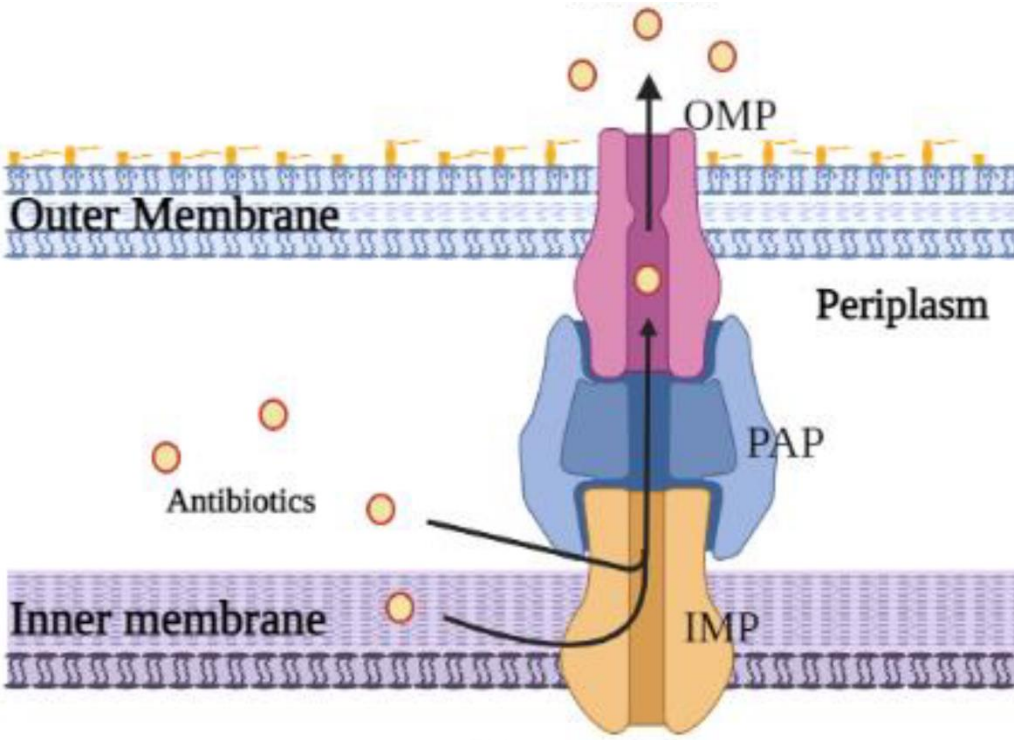
The efflux pumps in the host-pathogen interaction

Efflux pumps: what are they?

- Present in all living organisms
- Bacterial transport proteins involved in extrusion of substrates from the cellular interior to the external environment
- Single-component transporter or tripartite complex
- Classified into 7 superfamilies
- Most of them are well known as multidrug resistance (MDR) efflux pumps



- Allow the microorganisms to regulate their internal environment by removing toxic substances, including antimicrobial agents, metabolites and quorum sensing signal molecules.

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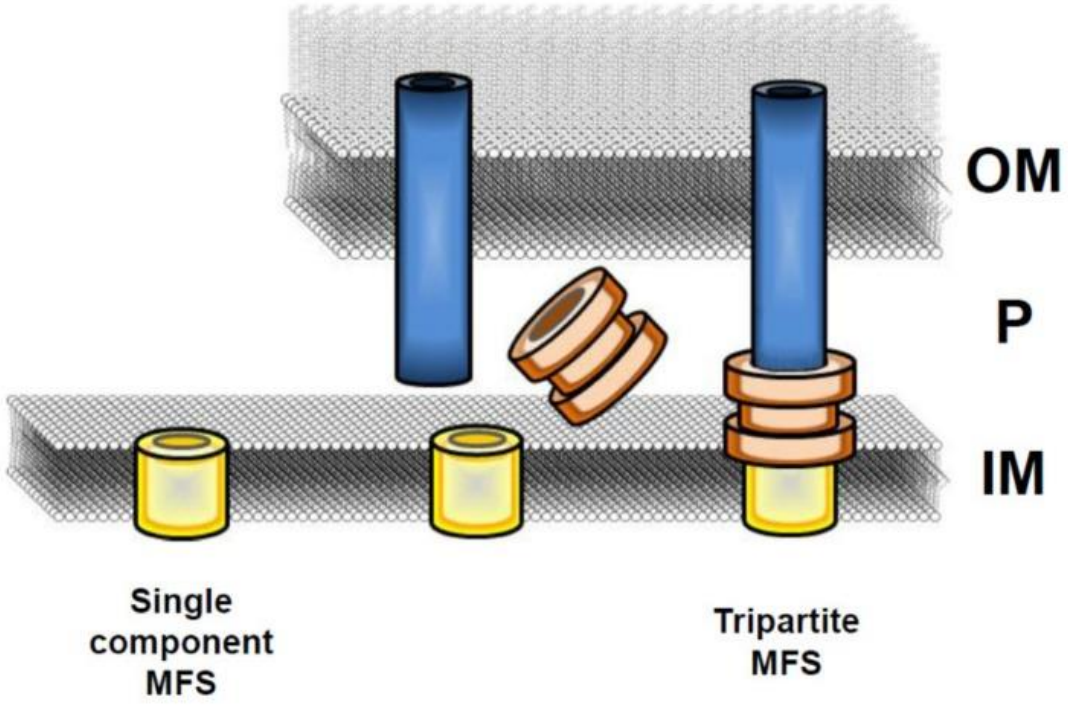

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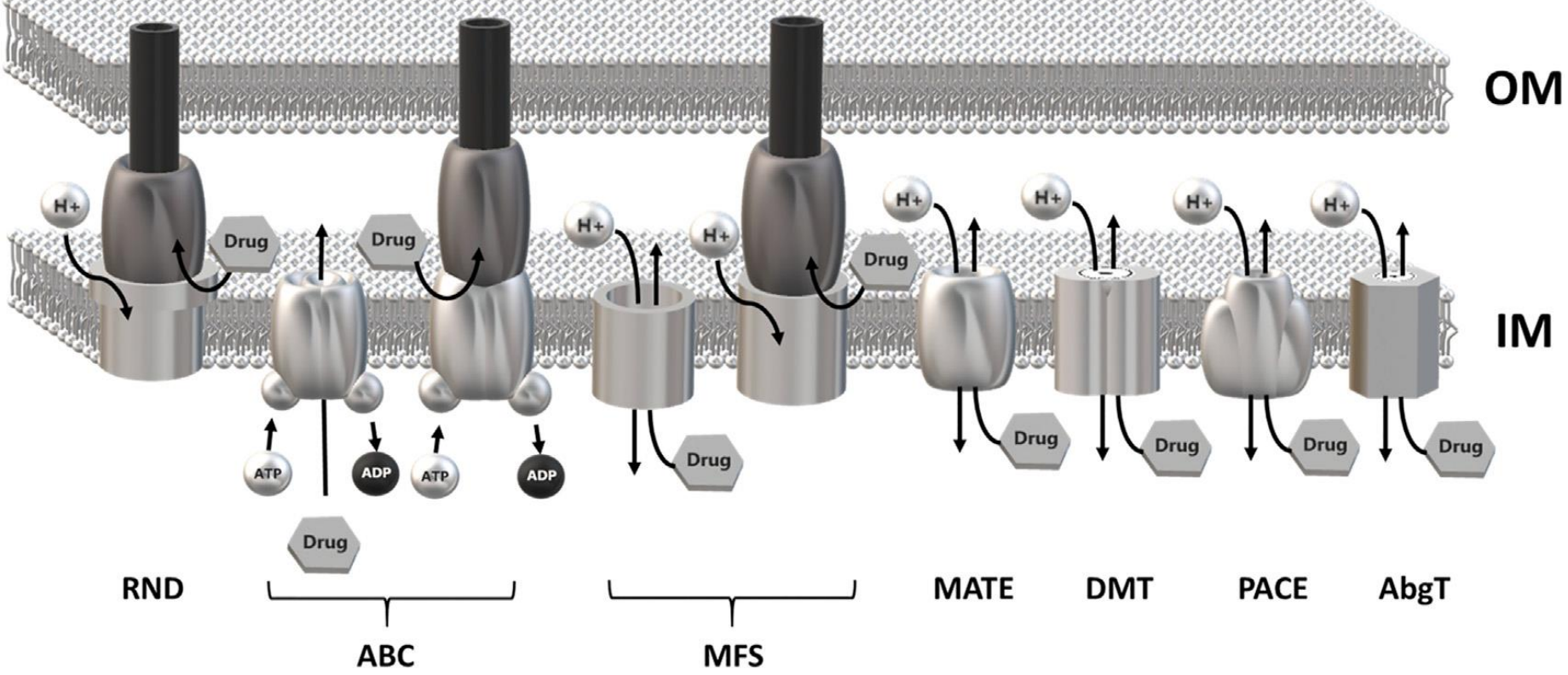

