *Salmonella* strains. Spleen loads at day 4 (virulent strains) or day 5 (strongly attenuated strains) post intravenous infection, normalized by inoculum size are shown. Statistical significance of differences to the control strain *Salmonella* Δ*rpoE* Δ*ompC* Δ*rcsD* as tested by one-way ANOVA is shown in color (red, $P< 0.0001$; black, $P> 0.05$; det.thr., detection threshold). **c**, Domain structure and transposon insertion site in PNPase. The yellow region was deleted in the N-terminal truncation mutant *pnp* Δ2-199 (aα, all α-helical domain AAHD; KH, RNA binding domain; PH1, RNase PH-

like domain 1; PH2, PH1, RNase PH-like domain 2; S1, RNA binding domain). **d**, Protein abundance differences between *Salmonella* Δ*rpoE* Δ*ompC* Δ*rcsD pnp* Δ2-199 (Δ4) and *Salmonella* Δ*rpoE* Δ*ompC* Δ*rcsD*. The dashed line represents a Benjamini-Hochberg false-disovery rate q = 0.05. *Salmonella* pathogenicity island 2 (SPI-2) - associated proteins are shown in red, envelope-associated proteins are shown in green. **e**, Protein abundance differences between *Salmonella* Δ*rpoE* Δ*ompC* Δ*rcsD pnp* Δ2-199 (Δ4) and wild-type.

Supplementary Figure 1: Protein abundance in various strains in vitro.

a, Abundance of porins OmpC (same data as in Figure 1e), OmpD, and OmpF. **b**, Fold differences in abundance of envelope-associated proteins between *Salmonella* Δ*rpoE* and wild-type (POR, porins; C/P/I, periplasmic chaperones, protease DegP, and periplasmic peptidyl-prolyl cis-trans isomerases). **c**, Fold differences in protein abundance in two different strain combinations. The dashed line represents complete reversal of Δ*rpoE*-dependent alterations in the *Salmonella* Δ*rpoE* Δ*ompC* strain. *Salmonella* pathogenicity island 2 (SPI-2) - associated proteins are shown in red, PhoP-associated proteins are shown in blue.

Supplementary Figure 2: Competitive indices from mixed infections.

The dashed lines represent wild-type levels of in vivo fitness. Values below 1 indicate attenuation of the mutant (***, P< 0.001; two-tailed t-test on log-transformed data).

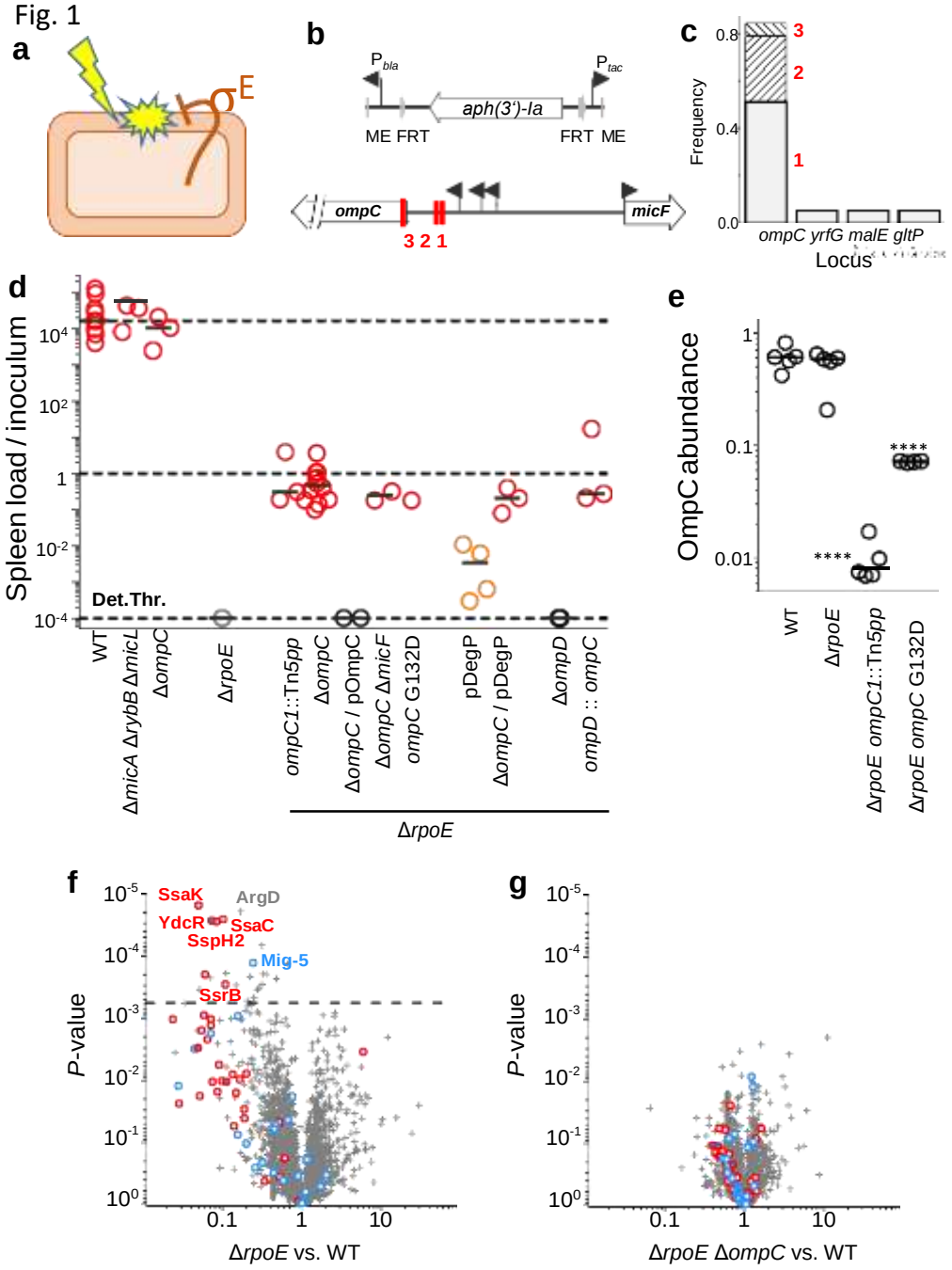
Supplementary Figure 3: In vitro activities of reporter fusions.

a, Activity of P_{katG}-*gfp-ova* in wild-type *Salmonella* (grey, black) or *Salmonella* Δ*ompC* (red) in MgMES (shaded) or MgMES containing 10 μM H_2O_2. **b**, Activity of P_{micA}-*gfp-ova* in wild-type *Salmonella* (grey, black) or *Salmonella* Δ*rpoE* (braun) in MgMES (shaded) or MgMES containing 5 mg l^{-1} polymyxin B.

Supplementary Figure 4: In vitro activities of reporter fusions.

Protein abundance differences between *Salmonella* Δ*rpoE* Δ*ompC* Δ*rcsD pnp* Δ2-199 (Δ4) and wild-type in mouse spleen. None of the proteins passed the q = 0.05 criterion.

Fig. 1


a
σE
b
Pbla
Ptac
aph(3')-Ia
ME FRT
FRT ME
ompC
micF
3 2 1
c
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0.0
3
2
1
ompC yrfG malE gltP
Locus
d
Spleen load / inoculum
10^4
10^2
1
10^-2
10^-4
Det.Thr.
WT
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ΔompC
ΔrpoE
ompC1::Tn5pp
ΔompC
ΔompC / pOmpC
ΔompC ΔmicF
ompC G132D
pDegP
ΔompC / pDegP
ΔompD
ompD :: ompC
ΔrpoE
e
OmpC abundance
1
0.1
0.01
****
****
WT
ΔrpoE
ΔrpoE ompC1::Tn5pp
ΔrpoE ompC G132D
f
P-value
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10^-4
10^-3
10^-2
10^-1
10^0
SsaK
ArgD
YdcR
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ΔrpoE vs. WT
g
P-value
ΔrpoE ΔompC vs. WT