- Classified into 7 superfamilies on the basis of their sequence similarity, substrate specificity, number of components (single or multiple), number of transmembrane-spanning regions, and energy source
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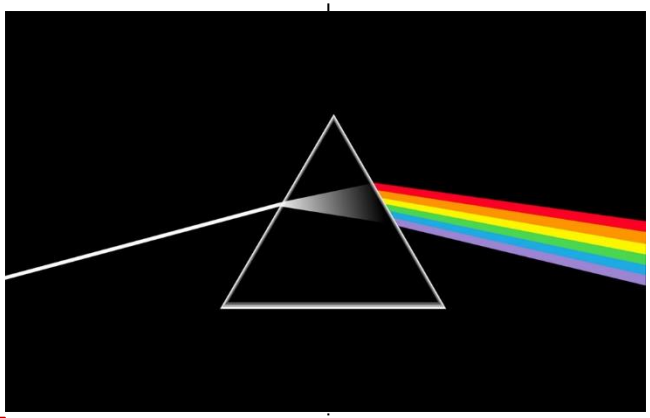
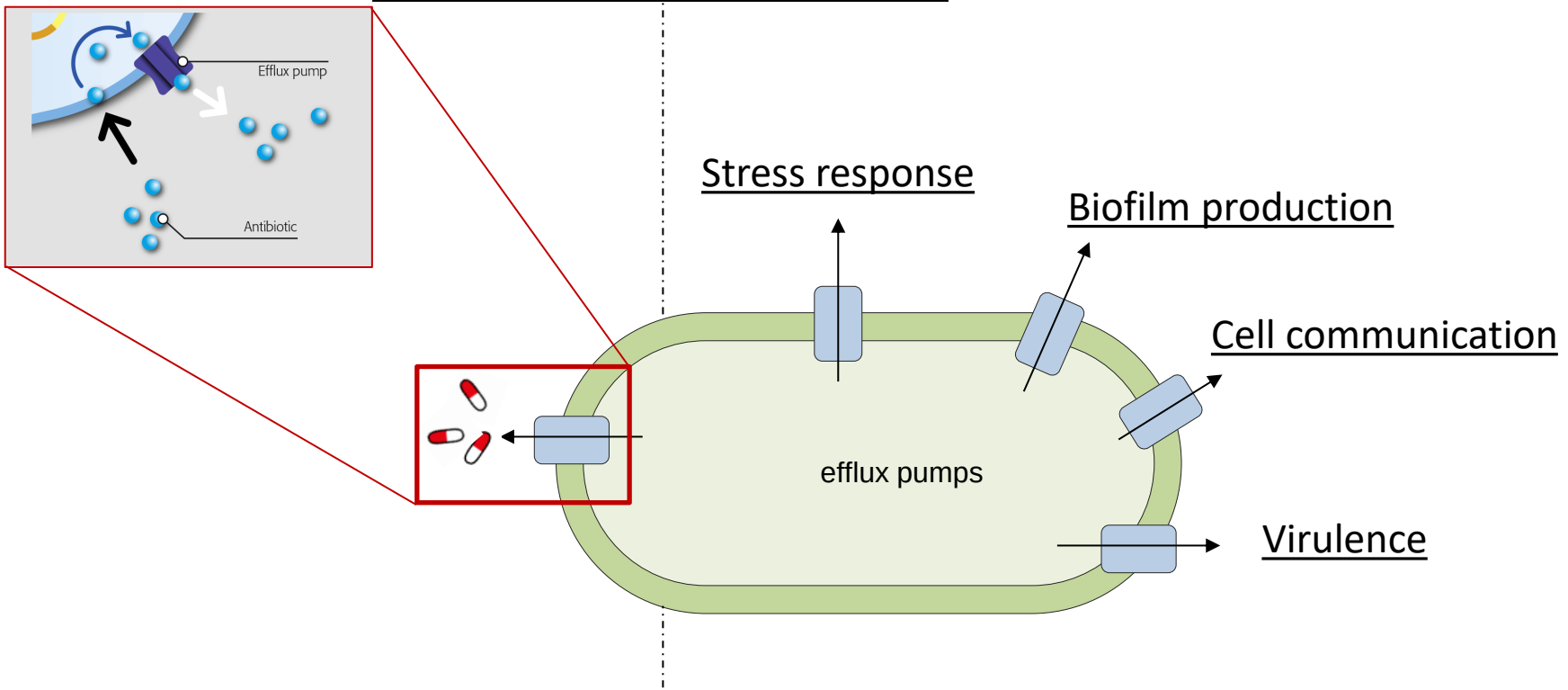
- Allow the microorganisms to regulate their internal environment by removing toxic substances, including antimicrobial agents, metabolites and quorum sensing signal molecules.

The multifaceted role of efflux pumps



Antibiotic resistance

Physiological roles



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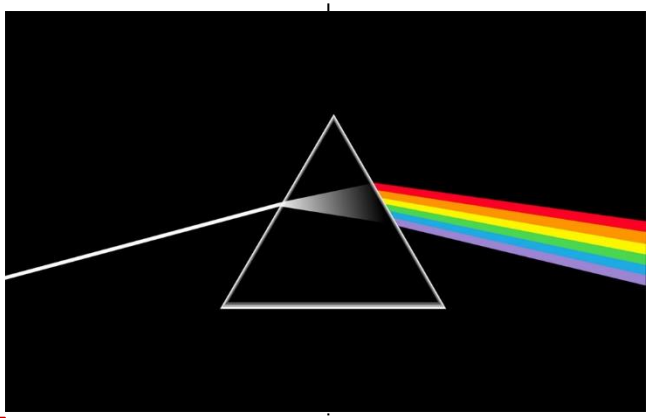

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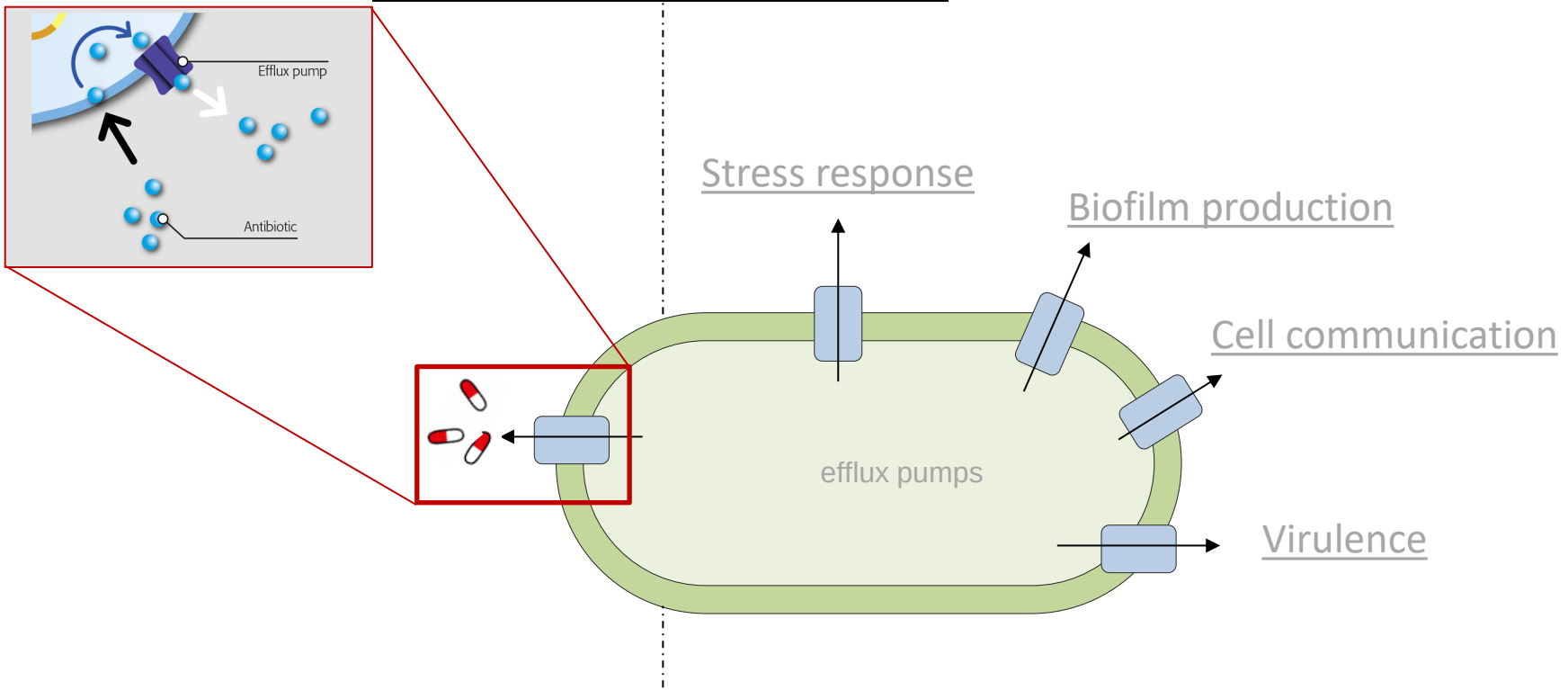
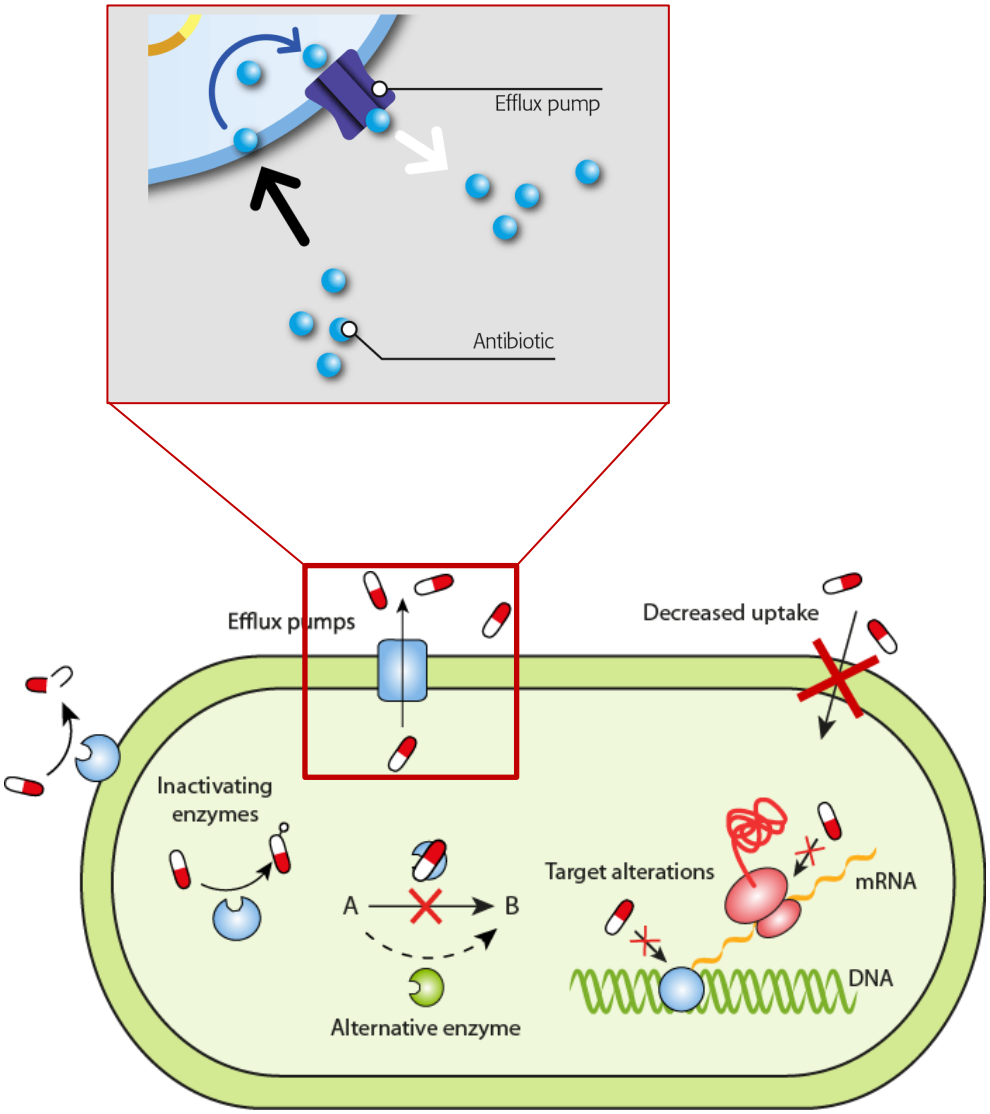


Multidrug-resistance efflux pumps



Bacterial efflux pumps actively transport many antibiotics out of the cell and are major contributors to the intrinsic resistance to antibiotics used to treat bacterial infections.

What about their physiological role?

Original image from: https://www.reactgroup.org/toolbox/understand/antibiotic-resistance/resistance-mechanisms-in-bacteria/

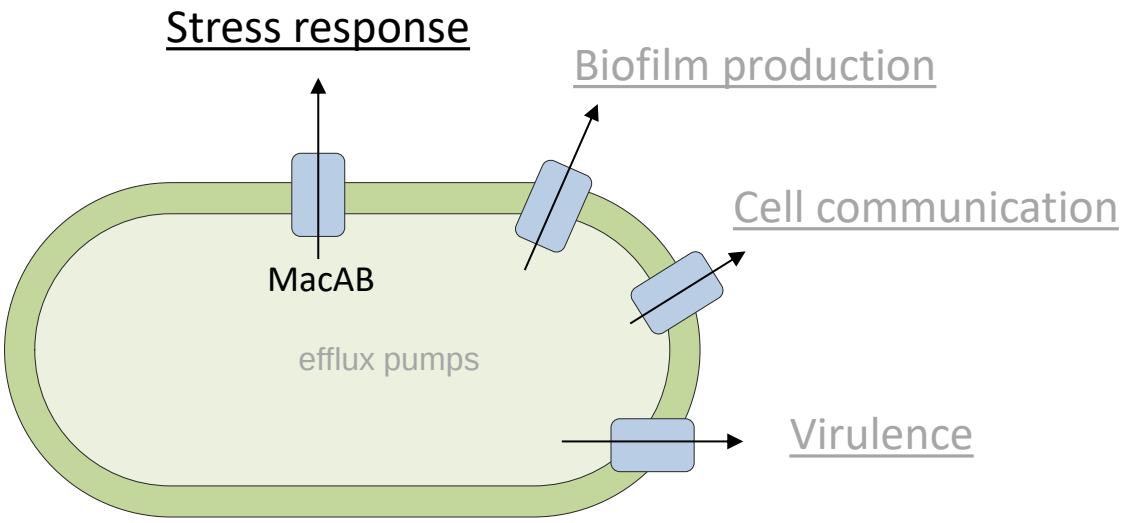
Efflux pumps and stress response

Le specie reattive dell'ossigeno (ROS) generate dal complesso NADPH ossidasi di membrana 2 (Nox2) rappresentano un'importante difesa delle cellule immunitarie innate contro le infezioni batteriche

Nox2 produce ROS che vengono rilasciati dai fagociti sia nell'ambiente extracellulare che nei fagosomi. Acido ipocloroso, superossido e perossido di idrogeno (H_2O_2) sono generati per uccidere i microrganismi.

I batteri patogeni hanno sviluppato diversi meccanismi per contrastare gli effetti dannosi dei **ROS**:

- detossificazione diretta dei ROS tramite enzimi come le superossido dismutasi (SOD), le catalasi e le perossidasi
- siderofori catecolati, metaboliti batterici prodotti in risposta alla carenza di ferro

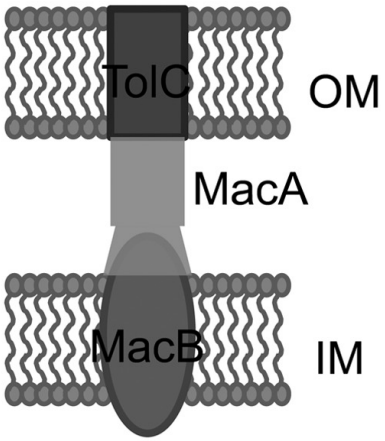


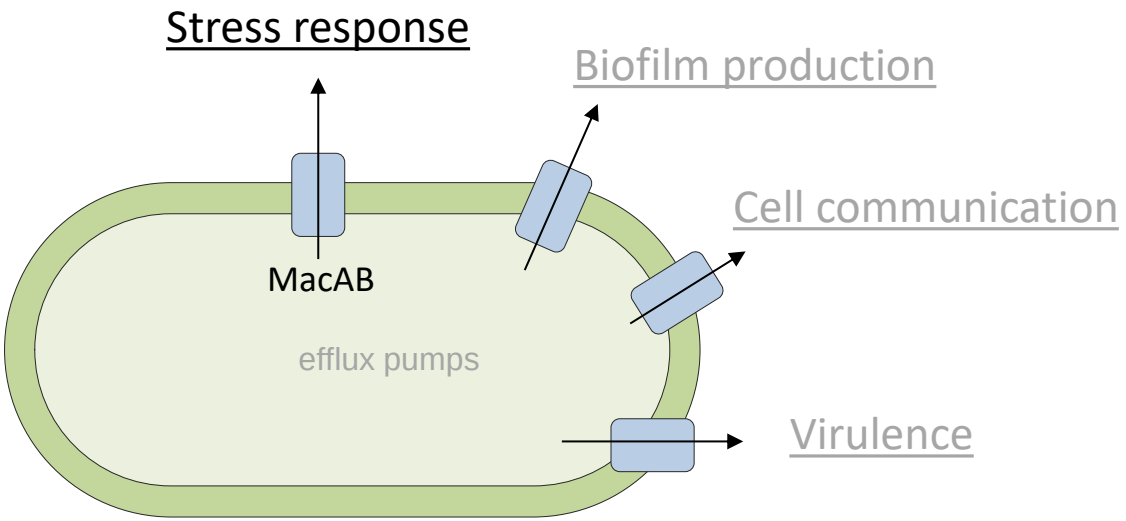
Efflux pumps and stress response

The ABC-Type Efflux Pump MacAB Protects *Salmonella enterica* serovar Typhimurium from Oxidative Stress

Lydia M. Bogomolnaya,[a,b] Katharine D. Andrews,[a] Marissa Talamantes,[a] Aimee Maple,[a] Yury Ragoza,[a] Andres Vazquez-Torres,[c] Helene Andrews-Polymenis[a]

Department of Microbial Pathogenesis and Immunology, College of Medicine, Texas A&M University System Health Science Center, Bryan, Texas, USA[a]; Institute of Fundamental Medicine and Biology, Kazan Federal University, Kazan, Russia[b]; Department of Microbiology, School of Medicine, University of Colorado at Denver, Aurora, Colorado, USA[c]





Efflux pumps and stress response

2013

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RESEARCH ARTICLE
Host-Microbe Biology