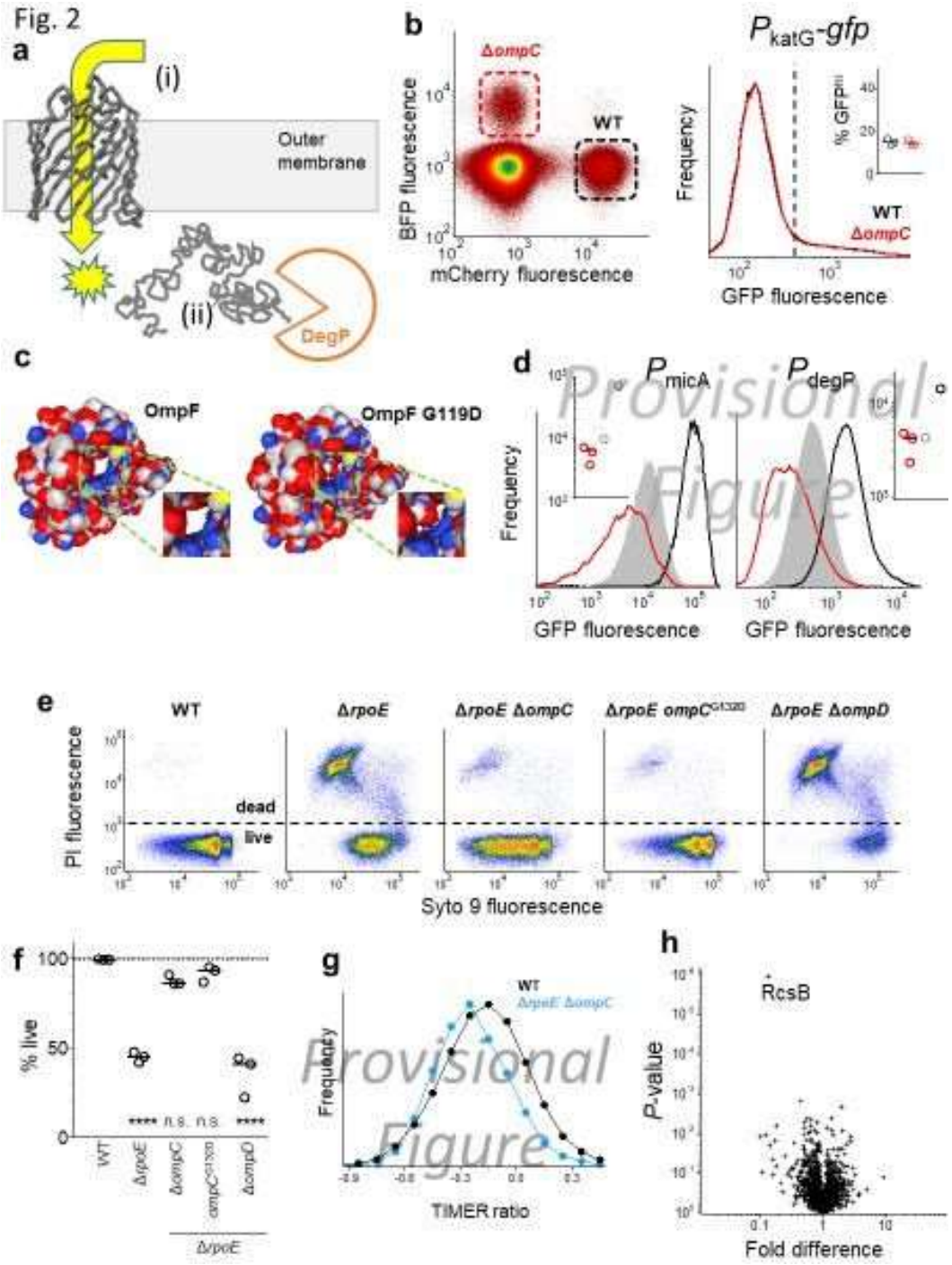
Fig. 2
a
(i)
Outer membrane
(ii)
DegP
b
ΔompC
WT
BFP fluorescence
mCherry fluorescence
PkatG-gfp
Frequency
% GFPIII
WT
ΔompC
GFP fluorescence
c
OmpF
OmpF G119D
d
PmicA
PdegP
Frequency
GFP fluorescence
GFP fluorescence
Provisional Figure
e
WT
ΔrpoE
ΔrpoE ΔompC
ΔrpoE ompCG119D
ΔrpoE ΔompD
PI fluorescence
dead
live
Syto 9 fluorescence
f
% live
n.s.
n.s.
****
****
WT
ΔrpoE
ΔompC
ompCG119D
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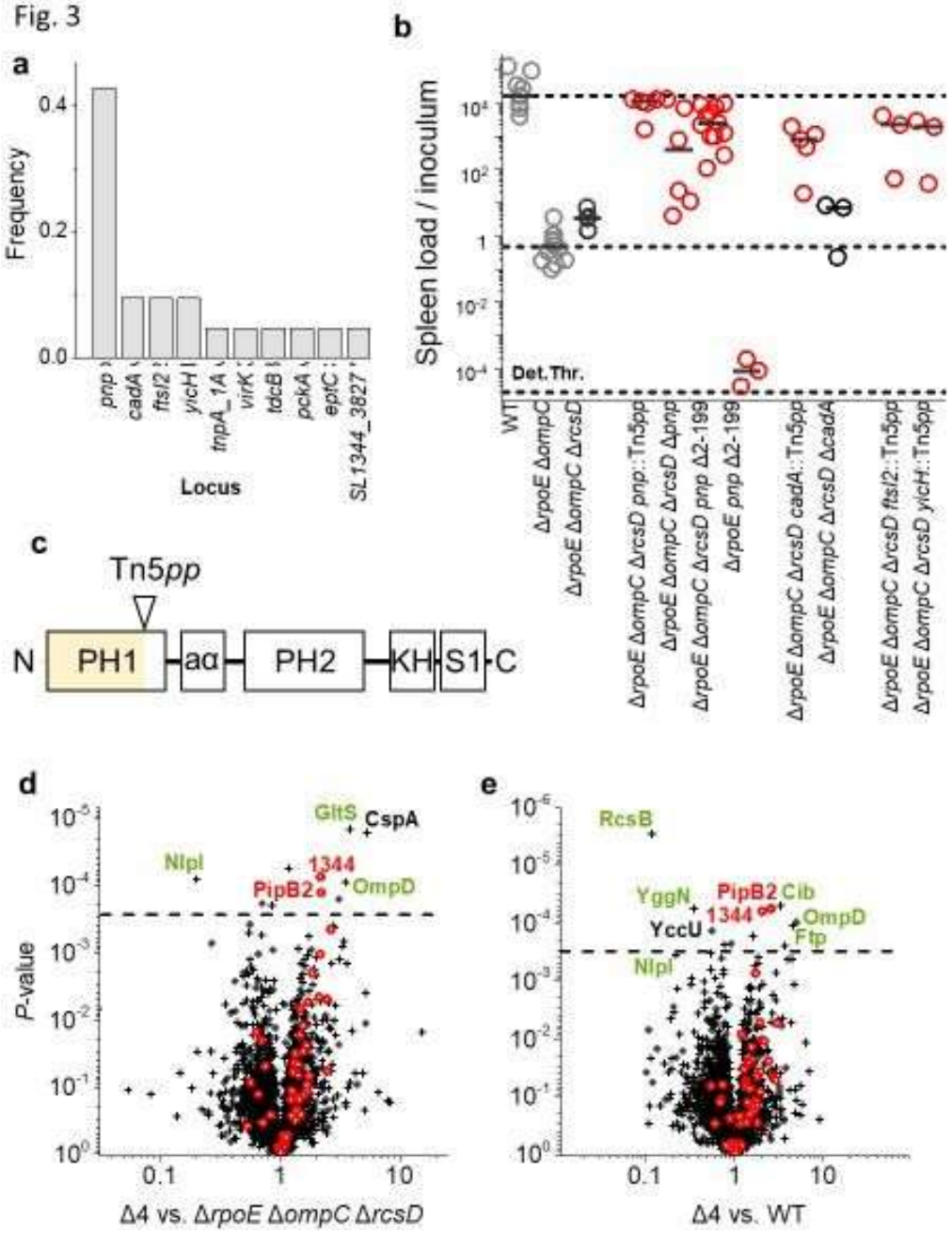
g
WT
ΔrpoE ΔompC
Frequency
TIMER ratio
Provisional Figure
h
RcsB
P-value
Fold difference

Fig. 3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ftsI/2
yicH
tnpA_1A
virK
tdcB
pckA
eptC
SL1344_3827
Locus
b
Spleen load / inoculum
Det. Thr.
WT
ΔrpoE ΔompC
ΔrpoE ΔompC ΔrcsD
ΔrpoE ΔompC ΔrcsD pnp::Tn5pp
ΔrpoE ΔompC ΔrcsD Δpnp
ΔrpoE ΔompC ΔrcsD pnp Δ2-199
ΔrpoE pnp Δ2-199
ΔrpoE ΔompC ΔrcsD cadA::Tn5pp
ΔrpoE ΔompC ΔrcsD ΔcadA
ΔrpoE ΔompC ΔrcsD ftsI/2::Tn5pp
ΔrpoE ΔompC ΔrcsD yicH::Tn5pp
c
Tn5pp
N
PH1
aα
PH2
KH
S1
C
d
P-value
GltS
CspA
NlpI
1344
PipB2
OmpD
Δ4 vs. ΔrpoE ΔompC ΔrcsD
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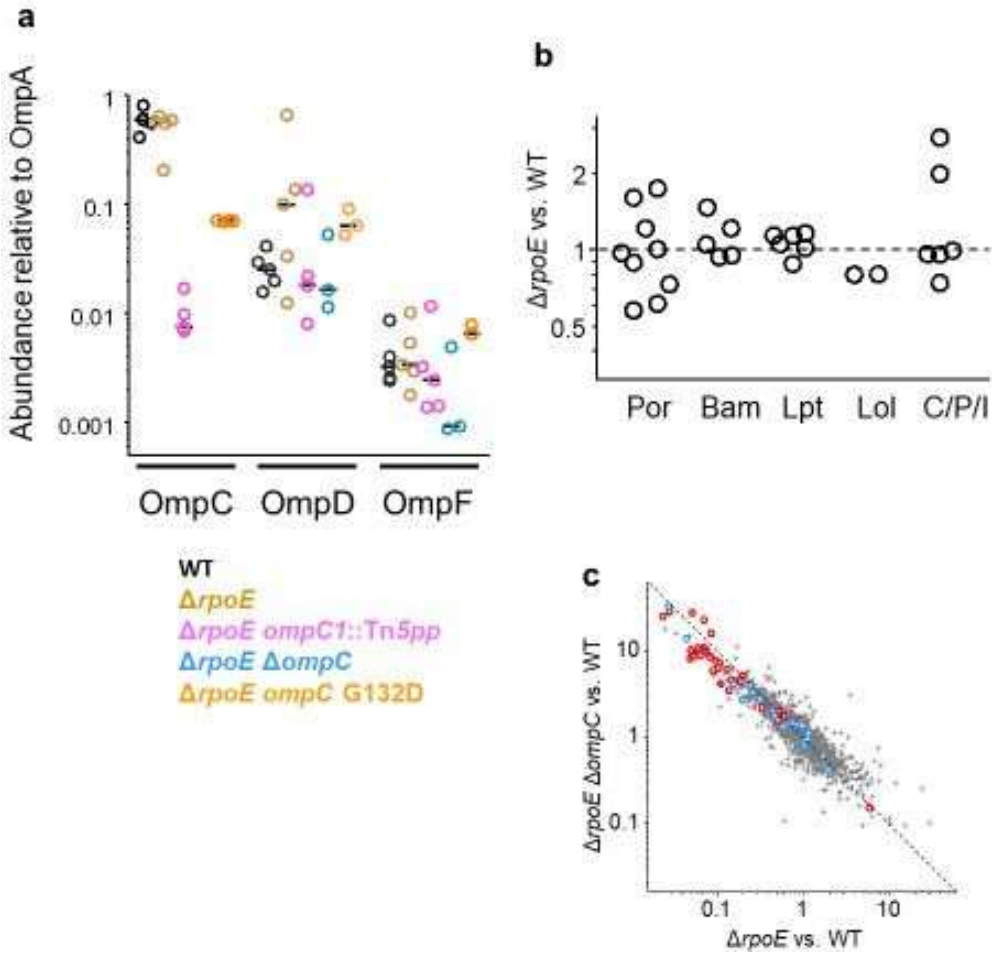
YccU
Ftp
NlpI
Δ4 vs. WT

Suppl. Fig. 1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0.001
OmpC
OmpD
OmpF
WT
ΔrpoE
ΔrpoE ompC1::Tn5pp
ΔrpoE ΔompC
ΔrpoE ompC G132D
b
ΔrpoE vs. WT
2
1
0.5
Por
Bam
Lpt
Lol
C/P/I
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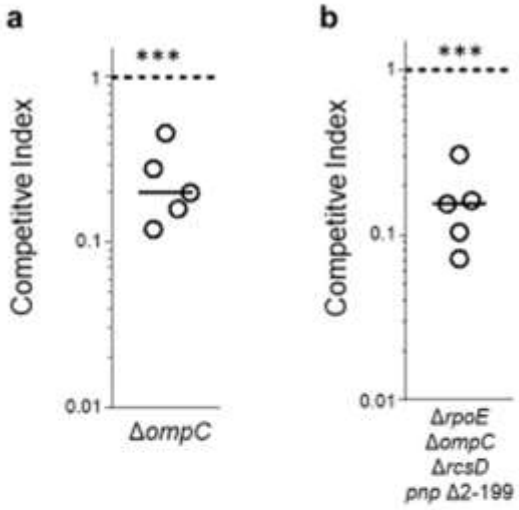
1
0.1
0.1
1
10
ΔrpoE vs. WT

Suppl. Fig. 2


a
b
***
***
Competitve Index
Competitve Index
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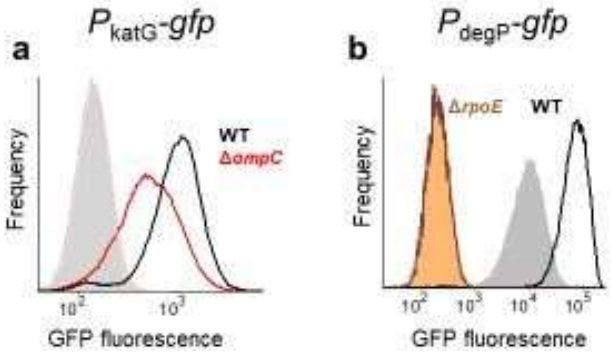
0.01
1
0.1
0.01
ΔompC
ΔrpoE
ΔompC
ΔrcsD
pnp Δ2-199