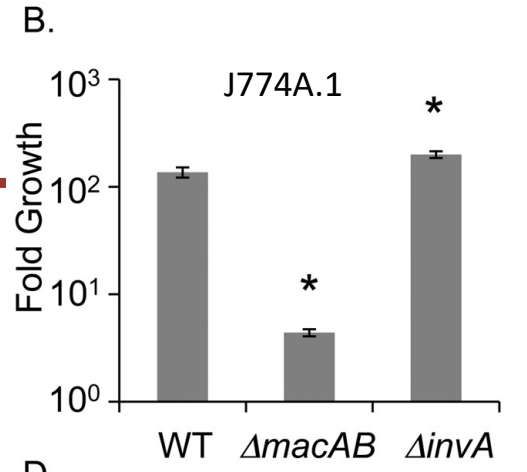
2020

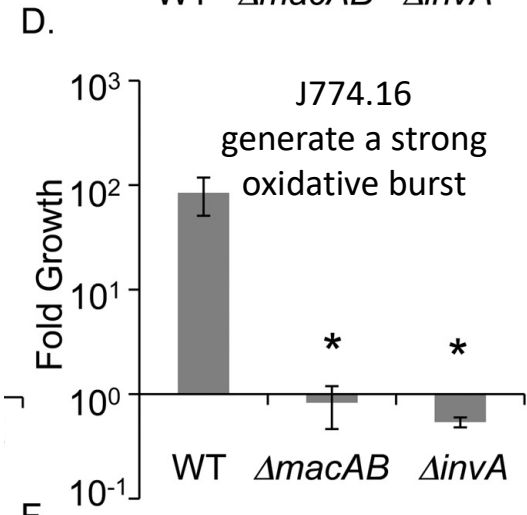
Linearized Siderophore Products Secreted via MacAB Efflux Pump Protect *Salmonella enterica* Serovar Typhimurium from Oxidative Stress

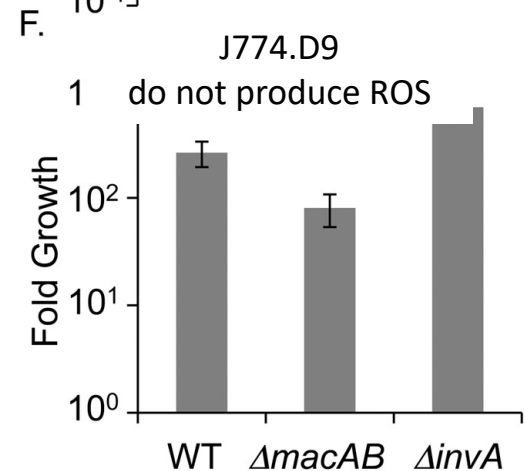
L. M. Bogomolnaya,[a,b,c] R. Tilvawala,[a,d] J. R. Elfenbein,[e,f] J. D. Cirillo,[a] H. L. Andrews-Polymenis[a]

- MacAB is required for intracellular growth in macrophages
- MacAB is required for survival in the inflamed intestine
- MacAB is required for resistance to hydrogen peroxide

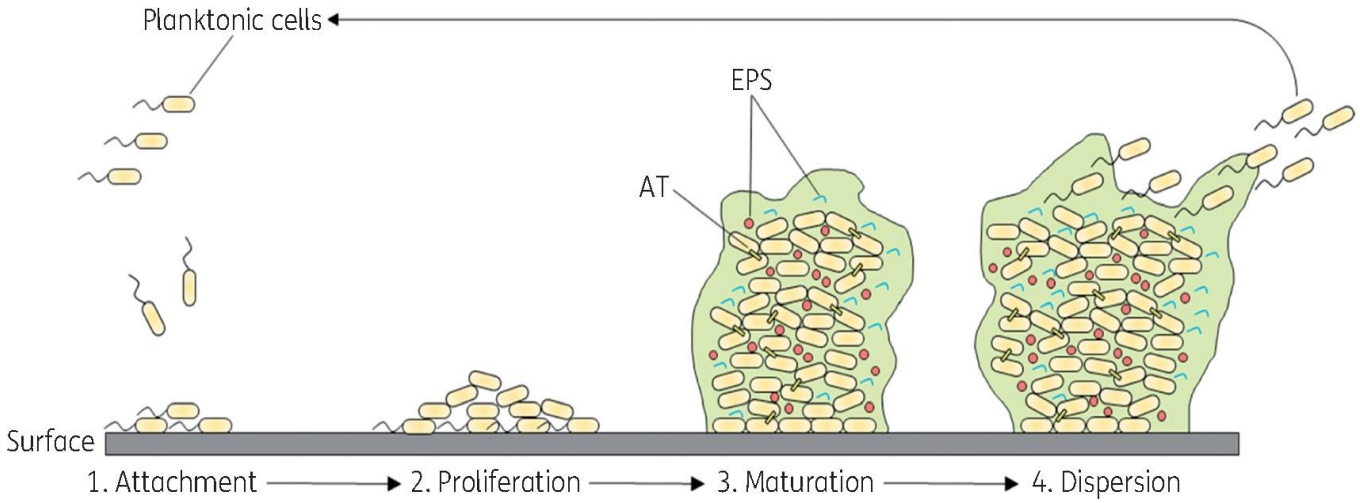
MacAB participates in the excretion of a linearized enterobactin trimer that induces protection against ROS-mediated killing



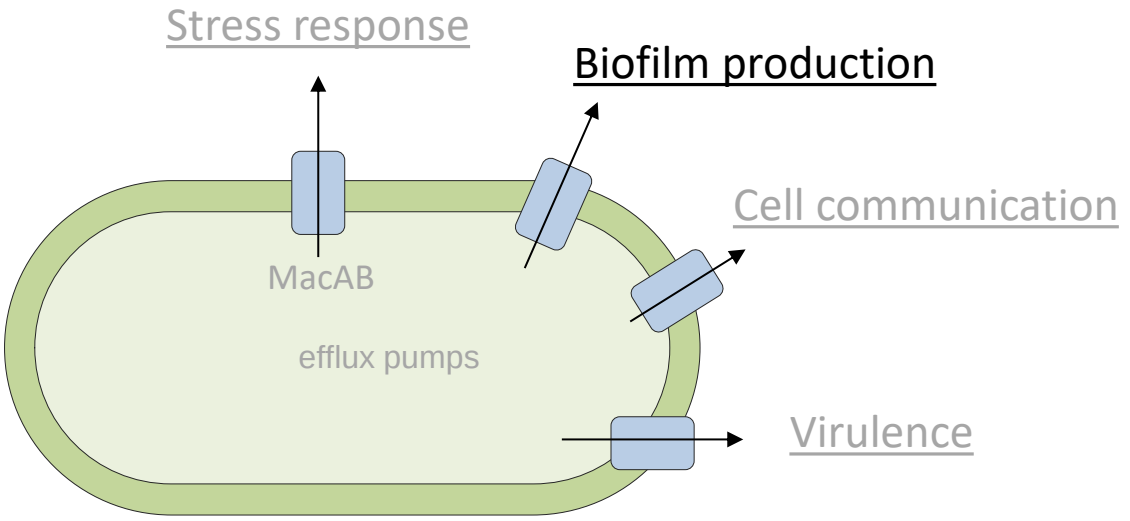




Efflux pumps and biofilm formation



Biofilms are collections of sessile microorganisms associated with a surface and enclosed in a self-produced matrix of extracellular polymeric substances (EPSs)



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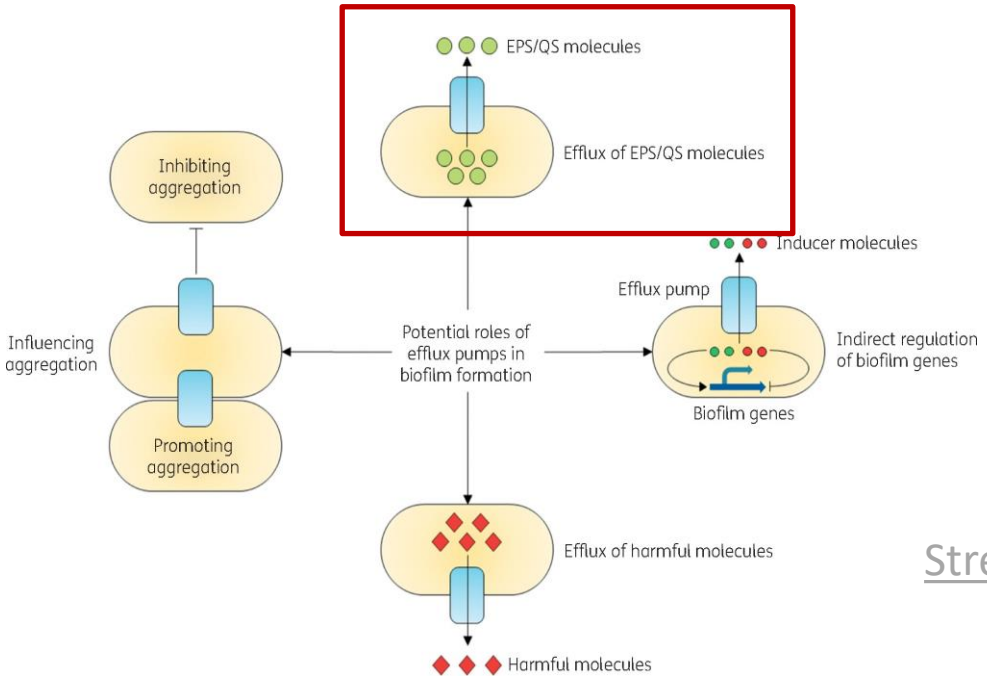

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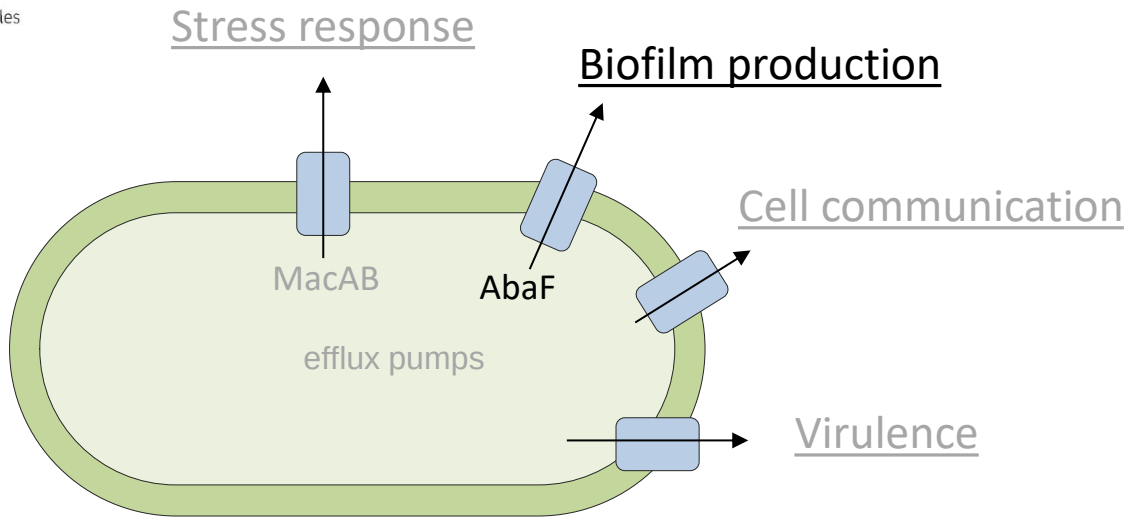
PHYSIOLOGY AND BIOTECHNOLOGY
December 1, 2008 Volume 74 Issue 23
https://doi.org/10.1128/AEM.01310-08

Inactivation of Efflux Pumps Abolishes Bacterial Biofilm Formation

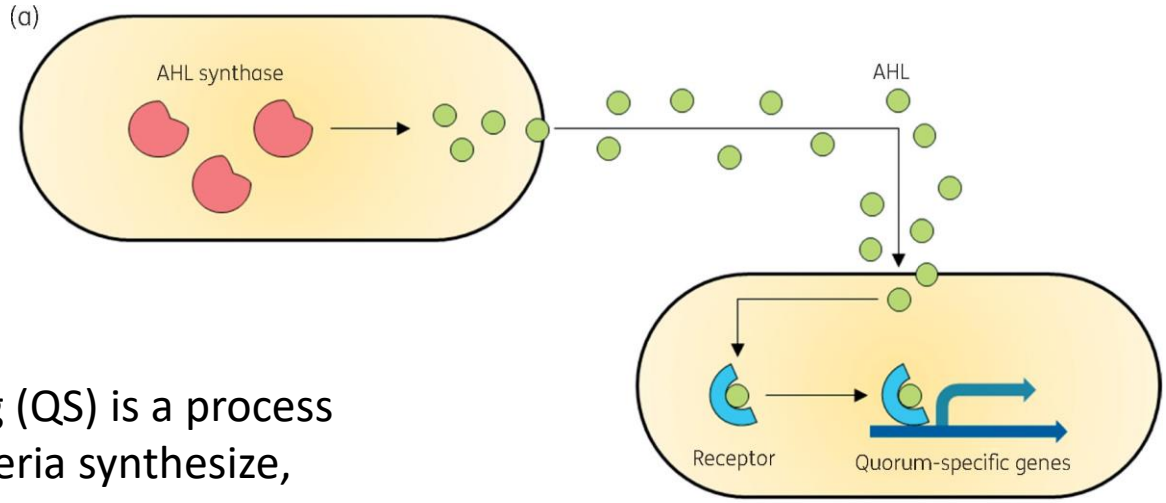
Malin Kvist, Viktoria Hancock, Per Klemm*

Microbial Adhesion Group, Department of Systems Biology, Technical University of Denmark, Lyngby, Denmark

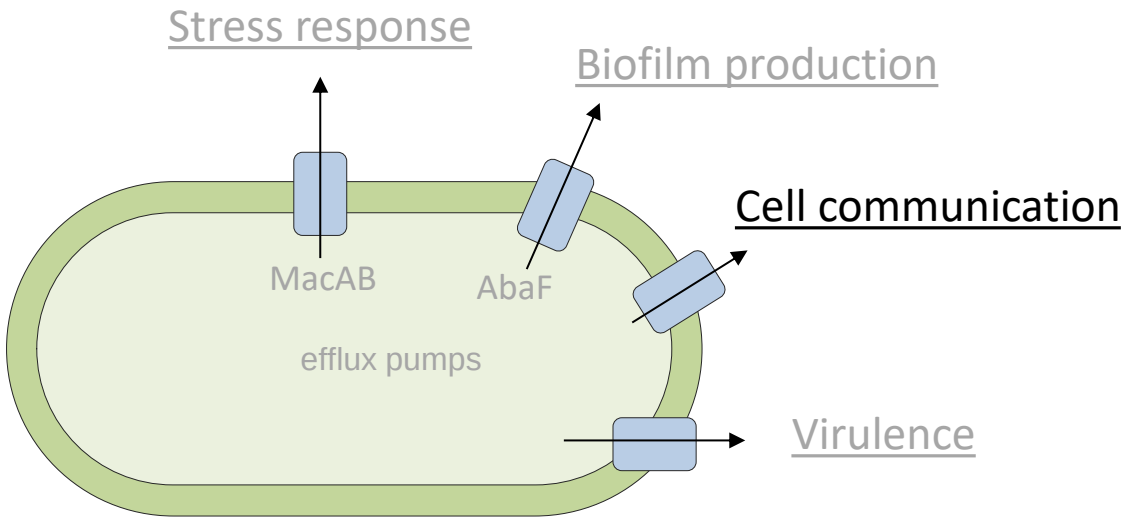
AbaF of *Acinetobacter baumannii* is involved in the extrusion of biofilm material



Efflux pumps and quorum sensing



Quorum sensing (QS) is a process whereby bacteria synthesize, recognize and respond to extracellular signalling molecules known as autoinducers (AIs) to mediate intercellular communication.

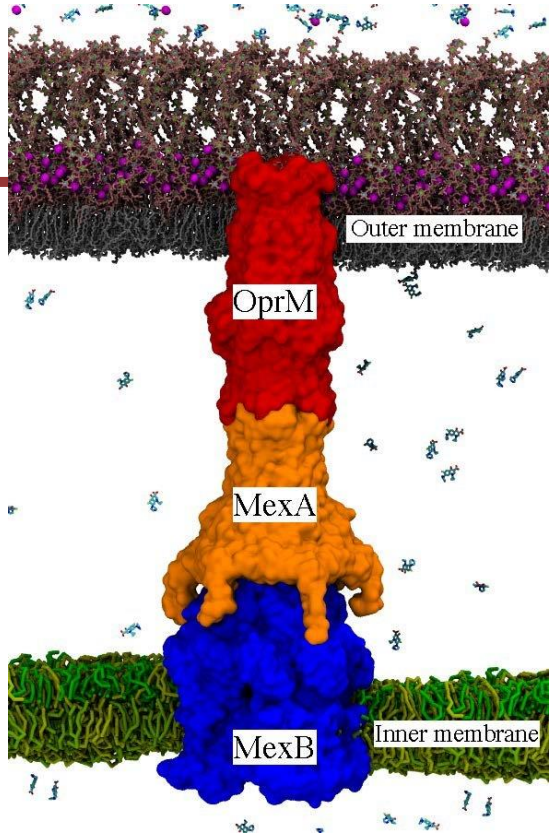


Efflux pumps and quorum sensing

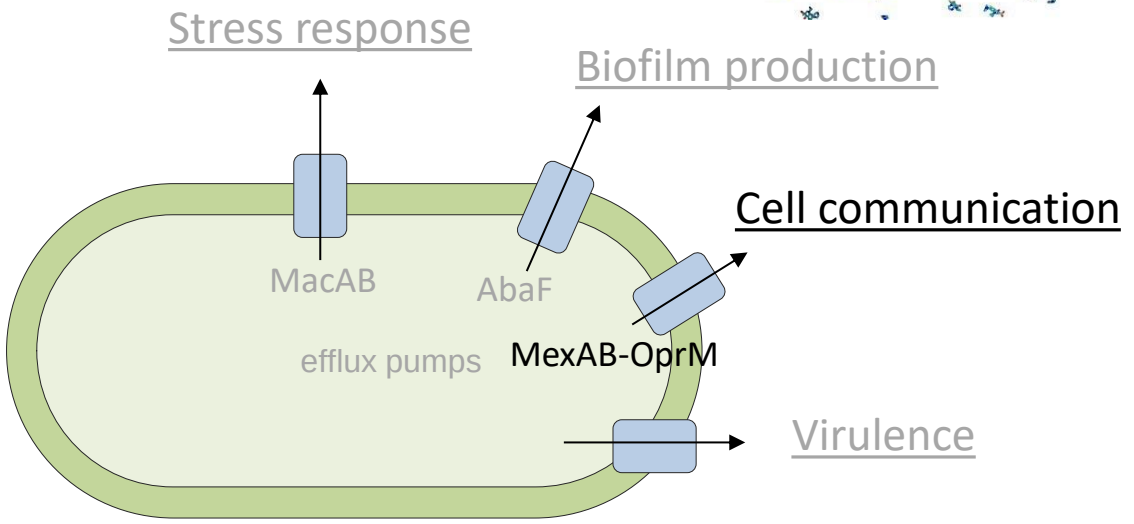
AMERICAN SOCIETY FOR MICROBIOLOGY | Antimicrobial Agents and Chemotherapy
MECHANISMS OF ACTION: PHYSIOLOGICAL EFFECTS
Volume 48 Issue 4

Enhancement of the *mexAB-oprM* Efflux Pump Expression by a Quorum-Sensing Autoinducer and Its Cancellation by a Regulator, MexT, of the *mexEF-oprN* Efflux Pump Operon in *Pseudomonas aeruginosa*

Hideaki Maseda[1,*], Isao Sawada[2], Kohjiro Saito[1], Hiroo Uchiyama[2], Taiji Nakae[1], Nobuhiko Nomura[2,*]



MexAB-OprM exports quorum-sensing mediators, acylhomoserine lactones (AHSLs), which induce the production of cell density-dependent virulence factors.