Suppl. Fig. 3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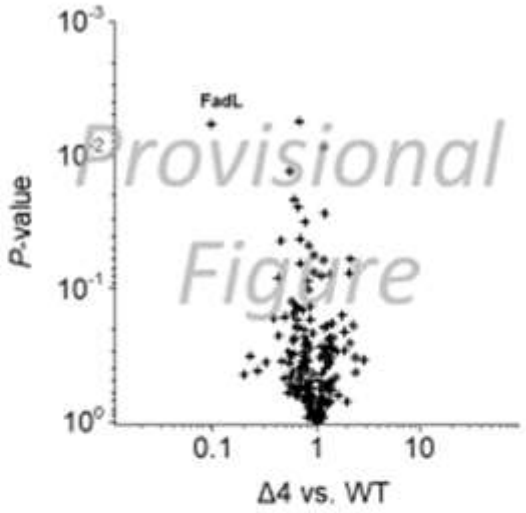
P_{katG}-gfp
Frequency
WT
ΔompC
10^2
10^3
GFP fluorescence
b
P_{degP}-gfp
Frequency
ΔrpoE
WT
10^2
10^3
10^4
10^5
GFP fluorescence

Suppl. Fig. 4



3.2 Limited role of efflux in multi-drug resistance of clinical *Escherichia coli* and *Pseudomonas aeruginosa* strains

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Limited role of efflux in multi-drug resistance of clinical *Escherichia coli* and *Pseudomonas aeruginosa* strains

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ABSTRACT
Extensive efforts have been devoted to developing efflux inhibitors for combatting multi-drug resistant (MDR) bacterial pathogens. However, we show here that MDR clinical isolates of *Escherichia coli*, a major threat to human health worldwide, largely retain high-level resistance against old and new antimicrobials even after genetic disruption of efflux. Additional mutagenesis revealed efflux-independent mechanisms including antibiotic-modifying enzymes and target gene mutations as primary causes of resistance against all tested classes of clinically relevant antibiotics. Genetic inactivation of major efflux systems also failed to restore susceptibility in diverse MDR *Pseudomonas aeruginosa* clinical isolates. Novel control strategies for MDR pathogens are urgently needed, but efflux inhibition might have limited impact.

Multi-drug resistant (MDR) Gram-negative bacterial pathogens represent a major global threat to human health. MDR pathogens drive clinical usage of last-resort antibiotics such as carbapenems and colistin further amplifying resistance development and the emergence of pan-resistant pathogens [1]. As a consequence, deaths attributable to antimicrobial resistance may rise sharply, although the global burden of MDR remains difficult to estimate [2]. Resistance is multi-factorial but one promiscuous mechanism covering diverse antibiotic classes is the expression of so-called resistance-nodulation-division (RND) superfamily exporters, which mediate active efflux of small molecules including many antibiotics [3-6]. In laboratory strains of various Gram-negative bacterial pathogens, upregulation of RND efflux systems increases resistance to diverse antibiotics, while genetic inactivation of such systems renders mutants hypersensitive. Overexpression of RND efflux systems is observed in many MDR clinical isolates suggesting that efflux might be involved in increasing resistance [7]. Based on these observations, academia and industry have devoted major efforts to develop efflux inhibitors, hoping that such inhibitors could broadly restore sensitivity to old antimicrobials, and/or break the intrinsic resistance of Gram-negative bacteria against drugs that are efficacious against Gram-positive bacteria lacking RND efflux systems.

Surprisingly, however, the foundation for these efforts has been rather weak. Almost all evidence for the impact of efflux comes from non-representative laboratory strains, while the quantitative contribution of efflux to clinical MDR remains unclear due to technical difficulties. Common methods for genetically inactivating efflux remain ineffective for MDR clinical strains. Efflux inhibitors are widely used to assess efflux contributions in clinical strains. However, currently available inhibitors are hampered by their selective efflux inhibition for certain drugs at low concentrations, and pleiotropic effects on cell envelope integrity and overall bacterial physiology at high concentrations, impairing conclusive interpretation [5]. Efflux system gene expression and sequences can be readily determined, but

overexpression and sequence polymorphisms of these genes poorly correlate with resistance levels in clinical isolates [8,9].

As part of the Innovative Medicines Initiative (IMI) Translocation project [10], we developed in this study a method that enables generation of genetically defined efflux mutants in diverse MDR clinical isolates. We employed a suicide plasmid (Figure 1a) and two consecutive single cross-overs using a positive selection marker (thiopurine-S-methyltranferase Tpm conferring resistance to tellurite [11]) that works even in MDR isolates, and a negative selection marker (levansucrase SacB conferring susceptibility to sucrose) for gene deletion events (see ONLINE METHODS). We used this method to study the role of efflux in two major MDR pathogens that represent particularly serious threats [1], *Escherichia coli* expressing extended-spectrum β-lactamases (ESBL-*E. coli*) [12], and multi-resistant *Pseudomonas aeruginosa* [13].

We selected ESBL-*E. coli* clinical isolates that were obtained at the University Hospital Basel from patient blood, sputum, surface swabs, urine, or fecal samples. All isolates were non-susceptible to agents of three or more antimicrobial categories thus fulfilling the standard definition for MDR status [14]. Whole genome sequencing revealed that these isolates are genetically diverse with hundreds of allelic differences, and cover seven different multi-locus sequence types (STs) with the expected dominance of ST 131 [15] (Figure 1b). These strains carry various β-lactamases and other highly heterogeneous resistance determinants (Figure 1c).

To determine the contribution of efflux, we aimed at deleting *tolC* which encodes the outer membrane channel required for the function of all known *E. coli* RND efflux systems. For several strains, we could obtain clean deletions of *tolC* within three days. However, for many others we had to screen hundreds of clones to obtain the desired mutants (see ONLINE METHODS). Altogether, we managed to obtain *tolC* mutants for 18 out of 24 ESBL-*E. coli*

isolates. We are not aware of any genetically defined efflux mutant of ESBL-*E. coli* clinical isolates in previous literature.

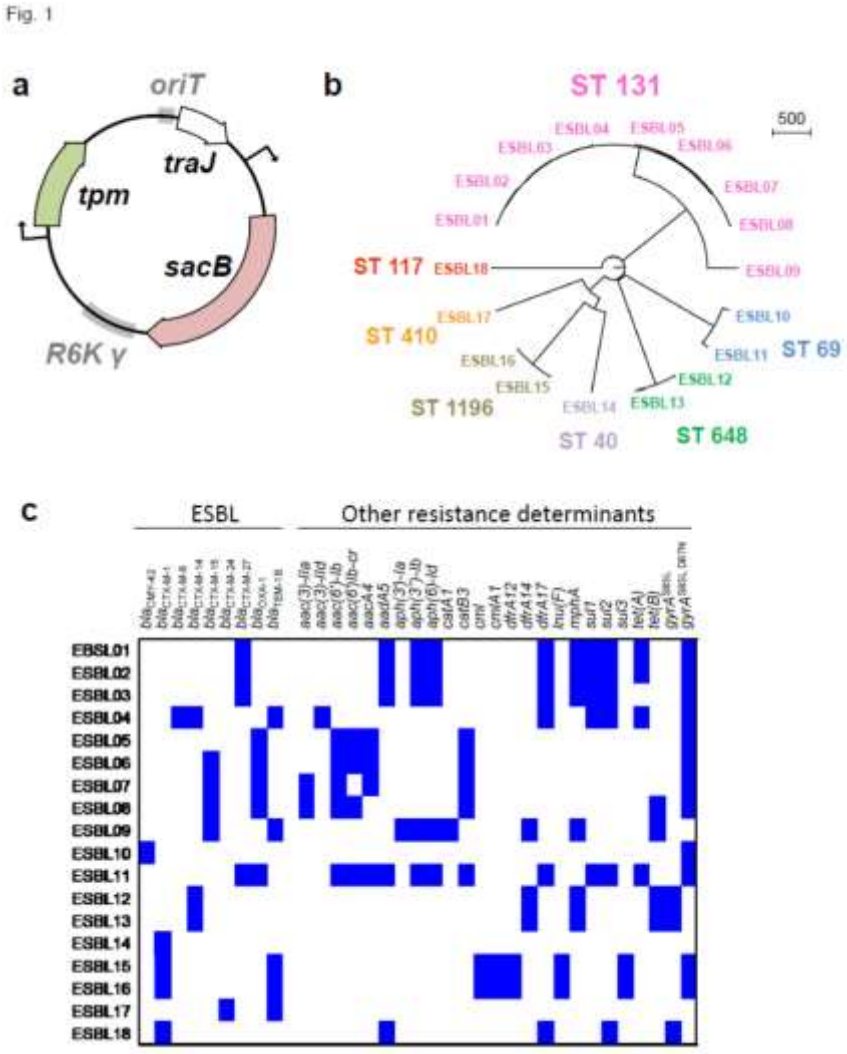


Figure 1: Gene deletion plasmid and diversity of clinical *Escherichia coli* strains.

(**a**) Plasmid for deleting genes in MDR bacterial pathogens. The plasmid carries the *R6K γ* origin of replication which depends on the replication protein π (encoded by *pir*) which is absent in almost all clinical strains; *tpm* encoding thiopurine-S-methyltranferase, which confers resistance to tellurite (most MDR clinical isolates are sensitive to tellurite); the origin of conjugational transfer *oriT*; *traJ* encoding the transcriptional activator for conjugational transfer genes; *sacB* encoding levansucrase conferring sensitivity to sucrose. The hooked arrows represent promoters.

(**b,c**) Analysis of clinical *Escherichia coli* strains expressing extended-spectrum β-lactamases by whole genome sequencing. (**b**) Tree illustrating the relationship between 18 strains for

which we could obtain Δ*tolC* mutants, based on the cgMLST (core genome multi-locus sequence typing) allelic profiles. The scale bar represents 500 allelic differences. The tree is colored according to MLST sequence types (STs). (**c**) Occurrence of acquired antimicrobial resistance genes as detected by ResFinder [16] and inspection of *gyrA* alleles.

To determine efflux activities of *E. coli* isolates and their respective *tolC* mutants, we used a Nile Red-based assay [17] that is especially suitable for comparing efflux in diverse isolates [18] (Figure 2a,b). Seventeen ESBL-*E. coli* isolates showed rapid efflux with kinetics in the range of previous reported data [17] (examples are shown as black lines in Fig. 2a). Deletion of *tolC* totally abolished efflux in 15 strains and dramatically slowed down efflux in two other strains (Figure 2a,b). The molecular mechanism of residual slow glucose-dependent Nile Red fluorescence loss in these two *tolC* mutants remain unknown, but may involve outer membrane efflux proteins that could partially compensate for TolC [19], or other efflux mechanisms that do not depend on a particular outer membrane efflux protein. ESBL18 and ESBL18 *ΔtolC* showed declining fluorescence signals in energy-depleted cells even before re-energization with glucose, preventing quantitative analysis of energy-dependent efflux (dotted orange line in Figure 2a). Nevertheless, our data indicate complete inactivation of efflux as a result of *tolC* deletion in 15 out of 18 clinical isolates.