Efflux pumps and virulence

Table 2. List of Tripartite Systems That When Deleted or Inactivated Result in Attenuated Virulence in Their Cell/Host Model of Infection[a]

Microorganism	Efflux pump family	Efflux system	Cell/host infection model	ref(s)
S. Typhimurium	RND	AcrAB-TolC	Human epithelial cells, murine macrophages, Galleria mellonella, mouse, chicken	101, 158−160
		MdtABC	Mouse	101
		MdsABC	Mouse	101
	ABC	MacAB-TolC	Mouse	101, 138, 139
		SitCDF	Cattle and bovine enterocytes	156
		MexGHI-OpmD	Rat	83
S. maltophilia	RND	SmeYZ	Mouse	114
B. pseudomallei	RND	BpeAB-OprB	Human epithelial cells and macrophages	169
B. burgdorferi	RND	BesABC	Mouse	32
C. jejuni	RND	CmeABC	Acanthamoeba polyphaga, Chicken	170
				143
S. flexneri	MFS	EmrKY	Human macrophages	144
L. pneumophila	ABC	LssBD-TolC	Ameoba and human macrophages	157
V. cholerae	RND	VexAB-TolC, VexCD-TolC, VexIJK	Mouse	120
Rhizobium anatipestifer	RND	RaeEF-RopN	Duck	99
E. amylovora	RND	MdtABC	Apple rootstock	147
		MdtUVW		
		AcrAB		171
R. solanacearum	RND	AcrAB	Tomato plant	148

[a]ABC, ATP-binding cassette; MFS, major facilitator superfamily; RND, resistance-nodulation-division.

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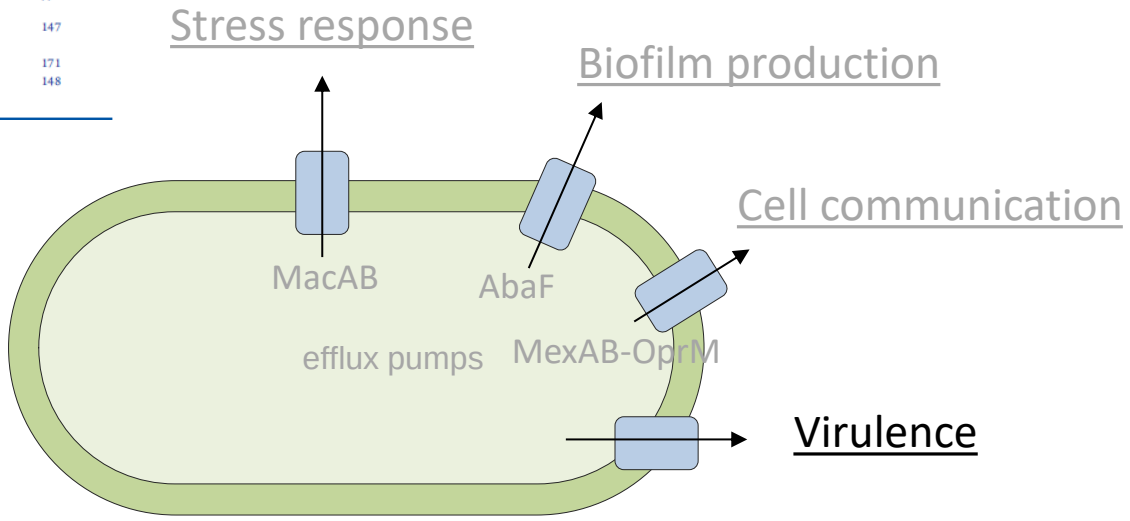

Efflux pumps and virulence

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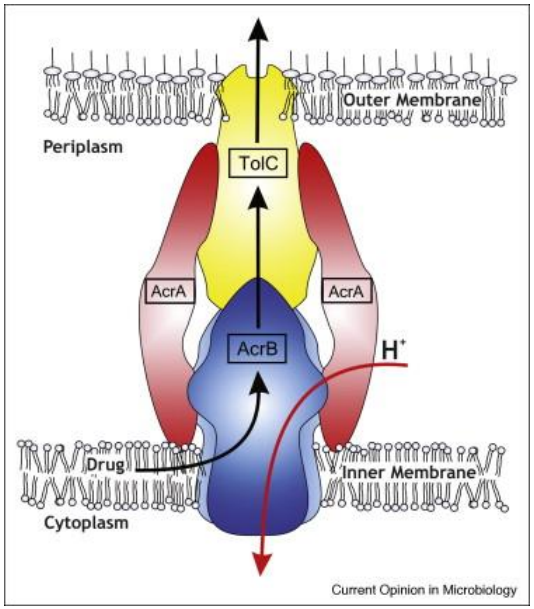
Lack of AcrB Efflux Function Confers Loss of Virulence on *Salmonella enterica* Serovar Typhimurium

Xuan Wang-Kan,[a] Jessica M. A. Blair,[a] Barbara Chirullo,[b] Jonathan Betts,[c] Roberto M. La Ragione,[c] Alasdair Ivens,[d] Vito Ricci,[a] Timothy J. Opperman,[e] Laura J. V. Piddock[a]



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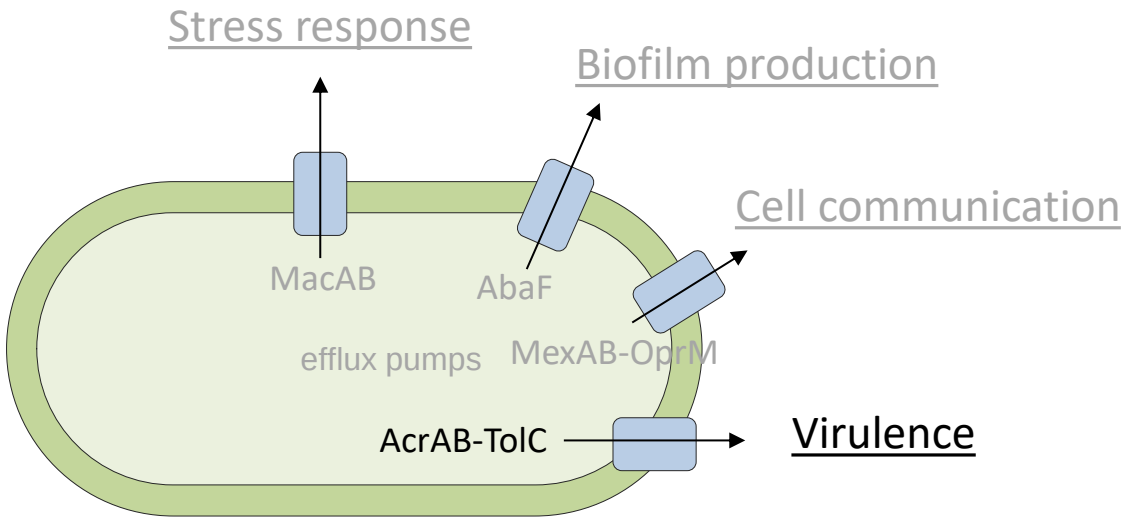

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		SitCDF	Cattle and bovine enterocytes	156
K. pneumoniae	RND	AcrAB-TolC	Mouse	51
E. coli	ABC	MacAB-TolC	Galleria mellonella and murine mammary glands	161
	RND	MdtB	Mouse spleen	161
		MdtEF	Human macrophages	162
A. baumannii	RND	AdeABC	*Galleria mellonella*	163
S. flexneri	MFS	EmrKY	Human macrophages	144
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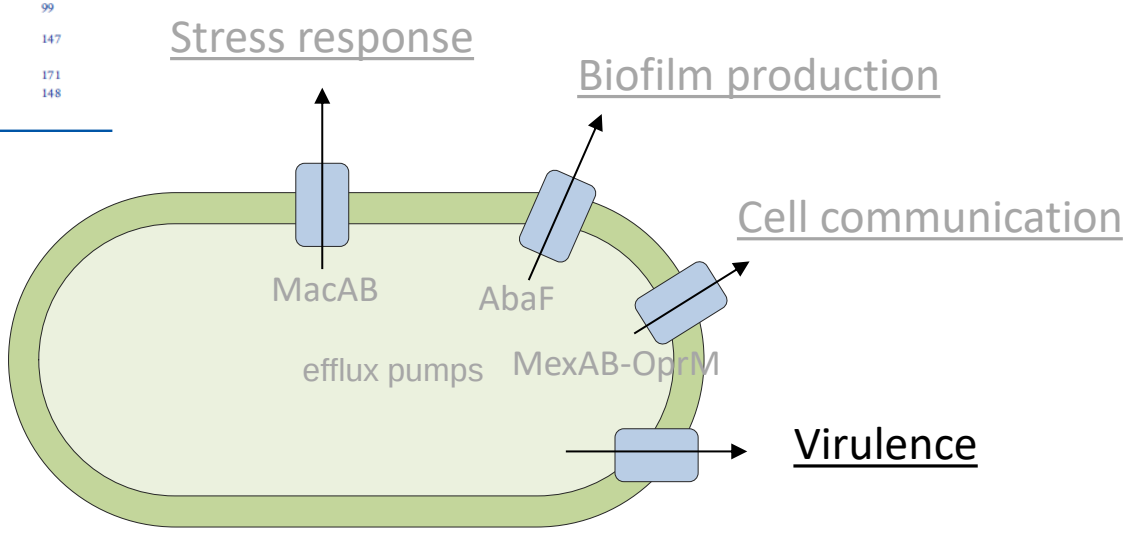
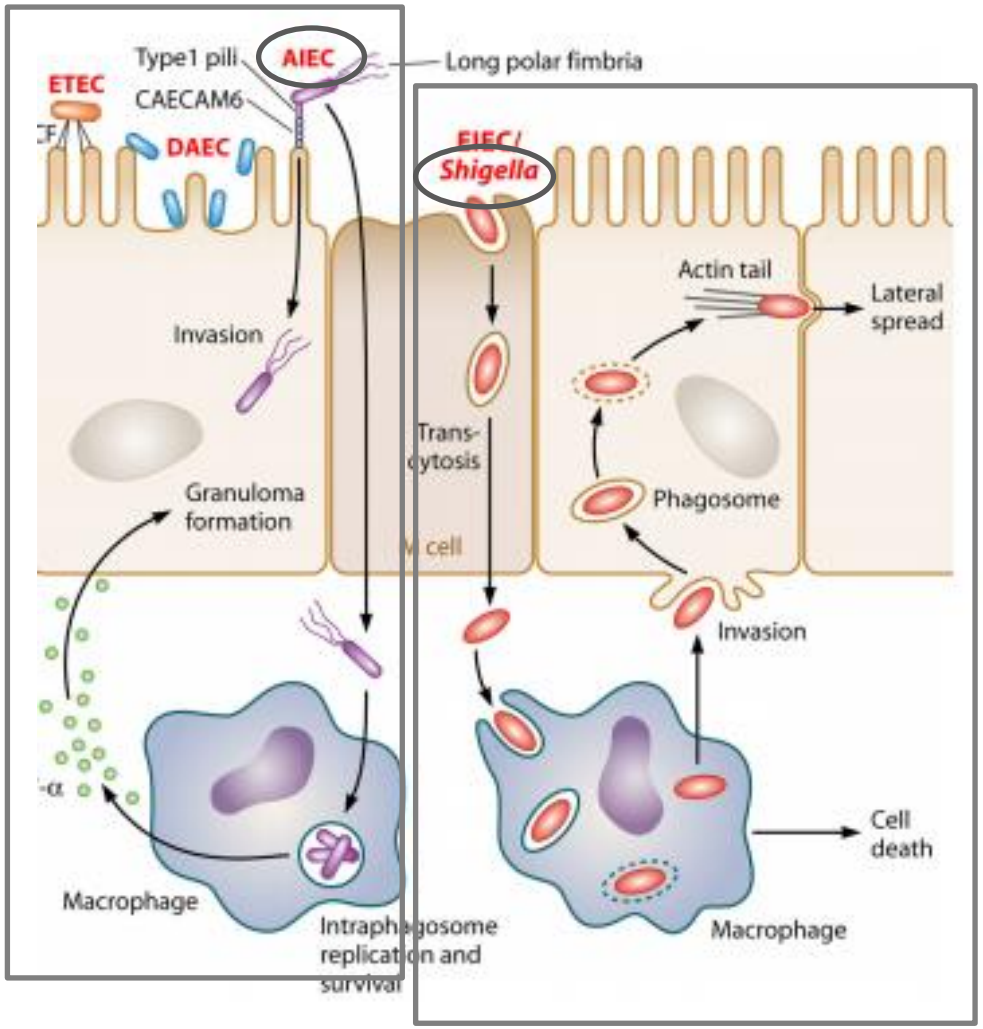


Role of multidrug efflux pumps during intracellular life of:

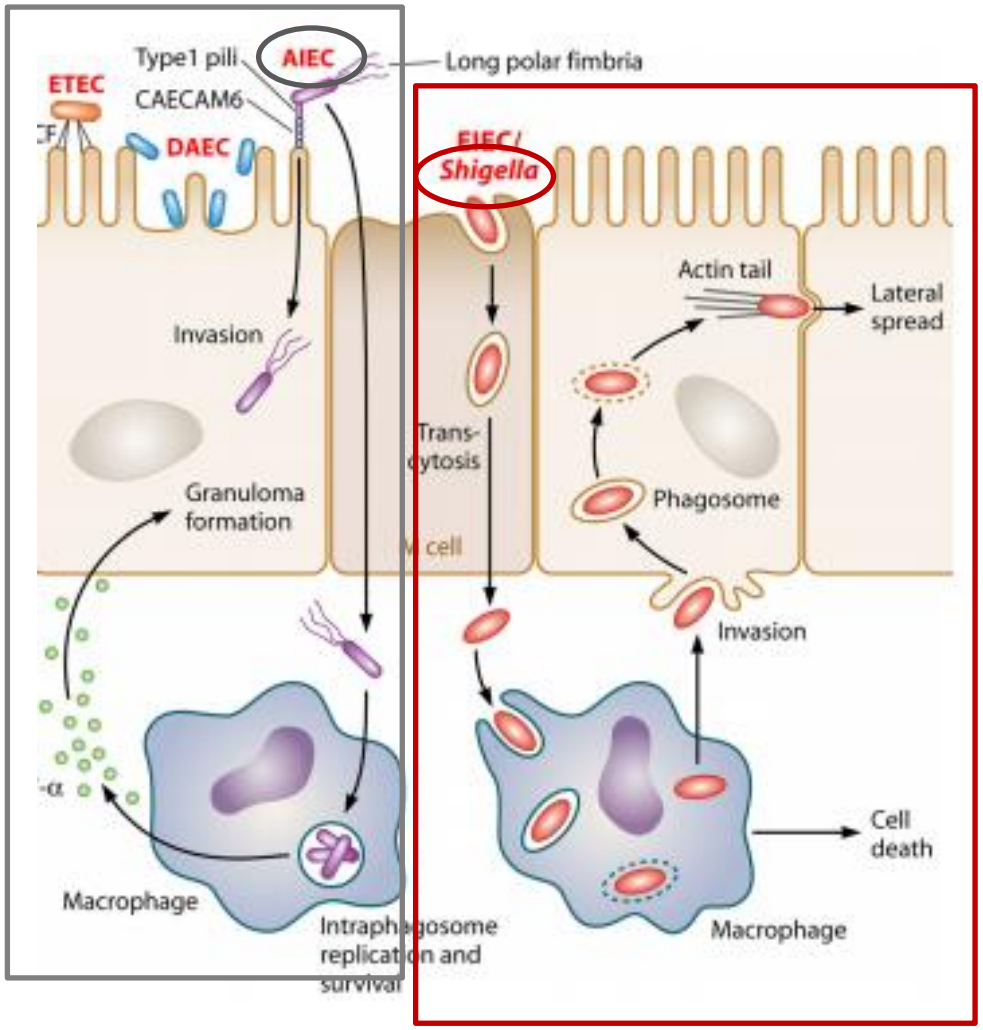
Adherent Invasive *Escherichia coli*



Shigella flexneri

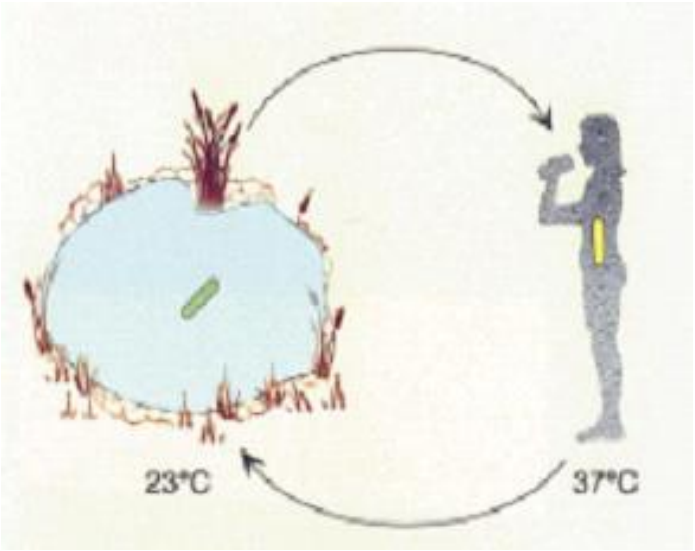
Role of multidrug efflux pumps during intracellular life of *Shigella flexneri*