We tested the strains for susceptibility to therapeutically relevant antimicrobials according to EUCAST (v 6.0; available at http://www.eucast.org/clinical_breakpoints/) using commercial phenotyping systems (Vitek 2 and E-tests, bio-Mérieux). We represent the data as minimal inhibitory concentrations that prevent growth (MIC) (Figure 2c-d). High MIC values corresponding to probable clinical treatment failures ("resistant" according to EUCAST) are shown in red, while likely efficacious treatment is represented by the blue regions ("sensitive"). If RND efflux represented a major contribution to clinical antimicrobial resistance, we would expect our efflux mutants to show markedly decreased MIC values, compared to their parental clinical isolates. Deletion of *tolC* in ESBL-*E. coli* had indeed some

impact on MIC values in various isolates (arrows in Figure 2c), in some cases similar to what has been observed in fully susceptible laboratory strains [3-6]. However, such changes were almost always too small to convert highly resistant strains into susceptible ones (i.e., moving from the red to the blue areas). For β-lactam antibiotics, changes occurred sometimes even in the opposite direction (i.e., getting more resistant) as previously reported in an efflux inhibitor study [20]. This could be a consequence of pleiotropic effects of efflux inactivation (see below).

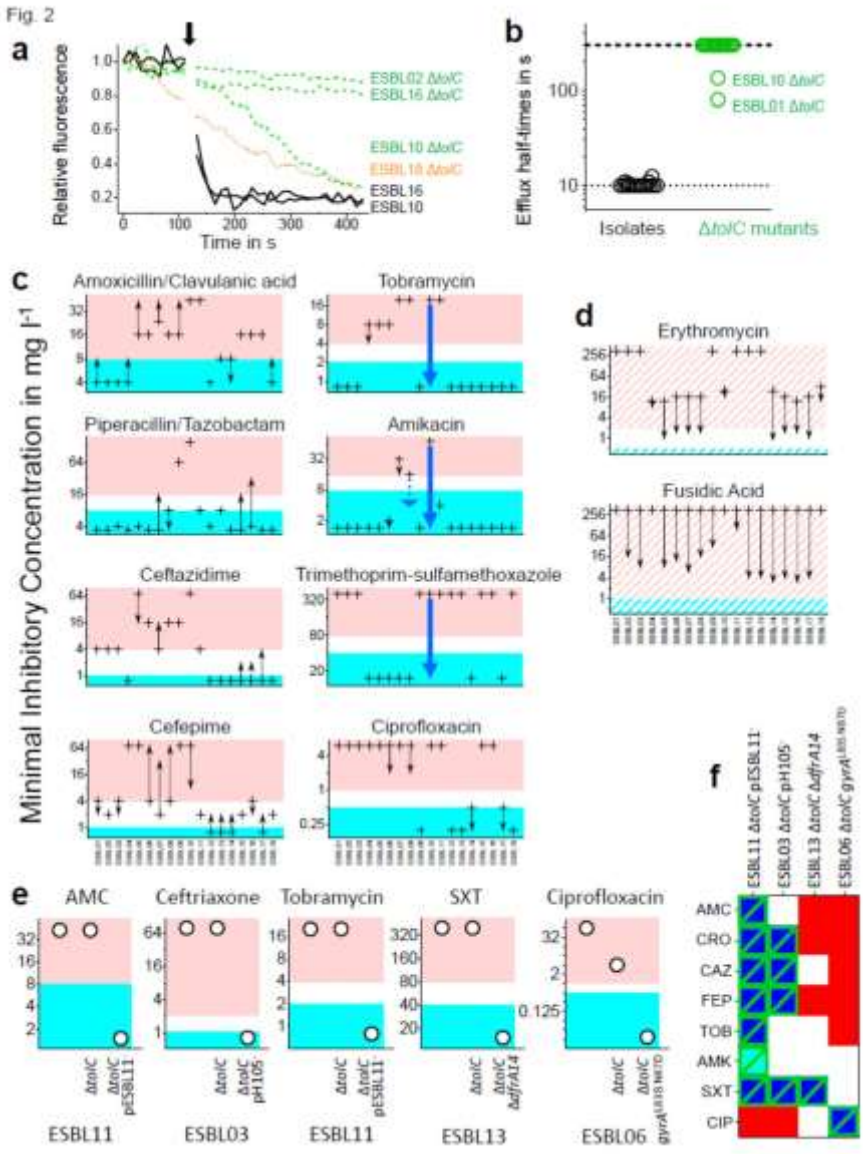


Figure 2: Impact of genetic inactivation of efflux on antimicrobial susceptibility in ESBL-*E.coli*.

(**a**,**b**) Efflux activities in clinical isolates and corresponding Δ*tolC* mutants. (**a**) Energy-depleted cells were loaded with Nile red. Cells were then re-energized with glucose (arrow, 120 s), and efflux was measured as decrease in Nile red fluorescence (which is lower in aqueous solution compared to bacterial membranes). All isolates except ESBL18 showed rapid energy-dependent efflux (examples shown in black), whereas all Δ*tolC* mutants showed no, or much slower, fluorescence loss upon energization (examples shown in green). ESBL18 and its Δ*tolC* mutant (orange dotted line) lost fluorescence in an energy-independent manner (i.e., even prior to glucose addition) preventing quantitative analysis of efflux. (**b**) Time intervals after energization until 50% of fluorescence intensity was lost. Time resolution prevented measurement of half-times below 10 s (dotted line) or above 300 s (dashed line). (**c**,**d**) Minimal inhibitory concentrations that prevent growth (MICs) of ESBL-expressing *Escherichia coli* isolates and corresponding Δ*tolC* mutants. Data are shown for common therapeutically used antimicrobials (**c**), and for drugs that are ineffective against *Escherichia coli* (**d**). Crosses represent values for parental isolates. The impact of Δ*tolC* deletion is represented by arrows. If there is no arrow, the mutant MIC remained at the parental level. MIC ranges corresponding to clinical resistance (red) or susceptibility (blue) according to EUCAST breakpoints are shown as background. Breakpoints shown in (**d**) are estimates based on values for other bacterial pathogens. The thick blue arrows mark conversion of clinical resistance to susceptibility as a result of genetic inactivation of major efflux systems, while the dotted blue line for ESBL08 and amikacin should still be reported as "intermediate" (see text). MIC values outside the measurement range are shown above the highest tick, or below the lowest tick, respectively.

(**e,f**) Impact of additional inactivation of specific resistance determinants in efflux-deficient strains. (**e**) Comparison of susceptibility of parental strains, their Δ*tolC* mutants, and various double mutants (AMC, Amoxicillin/Clavulanic acid; SXT, Trimethoprim-sulfamethoxazole). We determined susceptibility to ciprofloxacin using a broth microdilution technique to cover the nanomolar concentration range. (**f**) Antimicrobial spectrum changes in double mutants (blue, switch from "resistant" or "intermediate" in the *tolC* mutant to "sensitive" in the double mutant; light blue, "sensitive" status further enforced; white, "sensitive" unaltered; red, "resistant" unaltered; AMK, Amikacin; CAZ, Ceftazidime; CIP, Ciprofloxacin; CRO, Ceftriaxone; FEP, Cefepime; TOB, Tobramycin; TZP, Piperacillin/Tazobactam). The green boxes indicate resistance mechanisms that were specifically inactivated in each of the four double mutants.

The only exception was isolate ESBL10 that became susceptible to the aminoglycosides tobramycin and amikacin, and trimethoprim-sulfamethoxazole upon *tolC* deletion (thick blue arrows in Figure 2c). Interestingly, ESBL10 was the only isolate that showed resistance to these drugs but lacked antibiotic-specific resistance determinants (such as *aac(6')-Ib* and *aac(6')-Ib-cr* encoding aminoglycoside modifying enzymes, of *dfrA* alleles and *sul* alleles resistant to trimethoprim or sulfamethoxazole, repectively; Figure 1c). This apparent lack of specific resistance mechanisms would be compatible with an important role of general mechanisms such as efflux. On the other hand, ESBL10 *tolC* remained resistant to agents in three clinically relevant antimicrobial categories and thus retained MDR status. Another mutant, ESBL08 Δ*tolC*, showed diminished MIC for amikacin (MIC 4 mg l^{-1}) but retained tobramycin resistance. Under these circumstances, the amikacin MIC may not be a reliable predictor of clinical activity [21] and the strain should be reported as "intermediate" for amikacin according to EUCAST expert rules (http://www.eucast.org/expert_rules_and_intrinsic_resistance/) [22] (dotted blue arrow in Figure 2c).

The modest impact of efflux inactivation was initially surprising, but did not necessarily indicate that efflux had no role in MDR. Inactivating efflux can cause pleiotropic side effects including suppression of outer membrane porin F (OmpF) [20,23-25]. Such pleiotropic effects might provide compensatory resistance mechanisms not present in the wild-type isolates, thus replacing one mechanism (efflux) by another (i.e., poor porin-dependent drug entry) with little overall change in MIC (or even increased resistance as observed for β-lactams [20]). Alternatively, the non-impressive Δ*tolC* phenotypes could also be due to the large sets of antibiotic-modifying enzymes, acquired antibiotic-resistant target alleles such as *dfrA14*, and mutated chromosomal target genes such as $gyrA^{S83L\ D87N}$ in our isolates (Figure 1c). To determine the quantitative contributions of efflux/compensatory side effects *vs*.

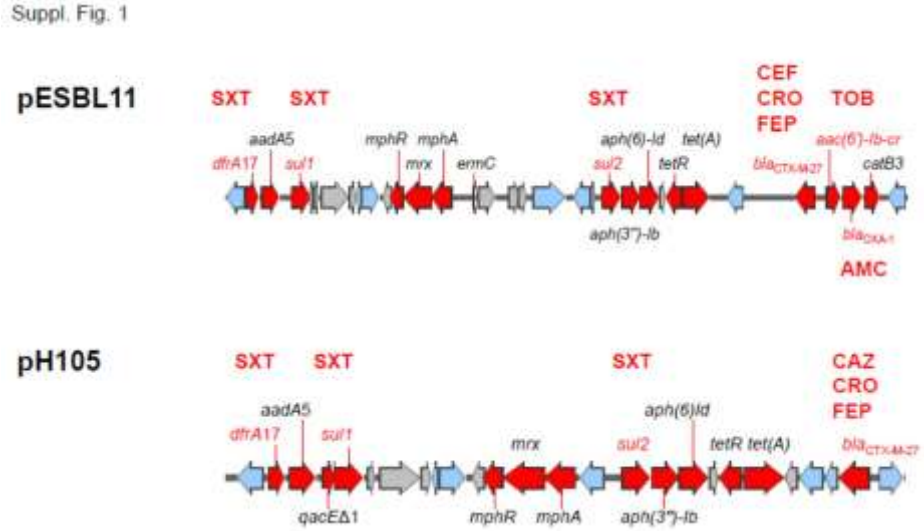
efflux-independent antibiotic-specific mechanisms, we constructed double mutants in isolates from three different *E. coli* sequence types.

To inactivate multiple resistance mechanisms simultaneously, we cured plasmids carrying multiple antibiotic-modifying genes and acquired antibiotic-resistant target alleles (Supplemental Figure 1) in two strains yielding double mutants ESBL11 Δ*tolC* pESBL11^{-} and ESBL03 Δ*tolC* pH105^{-} (ESBL03 contained a plasmid with 99.95% sequence identity to pH105 [26]). For comparison, we constructed two additional double mutants in which a single resistance determinant was targeted (ESBL13 Δ*tolC* Δ*dfrA14*, potentially restored susceptibility to trimethoprim; ESBL06 Δ*tolC gyrA*$^{L83S\ N87D}$, potentially restored susceptibility to ciprofloxacin). Susceptibility measurements showed that all four double mutants specifically lost resistance phenotypes in strict concordance with the respective targeted mechanisms (Figure 2e,f). We observed striking MIC changes in all five tested clinically relevant antimicrobial categories, in marked contrast to the mostly modest changes observed for Δ*tolC*. These results indicated that (i) antibiotic-modifying enzymes and resistant target alleles mediated high-level resistance to diverse classes of antibiotics even in absence of efflux, and (ii) pleiotropic effects of the *tolC* deletion did not confer clinically relevant

resistance to any of the tested antibiotics. Together, this evidence suggested a limited role of RND efflux for MDR of ESBL *E. coli* clinical isolates.

Supplemental Figure 1: Plasmid-encoded antimicrobial resistance genes.

(**a,b**) Gene clusters that contained all detected antimicrobial resistance genes on plasmids



present in ESBL11 (pESBL11; 30,000 bp of a total plasmid sequence of 148,945 bp are shown) and ESBL03 (pH105; 20,000 bp of a total plasmid length of 134,920 bp). Gene names in red indicate genes conferring resistance to clinically relevant antibiotics that were tested in this study (AMC, Amoxicillin/Clavulanic acid; CAZ, Ceftazidime; CRO, Ceftriaxone; FEP, Cefepime; SXT, Trimethoprim-sulfamethoxazole SXT; TOB, Tobramycin). AAC(6′)-Ib-cr can modify both tobramycin and amikacin. However, strain ESBL11 was only resistant to tobramycin while amikacin MIC remained below the EUCAST breakpoint (Figure 2c). Similar findings have been obtained for many *Enterobacteriaceae* that possess AAC(6′)-Ib [21]. AAC(6′)-Ib-cr can also cause low-level resistance to ciprofloxacin [27], but in ESBL11 which carries also a high-resistance chromosomal *gyrA*$^{S83L\ D87N}$ allele (Figure 1c), AAC(6′)-Ib-cr likely provided only a modest contribution to overall ciprofloxacin resistance. Gene names in black indicate other resistance genes. Transposable elements are shown in blue, and other genes are grey.

Efflux may have a more important role during initial encounters with new drugs, at a time when specific resistance mechanisms might yet not have evolved [3-6]. To evaluate this possibility, we tested erythromycin and fusidic acid, two drugs that are used against Gram-

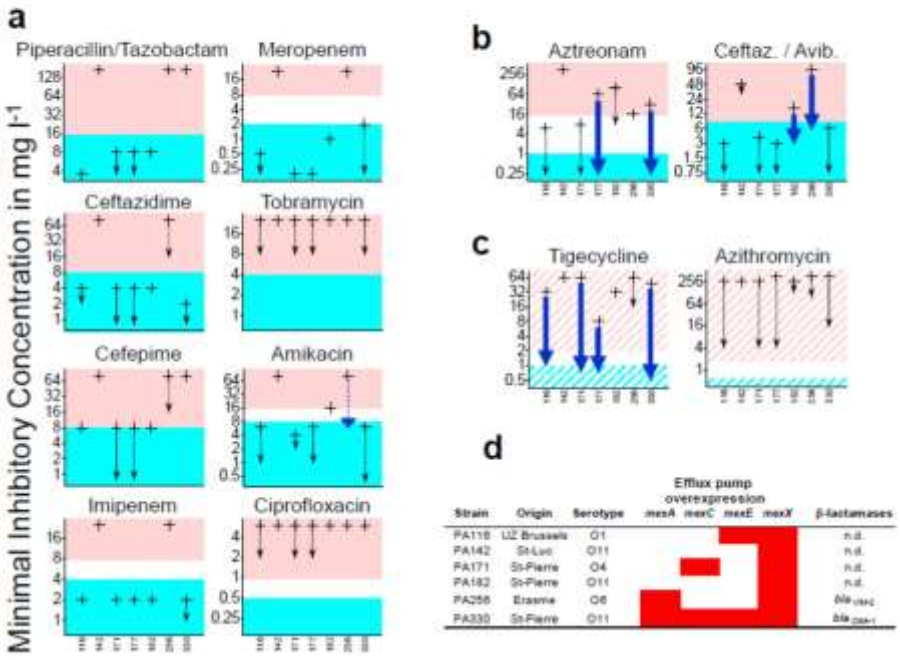
positive bacteria but are ineffective against *E. coli* because of RND efflux-mediated intrinsic resistance [5]. Indeed, *tolC* deletion greatly increased sensitivity to these drugs in several MDR ESBL-*E. coli* clinical isolates (Figure 2d), but a sizable fraction showed no or only weak sensitization upon efflux inactivation. These data suggest that even without specific selection pressures, several MDR clinical isolates already carried potent resistance mechanisms to "new" drugs limiting the impact of efflux inhibition.

We also selected several MDR *P. aeruginosa* isolates from four different Belgian hospitals [28] that had divergent serotypes, antibiograms and efflux pump expression patterns (Supplemental Figure 2a-d). In seven out of 12 tested *P. aeruginosa* strains, we were able to delete *oprM* encoding the outer membrane channel of major efflux systems MexAB and MexXY (except for rare isolates of the taxonomic outlier PA7 group [29], in which MexXY uses OprA), as well as minor systems MexMN, MexVW, and partially MexJK. For some extensively MDR *P. aeruginosa* isolates, there was a high background growth even at 200 mg l^{-1} tellurite hampering positive selection for ex-conjugants. One genetically defined efflux mutant for a clinical MDR *P. aeruginosa* isolate from the rare outlier PA7 group was reported previously [30].

Similar to our findings for ESBL *E. coli*, MDR *P. aeruginosa* Δ*oprM* mutants showed mostly non-impressive alterations in antimicrobial susceptibility. Strain PA256 Δ*oprM* had a diminished MIC value for amikacin (6 mg l^{-1}) but remained resistant to tobramycin. Under these circumstances, the amikacin MIC may not be a reliable predictor of clinical activity, and the strain should be reported as "resistant" for amikacin [22] (dotted blue arrow in Supplemental Figure 2a). Deletion of *oprM* had more remarkable effects on *P. aeruginosa* susceptibility to aztreonam (which is rarely used against this pathogen) and a ceftazidime / avibactam combination that was approved in 2015, after isolation of the *P. aeruginosa* strains used in this study [28] (Supplemental Figure 2b). However, several mutants retained resistance

suggesting the presence of potent alternative (pre-existing) resistance mechanisms. We also tested two drugs, tigecycline and azithromycin, that cannot be used therapeutically against *P. aeruginosa* because of high intrinsic resistance. In laboratory strains, inactivation of just one OprM-dependent efflux pump, MexAB, largely overcomes this intrinsic resistance [5]. Similarly, *oprM* deletion dramatically increased sensitivity to both "new" drugs in some of our clinical isolates (Supplemental Figure 2c). Susceptibility breakpoints for these two drugs have not been established for *P. aeruginosa* but using EUCAST breakpoints for other bacterial pathogens as tentative first approximations, several *oprM* mutants might indeed have

Suppl. Fig. 2



become sensitive to clinically achievable tigecycline concentrations (hatched blue area). On the other hand, several mutant strains retained high resistance against both tigecycline and azithromycin.

Supplemental Figure 2: Impact of *oprM* deletion on MDR *P. aeruginosa* clinical isolates. (**a**,**b**,**c**) Minimal inhibitory concentrations that prevent growth (MICs) of *P. aeruginosa* strains and their Δ*oprM* mutants. Data are shown for common therapeutically used antimicrobials (**a**), for rarely used or just recently approved drugs (**b**) (Ceft. / Avib., Ceftazidime /

Avibactam), and for drugs that are ineffective against *P. aeruginosa.* (**c**). Crosses represent values for parental isolates. The impact of *oprM* deletion is represented by arrows. If there is no arrow, the mutant MIC remained at the parental level. MIC ranges corresponding to clinical resistance (red) or susceptibility (blue) according to EUCAST breakpoints are shown as background. Breakpoints for Ceftazidime / Avibactam (**b**) were based on values for Ceftazidime. Breakpoints shown in (**c**) are estimates based on values for other bacterial pathogens. The thick blue arrows mark conversion of clinical resistance to susceptibility as a result of genetic inactivation of major efflux systems, while the dotted blue line for PA256 and amikacin should still be reported as "resistant" (see text). MIC values outside the measurement range are shown above the highest tick, or below the lowest tick, respectively. (**d**) Strain characteristics of highly resistant *Pseudomonas aeruginosa* clinical isolates (n.d., not detected).

Deletion of *oprM* inactivates the clinically most relevant RND efflux systems in *P. aeruginosa*, MexAB and MexXY [31], which represent the primary targets for efflux inhibitor development programs against MDR *P. aeruginosa*. In addition, *P. aeruginosa* encodes various other RND efflux systems that are independent of OprM [5]. It is possible that such other efflux systems masked the effect of *oprM* deletion in some of our mutants (as it has been shown for one single isolate of the rare PA7 group [30]), but the most important alternative pumps, MexCD-OprJ or MexEF-OprN, were not overexpressed in our most refractory strains 142 and 256 (Supplemental Figure 2d). In general, it might be challenging to develop promiscuous but safe inhibitors/inhibitor combinations for all relevant *P. aeruginosa* efflux systems, given the toxicity of several otherwise promising inhibitors [3-6].

Together, our genetically defined mutants show that major RND efflux systems contribute to the MDR phenotype and intrinsic resistance of many clinical ESBL-*E. coli* and *P. aeruginosa* strains. However, the effect size is often moderate due to potent efflux-independent resistance mechanisms. A limited contribution of efflux to high-level resistance has been previously postulated based on observations with laboratory strains [5] and three

Salmonella isolates from pigs [32]. Our data indicate that efflux inhibitors might have limited impact on therapeutic success with old or even new antimicrobials against key human MDR pathogens, arguing against the main motivation for the extensive past and current development programs for such inhibitors. Efflux might play a more important role in strains with only low-level resistance (which we did not include in this study), but this would have little relevance for solving the urgent MDR crisis. Further research on RND efflux systems and their substrate selectivity is still essential to obtain a rational basis for developing efficacious novel drugs that escape efflux. Moreover, efflux systems can contribute to pathogen virulence [33] suggesting a potential role for efflux inhibitors in anti-virulence strategies.

We have determined the quantitative contribution of major efflux systems to resistance in diverse MDR clinical isolates of two major bacterial pathogens that have been at the focus of RND efflux research. Future studies could determine the role of efflux in clinical multi-drug resistance in additional pathogens using the methods developed in this study.

METHODS

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

D.B. designed the study with input from A.E. and C.K.; O.C. and J.M.B. constructed mutants; D.M., O.C., H.S.-S. and R.N. determined genome sequences; O.C. and P.M. determined efflux activities; A.E. and J.F. determined MIC values; P.S.A., V.T., and C.K. provided tools and expertise; D.B. wrote the manuscript with early input from O.C. and subsequently all authors provided advice and approved the final manuscript.

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4 Discussion

The aim of this work was to explore opportunities to synergize with the host immune system in order to fight pathogens infections more efficiently. More precisely, we aimed to identify target mutations that could rescue fitness of an avirulent *rpoE Salmonella* mutant by using an unbiased method. Our approach revealed that two deletions could reverse the *rpoE* phenotype *in vivo*.

4.1 Advantages and limitations of the techniques used

Transposon screening is an unbiased method to identify target genes. Due to outward facing promoters with strong or moderate activity (P_{tac} or P_{bla}, respectively), our transposon strategy was designed to identify up- or down-regulation of adjacent genes in addition to loss of function due to transposon insertion. Our library contains 50,000 clones. The *Salmonella* genome is 5'067'450 bp length – including plasmids – suggesting the transposon inserted approximately every 101 bp. Due to the relatively small number of clones in our library, we mainly obtained insertion inside genes resulting in loss-of-function mutations. This result is not surprising because statistically, the chances of obtaining insertions within a gene, resulting in its disruption, are higher than the ones to up- or down-regulate one. For the same reason, it is not surprising that none of the recovered clones had an insertion of the transposon in σ^E-dependent genes. This raises the possibility of a "hot spot" for transposon insertion in the input library. Sequencing the input library could have been an option in order to determine *in vitro* enrichment of any transposon insertion. Generating a transposon library bears some intrinsic limitations, as insertions in small genes are less likely to occur. Additionally, insertions in essential genes would be deleterious and, therefore, be negatively selected and lost.

The animal model we used in our study was a systemic infection in susceptible BALB/c mice. The main advantage of this animal model is to study the pathogen adaption in a complex living mammalian organism with high diversity of cells type and high complexity of molecular interactions. The use of susceptible mice had two main advantages: (i) in the clinics, immunocompromised patients are subject to pathogens with a high mutation frequency rate (160) and (ii) we could technically obtain sufficient amounts of bacteria allowing further characterization. However, we must consider that murine and human physiology/immunology are different, potentially limiting the predictive power for human patients.

4.2 Rescue the survival of an avirulent mutant by deleting a major porin

In this first transposon screen, we obtained only loss-of-function mutations meaning either (i) most σ^E-dependent genes may have an individually limited effect on virulence or (ii) our library missed appropriate insertion sites upstream of key σ^E-dependent genes. Clones of the output library had mainly the transposon integrated in three different positions in the promoters' region of *ompC* (red stars in figure 10), which is also upstream of the sRNA *micF*, and the phosphotransferase *rcsD*. This insertion resulted in significantly lowering *ompC* expression as measured by proteomics. We focused on the dominant clone (the circled one in Figure 10). Interestingly, in the dominant clones, the genes hit by the transposon and its adjacent genes are directly or indirectly linked to the pathogen envelope.

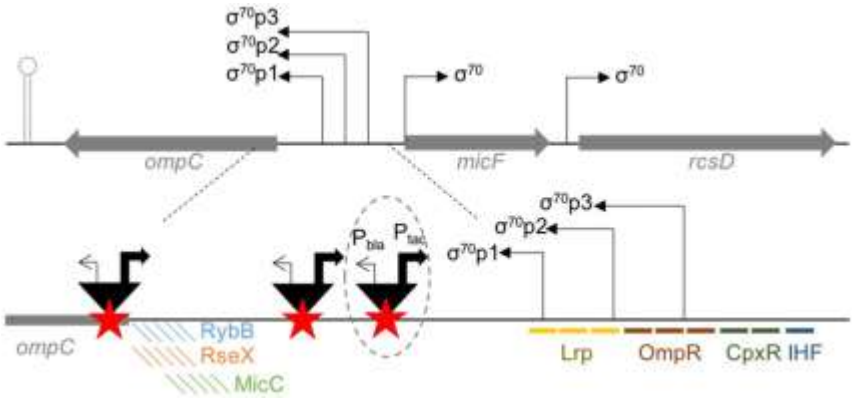

Figure 10. Major transposon integration sites in the Δ*rpoE* output library.

4.2.1 MicF

As P_{tac} was facing towards the sRNA *micF*, we hypothesized that one transposon role was to increase *micF* expression. *micF* encodes a small regulatory RNA targeting several mRNAs, whose genes are implicated in diverse function (161): the biofilm formation regulator protein BssS, the lipid A deacylase LpxR, a hypothetical periplasmic protein YahO, the leucine-responsive transcriptional regulator Lrp, and another major porin, OmpF. Unfortunately, due to technical reasons, we were unable to directly quantify *micF* expression by qRT-PCR. Additionally, Δ*rpoE* Δ*ompC* Δ*micF* bacteria exhibit the same *in vivo* fitness as Δ*rpoE* Δ*ompC*. Finally, P_{tac} is located 280 bp from the *micF* promoter. This would produce a long, potentially inefficient transcript. Together, these data suggest that the beneficial effect of the transposon is not linked to *micF* expression.

4.2.2 OmpC

ompC encodes a major *Salmonella* porin. Due to the presence of a terminator encoded downstream of *ompC*, that should stop transcription, we did not investigate adjacent downstream genes. Binding sites for the sRNAs RybB, RseX and MicC are downstream of P_{bla}. RybB is σ^E-activated and has

numerous mRNA targets. RseX and MicC down-regulates OmpA and OmpC levels, and OmpC and OmpD levels, respectively. Downstream of P_{tac} are diverse transcription factor binding sites such as Lrp – a MicF target, OmpR – part of the EnvZ/OmpR two-component system, CpxR – the response regulator of the envelope stress response system CpxAR, and IHF – the Integration Host Factor that down-regulates *ompC* transcription (162). These observations suggest that the transposon integrated into a highly regulated zone of the genome where many regulatory networks converge.

4.2.3 OmpC: "channel hypothesis"

OmpC level is tightly regulated, suggesting OmpC plays a crucial role *in vivo*. Surprisingly, *ompC* has no reported effect on *in vivo* fitness (22). However, our *in vivo* data showed a slight but still significant decrease in Δ*ompC* mutant *in vivo* fitness, indicating *Salmonella* requires OmpC for systemic infection. This discrepancy could be due to our more sensitive method (FACS). Our *in vivo* and *in vitro* proteomics data showed that OmpC is the second most abundant porin in *Salmonella*, following the structural porin OmpA. OmpC is important for acquisition of nutrients and/or specific molecules. OmpC is involved in the antibiotic passage *in vivo* and *in vitro* in *E. coli*, *S. Typhimurium*, *K. pneumoniae*, and *E. asburiae* as drug resistant strains were described to lose OmpC (163–167). These findings suggest that (i) OmpC may be a possible pore for undesired toxic molecules ("channel hypothesis"), and that (ii) pathogens can support the loss of OmpC within the host.

4.2.4 OmpC: "cargo hypothesis"

Another possible reason to explain why deletion of OmpC leads to partial rescue of *in vivo* fitness is that, during infection, different type of stresses may lead to the unfolding of outer membrane proteins (e.g. OmpC) followed by accumulation in the periplasmic space ("cargo hypothesis"). Overexpression of *degP*, encoding a σ^E-dependent protease, increases Δ*rpoE in vivo* fitness as we could recover a few bacteria from infected mice at day 5. Next, we combined *degP* and *skp* overexpression as these two proteins are part of the same pathway during OMP assembly *in vivo* (32). Preliminary data showed that *in vivo* fitness was not further improved.

These data led us to conclude that OmpC induces accumulation of periplasmic cargo that has a negative effect on bacterial *in vivo* fitness. As overexpression of *degP* in Δ*rpoE* Δ*ompC* reach the same *in vivo* fitness as Δ*rpoE* Δ*ompC* (without *degP* overexpression), we could not rule out that the "cargo hypothesis" is the only stress induced by OmpC. To further test the "cargo hypothesis", we could overexpress other proteases alone or in combination. Indeed, the OmpC-dependent cargo effect perhaps overwhelms DegP.

Additionally, cell fractionation, followed by proteomics comparative analysis between the cytoplasm and the periplasm could also be performed. If this hypothesis is correct, we should detect OmpC enrichment in the Δ*rpoE* bacterial periplasm.

This means that the "cargo hypothesis" partially explains the rescue phenotype but is not the sole explanation. To try to decipher between the two hypotheses, we first stained live/dead cells in MgMES pH 5.5 during stationary phase as OMPs are more prone to aggregation at low pH (168). We could show that Δ*ompC* rescued Δ*rpoE* survival, suggesting that either (i) OmpC itself is aggregated thus toxic, or (ii) OmpC allows the passage of a toxic molecule that lead to cargo formation in the periplasm. Most likely, OmpC is not a pore entrance only for H^+, as the latter would pass through OmpD as well, and Δ*ompD* does not restore Δ*rpoE* survival. Potential toxic molecules that could pass through OmpC are organic acid such as lactate, acetate, or citrate, which are not present in our MgMES media.

We then generated Δ*rpoE* OmpC^{G132D} – a mutant that should have a smaller OmpC pore size – and we tested its *in vitro* and *in vivo* fitness. This mutant exhibited Δ*rpoE* Δ*ompC*-like fitness and was able to rescue Δ*rpoE* survival at mildly acidic pH. Even though we checked by sequencing the promoters' region and the coding sequence, *in vitro* proteomic results demonstrated a decreased OmpC level in Δ*rpoE* OmpC^{G132D}, suggesting that the protein might be unstable, thus degraded.

Sequencing the output library could have been an option to not omit any target, to calculate each mutation frequency rate, and to perhaps better understand the rescue phenotype by linking all the information from the different transposon targets and the adjacent genes.

It seems difficult to decipher between the two hypotheses. A combination of the "channel" and the "cargo" hypotheses likely explains the survival advantage. It is still possible that other hypotheses exist.

4.2.5 OmpC: other hypotheses

Liu *et al.* have shown that Δ*ompC* survives better than the WT in human blood and serum (164). The authors suggested that OmpC is an immunogen as anti-OmpC antibodies were detected in human serum, thus suggesting that the immune system recognizes more, and attacks better a strain expressing *ompC*. A way to assess this hypothesis would be to test comparative complement killing of *rpoE* and *rpoE ompC*.

Our *in vitro* proteomics results showed an overall proteins level down-regulation in Δ*rpoE* compared to WT, especially a down-regulation in virulence genes such as SPI-2 and PhoP-dependent genes, as previously described. Decreased expression of virulence genes leads to decreased fitness, which might explain Δ*rpoE* phenotype. Interestingly, Δ*rpoE* Δ*ompC* proteomics profile already resembles more WT profile. Therefore, OmpC seems to induce a stress in Δ*rpoE* that may decrease virulence. This might

contribute to a decreased fitness of Δ*rpoE*. We can also conclude that deletion of *ompC* partially positively impact the expression of these virulence genes.

4.2.6 No involvement of other porins

Surprisingly, no major porins were up- or down-regulated in response to *ompC* deletion. Many examples exist of OmpC- and OmpF-deficient strains that become drug resistant *in vitro* and *in vivo* in *S. Typhi* and *E. coli* (169–172).

OmpD is 66% identical to OmpC. However, Δ*rpoE* Δ*ompD* did not rescue Δ*rpoE* survival *in vivo* nor *in vitro*. Replacing *ompC* by *ompD* in *ompC* locus did not rescue *in vivo* fitness as well as simple *ompC* deletion. Together, these data show that OmpD does not contribute significantly to perturbations that require σ^E for maintaining *in vivo* fitness. These results may suggest a pore specificity between porins and confirms again the presence of an OmpC-dependent stress in Δ*rpoE*.

4.2.7 Minor contribution of *rcsD*

Rcs is another envelope stress response system highly expressed in Δ*rpoE* bacteria (93). Overexpression of the Rcs system is deleterious for *Salmonella* (78). As *rcsD* is encoded downstream of *micF*, we deleted this phosphotransferase of the Rcs system. Deletion seemed to increase slightly Δ*rpoE* Δ*ompC* fitness. *In vivo* proteomics data showed that the main difference between Δ*rpoE* Δ*ompC* and Δ*rpoE* Δ*ompC* Δ*rcsD* is the decreased abundance of RcsB, the major DNA-binding protein of the Rcs sytem. Preliminary data showed that Δ*rpoE* Δ*ompC* Δ*rcsB* is avirulent. Together, these data suggest that a low basal activity of the Rcs system may be necessary. Also, it is important to note that this benefit is seen only in Δ*rpoE* Δ*ompC* bacteria as an Δ*rpoE ΔrcsD* double mutant is avirulent *in vivo*, highlighting again the crucial role of OmpC in survival.

4.3 Increase the *in vivo* fitness with a second mutation

To identify additional target(s) that could increase the *in vivo* fitness of Δ*rpoE* Δ*ompC* Δ*rcsD,* we performed two rounds of transposon screens. Indeed, after collecting the clones obtained from the first *in vivo* passage, no significant target was identified, and a second mouse was infected with roughly 200,000 clones for enrichment of clones with a fitness advantage. Among the million clones recovered from the spleen, four clones were found several times with the transposon inserted in *cadA*, *yicH*, *ftsI2*, or *pnp*. The most highly dominant clone was *pnp,* which was found nine times out of 20 sequenced clones. *cadA*, *yicH*, and *ftsI2* were found two times each. The target genes are discussed further below.

4.3.1 pnp

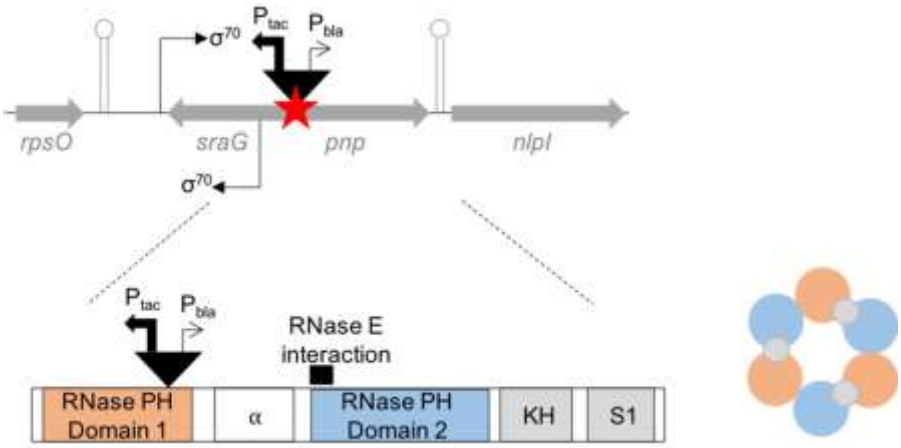

Figure 11. Transposon insertion site in *pnp* and adjacent genes (top left). Gene composition (bottom left) adapted from (173). *pnp* tructural organization (bottom right) adapted from (174)).

pnp encodes a major regulator termed polynucleotide phosphorylase (PNPase) that is involved in RNA processing and cold shock response. The transposon integrated 596 bp from the *pnp* start codon with P_{bla} looking toward the 3' end of *pnp* (Figure 11). Downstream of P_{tac} lies *sraG*, a sRNA that post-transcriptionally down-regulates *pnp* expression (175). As (i) P_{tac} could increase *sraG* expression, leading to a decreased in *pnp* expression, (ii) terminators are encoded upstream and downstream of *pnp*, and (iii) the transposon integrated in *pnp*, *pnp* is likely the transposon target. Downstream of *pnp*, *nlpI* encodes a lipoprotein. We cannot exclude that the transposon insertion affects *nlpI* expression as *pnp* and *nlpI* form an operon (176). *In vivo* proteomics data showed a decrease in NlpI level in Δ*rpoE* Δ*ompC* Δ*rcsD* pnpΔ2-199 compared to the WT. Interestingly, OMVs formation increased in Δ*nlpI* (177), allowing cells to better resist membrane-damaging agents. This might slightly contribute to the increased fitness.

However, *pnp* deletion in Δ*rpoE* Δ*ompC* Δ*rcsD* mutant bacteria led to unreproducible results *in vivo*. Mice over 12 weeks are more resistant to *Salmonella* infection due to a more mature immune system, thus decreasing the total number of output CFUs. Moreover, infecting with WT bacteria likely creates a favorable niche for the *Salmonella* mutant by overwhelming the host immune system. However, no correlation with mice age or presence of the WT in the inoculum was observed. We therefore generated a truncated mutant lacking the 5' *pnp* where the transposon inserted, deleting amino acids 2 to 199 in Δ*rpoE* Δ*ompC* Δ*rcsD*, termed Δ4. *In vivo* proteomics results showed a threefold decreased expression in PNPase, showing that this enzyme is still active. Interestingly, Δ4 mutant reproducibly exhibited a high level of *in vivo* fitness. The 5' *pnp* truncation may produce multiple effects; however,

due to its reproducible phenotype compared to the clone containing the transposon, we decided to further investigate Δ*rpoE* Δ*ompC* Δ*rcsD pnp* Δ2-199. *pnp* involvement is crucial in *in vivo* fitness only when OmpC is absent as Δ*rpoE pnp* Δ2-199 is strongly attenuated.

PNP contains repeated exoribonuclease domains called RNase PH domain 1 (orange in figure 11) and RNase PH domain 2 (blue in figure 11). Only domain 2 is active in RNA degradation (178). As we observed an increase in fitness of Δ*rpoE* Δ*ompC* Δ*rcsD* ΔNt-*pnp* bacteria, domain 1 might be essential in events aside from RNA degradation. RNase-PH forms a hexameric ring of three identical homodimers (right panel in figure 11). The ring-like structure is important for activity in the maturation of tRNA precursors as a mutant with a disrupted trimerization interface is not functional (179). Our truncated protein could be unable to fold into this ring structure, conferring a survival advantage. The principal role of tRNA is to link the mRNA and the amino acids sequence of proteins. It is difficult to predict why such a deletion would increase fitness.

PNPase interacts with the endoribonuclease RNase E to form the degradosome, a multienzyme complex that degrades mRNA. In Δ*rpoE* bacteria, mRNAs levels increase due to the absence of the sRNAs RybB, MicA and MicL. In Δ*pnp* bacteria, steady-state levels of mRNA increase (180). In our mutant, interaction with the degradosome is not modified and the RNase PH domain 2 is intact. If deleting the RNase PH domain 1 increases activity of the RNase PH domain 2, then the mRNA level should be WT-like. The high *in vivo* fitness level of Δ*rpoE* Δ*ompC* Δ*rcsD* ΔNt-*pnp* bacteria may be due to a "back-to-normal" steady-state level of mRNAs. An accumulation of mRNA could be deleterious for *Salmonella* due to steric hindrance in the cytoplasm by disturbing other crucial reactions. We could verify the mRNA accumulation by Northern Blot analysis. It could also be deleterious to be overwhelmed with mRNA as many unnecessary and/or inappropriate proteins are produced, causing energy loss and eventual death. We could also compare WT and mutant bacterial transcriptomes for a broader view and better understanding of the rescue phenotype. However, if deleting RNase PH domain 1 has no effect on the activity of RNase PH domain 2, then the steady-state level of mRNA would not be implicated in *in vivo* fitness.

As *pnp* has pleiotropic functions, it is challenging to identify a specific role in the survival advantage. The pleiotropic effect was already observed by proteomics, where the overall protein levels were almost restored to WT profile. Nevertheless, a 1,000 fold increase in *in vivo* fitness suggests that *Salmonella* can bypass σ^E, an absolutely crucial contributor to *in vivo* fitness and virulence, by a number of minor alterations, instead of using a completely distinct envelope maintenance program, under the precondition that the major "trouble-maker" OmpC is absent.

4.3.2 cadA

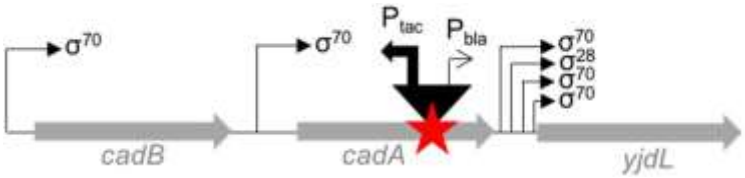

Figure 12. Transposon insertion site in *cadA* and adjacent genes.

Another target gene that was identified by our transposon screen was *cadA. cadA* encodes a lysine decarboxylase that consumes a proton when producing cadaverine, a polyamine, in the cytoplasm. Cadaverine is then exported to the periplasmic space by the lysine:cadaverine antiporter CadB. By consuming H^+, *cad*A increases cytoplasmic pH, protecting the cell from mild acid stress (181). The transposon inserted at 1534 bp from the *cadA* start codon with P_{bla} facing the 5' region of *cad*A and the downstream gene *yjdL*. *yjdL* encodes a dipeptide/tripeptide:H^+ symporter (182). *cadB* is encoded upstream of *cadA*. As P_{tac} and *cadB* are encoded in opposite directions, we cannot exclude that the transposon role is to repress *cadB* expression by antisense. If *cadA* and *cadB* are deleted, cells are less resistant to mildly acidic pH, which should not provide a survival advantage. It is possible that the transposon role is to up-regulate *yjdL* thanks to P_{bla} and to down-regulate *cadA* expression in the meantime. Nonetheless, this is unlikely as the accumulation of H^+ in the cytoplasm due to the lack of *cadA* would be worse by the action of YjdL, which imports dipeptide, and H^+ in the cytoplasm.

Thus, most likely, the transposon role is to disrupt *cadA* expression. However, Δ*rpoE* Δ*ompC* Δ*rcsD* Δ*cadA* bacteria did not reach WT fitness, indicating part of *cadA* may be necessary *in vivo*. The CadA protein is composed of three parts: an N-terminal domain, a major domain from 390 - 1635 bp, and a C-terminal domain (183). The transposon insertion at 1534 bp disrupts the major decarboxylase domain of *cadA* leaving intact the N- and the C-terminal domains. However, an internal ATG is lacking to initiate transcription: the N- and C-terminal domains are probably not expressed. We also cannot exclude a secondary mutation somewhere else in the genome that would explain Δ*rpoE* Δ*ompC* Δ*rcsD* *cadA::Tn5* phenotype.

4.3.3 yicH

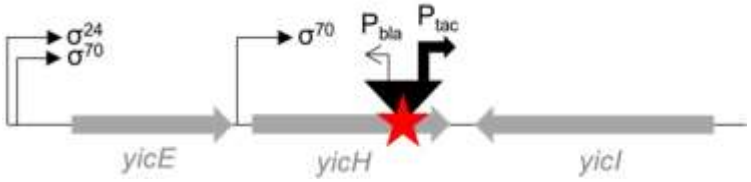

Figure 13. Transposon insertion site in *yicH* and adjacent genes.

yicH is a 1710 bp gene that is poorly described. It may encode a protein with ß-barrel structures (184), and is predicted to be an IMP (185). The transposon integrated at 1037 bp from the *yicH* start codon (Figure 13). P_{tac} faces the 5' region of *yicH* and the downstream gene *yicI*. *yicI* encodes – in the opposite direction – an enzyme involved in the carbohydrate metabolic process. We cannot exclude that the transposon role is to repress *yicI* by antisense expression through P_{tac}. Upstream of *yicH* is *yicE*, which encodes an H^+:xanthine symporter. As the distance between P_{bla} and *yicE* is 1191 bp, P_{bla} likely has no direct effect on *yicE* transcription, suggesting *yicH* is the transposon target.

According to the Membranome database (https://membranome.org/), the transmembrane domains of YicH are encoded within the first 81 bp, indicating the transposon has no effect on them. We could generate a truncated mutant and a knockout of *yicH* in order to characterize this gene and test its involvement in fitness. As *yicE* encodes a symporter and as *yicH* is predicted to encode an IMP, *yicH* may also encode a transporter that involves H^+. To test this hypothesis, we could test the passage of different molecules through this transporter by electrophysiology even though the panel of possible molecules is vast.

4.3.4 ftsI2

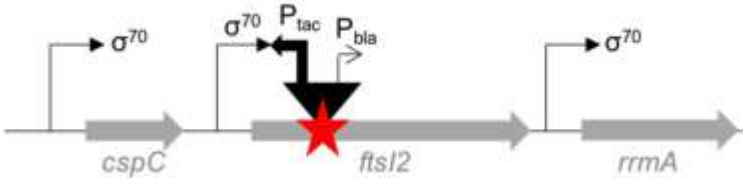

Figure 14. Transposon integration site in *ftsI2* and adjacent genes.

ftsI2 encodes a penicillin-binding protein (PBP) involved in cell wall structure. The transposon integrated at 164 bp from the start codon with P_{bla} facing the 5' region of *ftsI2*. *ftsI2* contains a domain encoding the PBP dimer and a domain encoding for the transpeptidase activity, both of which were intact after transposon insertion. Two internal methionines located downstream of the transposon could cause in-frame expression of the 3' part of *ftsI2*. The adjacent downstream gene is *rrmA*, which encodes a 23S rRNA methyltransferase. Upstream of *ftsI2* is *cspC*, a small gene encoding a stress protein involved in cold shock resistance. *cspC* expression is likely disrupted by P_{tac}, which is encoded in the opposite direction. It was shown recently that a *cspC* mutant was more sensitive to envelope stress *in vitro*, and double mutant *cspC cspE* was shown to be attenuated *in vivo* (186). Interestingly, ß-lactams bind to PBP, leading to an inhibition of cell wall synthesis. If the transposon insertion decreases *ftsI2* expression, ß-lactams cannot exert their deadly effect. Alternatively, *Salmonella* cell

wall integrity may be compromised, leading to a decrease in fitness, unless another PBP compensates. An increase in ß-lactam resistance was already observed in PBP point mutants. Two mechanisms were possible: (i) decreasing ß-lactam binding to PBP active site, (ii) evolving a ß-lactamase activity that degrades the antibiotic (187). To test these hypotheses, we could generate truncated mutants, test their *in vivo* fitness, and challenge them *in vitro* with ß-lactams.

4.3.5 Suppressor mutations of σ^E essentiality *in vitro*

In *E. coli*, *rpoE* is essential for viability unless unlinked suppressor mutations occur to ensure growth in the absence of *rpoE.* Mutations in *yhbW*, *ptsN*, and *ydcQ* were described in *E. coli*. *yhbW* encodes a putative luciferase-like monooxygenase of unknown function and was not investigated further by the authors (93). *ptsN* encodes a phosphotransferase enzyme involved in carbohydrate metabolism. It is interesting to note that this gene encodes the only suppressor mutation described thus far whose expression is σ^E-regulated (158). Overexpression of *ptsN* reduces cell envelope stress. Theoretically, we could have detected it with our approach due to the transposon promoters' activity. We may not have detected *ptsN* in our screen because *rpoE* is not essential for *Salmonella* viability, or because *ptsN* overexpression was not as dominant as *ompC* deletion. *ydcQ* encodes the antitoxin of a toxin-antitoxin system. It is surprising that the absence of *ydcQ* allows survival as it is the "antidote" of the system. YdcQ loss leads to a decreased in envelope stress response and in OMVs production. The toxin may trigger a signaling cascade that activates other, typically dormant stress response systems.

In *V. cholerae*, *rpoE* is also essential. *ompU*, which encodes the major porin, was identified as a significant suppressor mutation of *rpoE* essentiality (188). Interestingly, OmpU and OmpC share 67% identity, highlighting that major porin loss is not specific to *Salmonella*.

These suppressor mutations described above are highly diverse in terms of role. This shows that it is possible to circumvent σ^E essentiality *in vitro* by many ways. Our results demonstrated this is also possible *in vivo*. Indeed, under host attacks, the frequency rate of mutations might increase, emphasizing, once again, pathogen plasticity to ensure survival.

4.4 Implications of our results

By deleting *rpoE*, we aimed to simulate the effects of an *rpoE* specific inhibitor and the challenges posed by development of bacterial resistance. Would an *rpoE* inhibitor be useful in fighting *Salmonella* infection? Therefore, the intent of our approach was to synergize with the host immune system and seek suppressor mutations *in vivo*.

In our study, we could show that an avirulent strain Δ*rpoE*, mimicking a bactericidal antibiotic, could be rescued (fitness gain of about 100,000,000 fold) by only two further mutations. Furthermore, the deletion of *ompC* on top of the *rpoE* mutant may be considered as an attempt to mimic a bacteriostatic antibiotic, that was here again rescued by only one mutation. In clinics, frequency mutation rates are known to increase upon antibiotics treatment, even at sub-lethal concentrations (160). It seems straightforward for bacteria to become resistant to antibiotic. Indeed, a widely known clinical relevant mutation that give rise to resistance and implies a porin is the loss of OprD in *P. aeruginosa*, increasing the resistance to carbapenem. Additionally, as described above, pathogens showing already *ompC* null or down mutations are circulating in clinical isolates, making here already a first step towards resistance to a potential *rpoE* inhibitor. Our data reported here could have only been obtained by using an adapted *in vivo* model, because these data could not have been predicted with the actual *in vitro* conditions.

It was shown that the pathogen efflux system TolC-AcrAB is a potential target *in vitro* for antibiotics (29). However, the clinical relevance of the efflux system remains largely unknown. We inactivated TolC in ESBL-*E.coli* strains and we tested their antibiotic resistance profile. We observed non-significant slight changes, demonstrating that deleting efflux system in clinically relevant strains has no impact on drug resistance. Therefore, we also invalidated this system as a potential target for inhibitor.

Taken together, our study implications are the following: (i) *rpoE* and the efflux system are most likely not suitable target for inhibitor development, (ii) the commonly available *in vitro* conditions do not suit to target validation, and (iii) the necessity to use relevant *in vivo* model as well as clinically relevant strains.

5 Conclusion and future perspectives

In this study, we used a new strategy in order to fight pathogens infections more efficiently. Our aim was to identify potentially relevant inhibitor targets. We used a strain deleted of *rpoE*, a major regulon which encodes an envelope stress response factor. Δ*rpoE* exhibits small growth defects *in vitro* at low pH and during exponential phase. However, an *rpoE* mutant is completely cleared by the host immune system in our typhoid fever-like mouse model. Thus, our approach consisted in synergizing with the host immune system and seek suppressor mutations of *rpoE* essentiality *in vivo*.

By using transposon screens, we identified two loss-of-function mutations that rescued the fitness of Δ*rpoE in vivo*: a major *Salmonella* porin encoded by *ompC*, and a major regulator involved in RNAs processing encoded by *pnp*. While the exact molecular mechanisms underlying the fitness rescue are not completely clear yet, we could show that these two combined mutations allowed a 100,000,000 fold increase in fitness, which is remarkable.

Pathogens constantly mutate their genome to ensure survival. As this two-step mechanism would cause moderate fitness cost for bacteria, it is tempting to believe that these mutations can be rather easily acquired by pathogens in a patient. We thus invalidated *rpoE* as a potential inhibitor target. Our approach can be used in the future to evaluate the activity of potential inhibitor of other genes. Major regulator genes crucial for virulence could be tested before their potential inhibitor reach advanced clinical phase trial, saving time, energy, material, and investment.

Our approach highlights the importance of using *in vitro* conditions that mimic as best possible *in vivo* microenvironments encountered by pathogens during infection. Indeed, commonly used laboratory conditions often lack components complexity and fail to fully activate appropriate stress response genes. This renders the identification of suppressor mutations challenging and makes *in vitro* target validation unreliable. Therefore, our studies bring a new paradigm for evaluating target inhibitor *in vivo*.

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Young and dynamic scientist, I am looking for new challenges to move on in my career and continue to evolve. Thanks to my education at the Biozentrum in Infection biology, I gained a high knowledge and understanding of the host-pathogen interactions. I enjoy working in an international atmosphere.

Professional experiences

Since January 2014 (3 years 11 months)	Doctoral Research Assistant – Marie-Curie fellow Biozentrum, Infection Biology, Basel, Switzerland *In vivo* envelope stress response of *Salmonella* ❖ Utilization of a wide variety of techniques to study genes effects ❖ Attendance to many conferences on host-pathogen interactions
February 2013 (10 months)	Research Assistant – Erasmus Fellow – Master Project Biozentrum, Infection Biology, Basel, Switzerland Analysis of *Salmonella* virulence factor PhoP ❖ Learning of BSL2 working habits
November 2011 (5 months)	Research Assistant – Master Project University of Arkansas for Medical Sciences Department of Immunology and Microbiology, Little Rock, USA Refining the type IV secretion system substrate repertoire of *Coxiella* ❖ Discovery of abroad working habits
January 2011 (6 weeks)	Junior Research Assistant – Bachelor Project Monsanto SAS, Biotechnology laboratory, Nîmes, France Haplo-diploidization methods in melons ❖ Understanding of a company functioning

Education and Training

2014-Ongoing	PhD project, University of Basel, Infection Biology
2016	« Effective scientific communication » workshop
2015	« Presentation skills: The messenger is the message » workshop
2011-2013	Master degree, University of Montpellier 1, Biology
2009-2011	HND in Nutrition Science, Lycée Marie-Curie, Marseille

Prizes and honors

2017	Selected talk for the Swiss Society of Microbiology Congress in Basel
2014	Selected Marie-Curie fellow for PhD project
2013	Selected Erasmus fellow for Master project

Skills

Languages	French (mother tongue), English (C1), Spanish/German (Basics)
Communication	Excellent oral presentation skills
Team spirit	Collaboration with colleagues and Marie-Curie fellows
Leadership and management	Management of experiments with bachelor students; PhD retreat and weekly laboratory meeting schedule organization

Extracurricular activities

2017
PhD representative

Co-organization of the Biozentrum PhD retreat 2017

- Monthly connect with the Board to develop agenda, speaker's selection and sponsoring plans according to PhDs expectations
- Actively seek and engage with potential sponsors and build strong relationships with the selected sponsors
- Deal with any problem or request during the event

2017
1 week
eco-volunteer

Monitoring of the sea turtles' nests population in Tortuguero, Costa Rica

- Patrol the beach at night to protect nests from poachers
- Control sea turtles' health by body check, carapace measurements and eggs counting
- Survey the nests population by marking and counting nests
- Help preserve a safe environment for hatchlings by spontaneously organizing beach cleaning

2016
1 day
NGO volunteer

Alarming people on pesticides utilization especially in fruits cultivation

- Arouse people's curiosity by offering organic fruits juice
- Explain and discuss the arm of pesticides utilization
- Distribute flyers about pesticides and the NGO upcoming events

Hobbys

Traveling to discover different cultures and stay open-minded.
Reading, especially biographic books, to live a thousand lives.
Practicing crossfit regularly to surpass myself and relax afterwards.

Publications

P. Maturana, Joseph G. Graham, U. M. Sharma and D. E. Voth, (2013), Refining the plasmid-encoded type IV secretion system substrate repertoire of *Coxiella burnetii*. *Journal of Bacteriology.*

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