Adherent Invasive *Escherichia coli*



Shigella flexneri

Shigella: main features

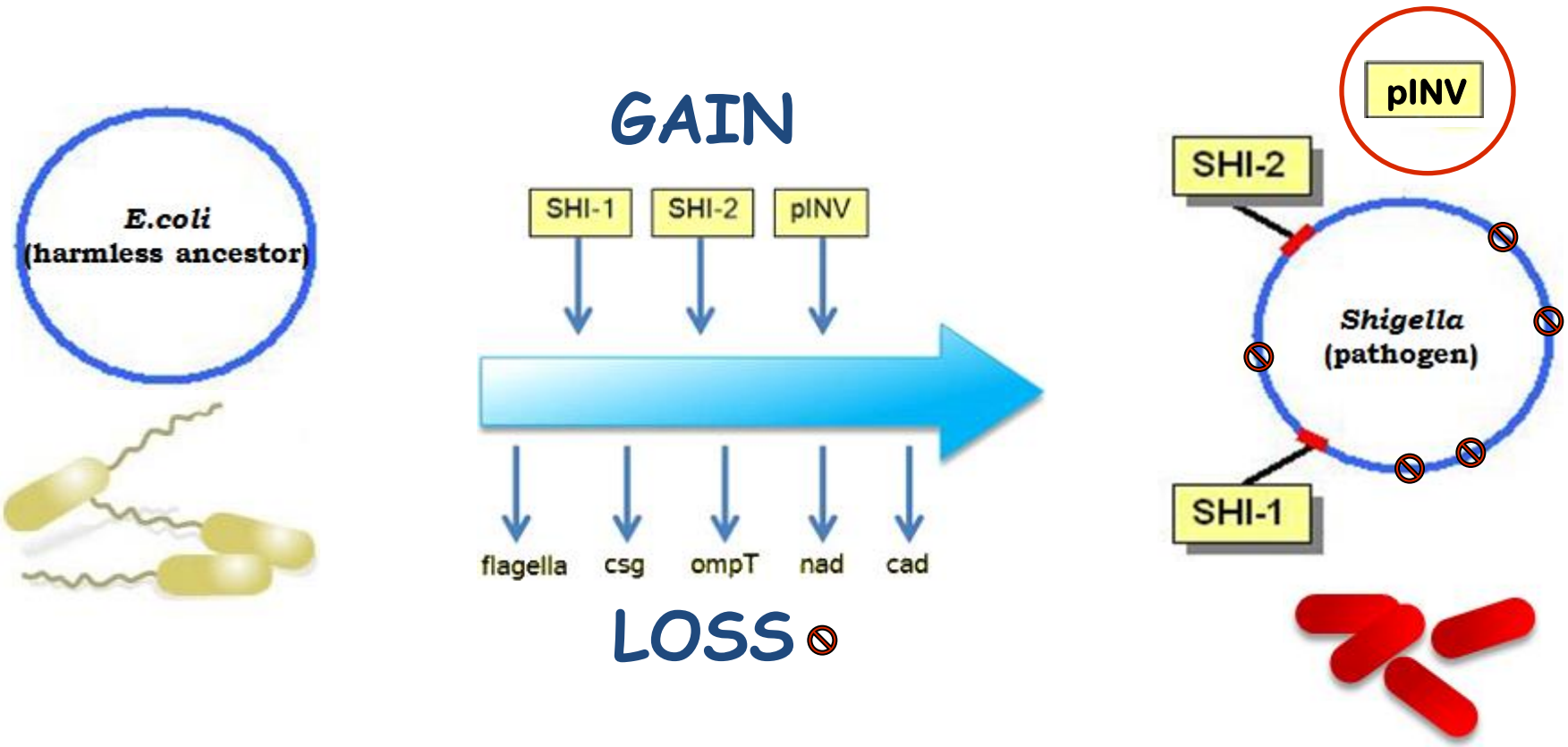


- Gram negative, facultative intracellular pathogen
- pathogen responsible for human dissentery, a highly infectious disease
- is able to survive in the outer environment and is acquired mainly from contaminated water
- *Shigella* shares high genome homology with its commensal ancestor *Escherichia coli*

Subgrouped into four "species":

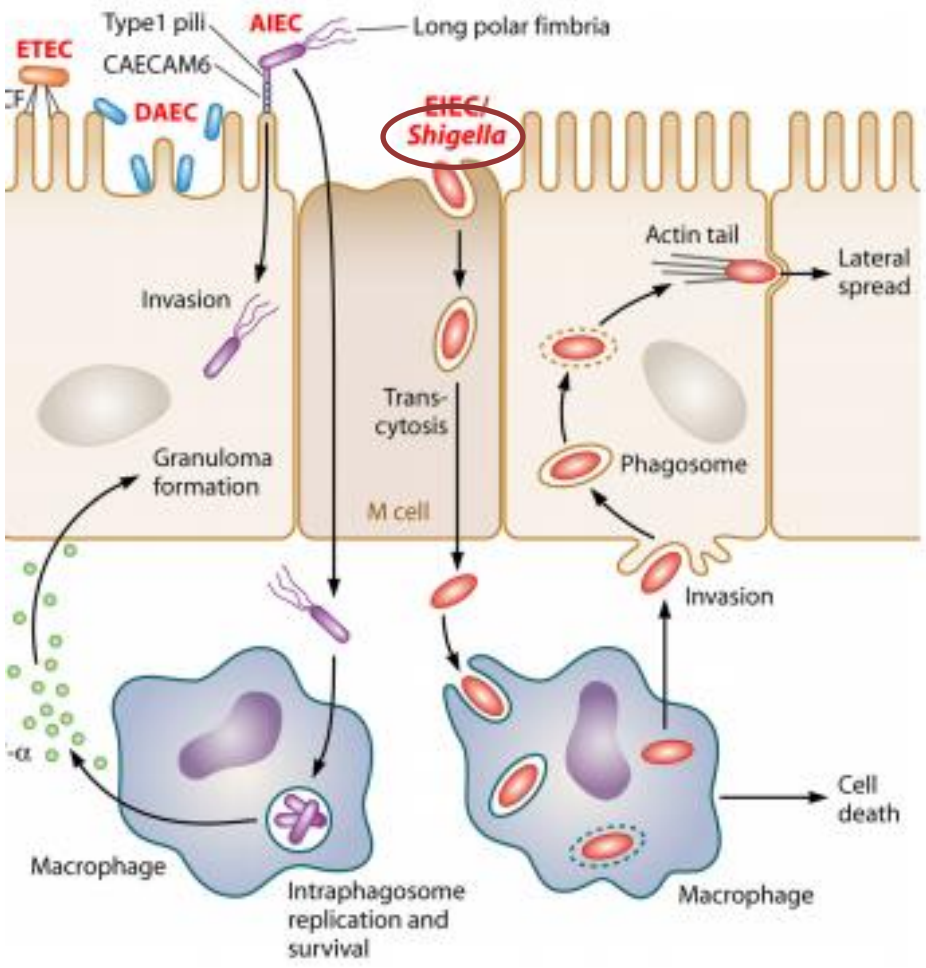
1. *Shigella flexneri*
2. *Shigella dysenteriae*
3. *Shigella boydii*
4. *Shigella sonnei*

The evolutionary pathway from *E. coli* to *Shigella*



The genomic reorganisation has enabled *Shigella* to trigger a virulence phenotype and survive in new niches within the human host

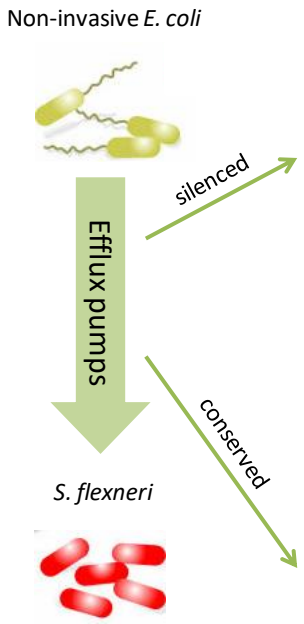
Shigella and the multi-step invasive process



Croxen *et al*., 2013

- invades **macrophages** and induces rapid cell death;
- Invade from the basolater side **enterocytes**, where intracellular replication and dissemination occurs;
- invasive program is **regulated** in response to environmental signals (pH, temperature, osmolarity, iron)

The efflux pumps conserved in the genome of *Shigella*

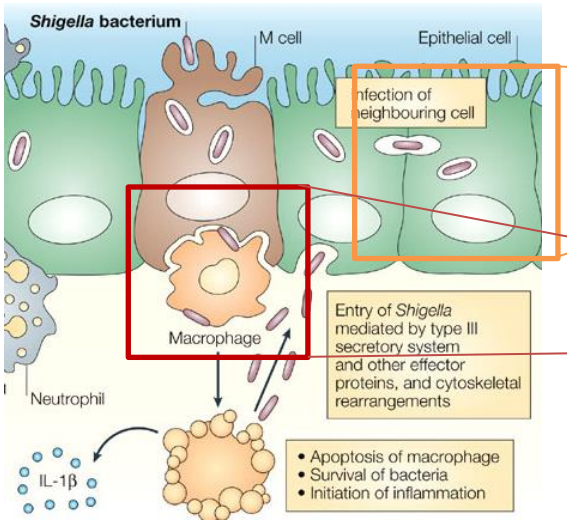


E. coli	*S. flexneri*
AcrEF	Not present
CusCFBA	IS element in *cusB* gene
MdtABCD	IS element upstream *mdtA* gene
MdtEF	*mdtE* gene disrupted
YceE	Not present
YjiO	Not present

E. coli	*S. flexneri*
AcrAB	AcrAB
AcrD	AcrD
Bcr	Bcr
EmrAB	EmrAB
EmrD	EmrD
EmrE	EmrE
EmrKY	EmrKY
Fsr	Fsr
MacAB	MacAB
MdfA	Cmr
MdtH	YceL
MdtJI (YdgFE)	MdtJI
MdtK	YdhE
MdtL	YidY

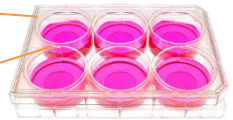
If the genomic reorganization through a virulence phenotype spared 14 efflux pump encoding operons, could these be important for survival in the host?

How to monitor the differential expression of efflux pumps during *Shigella* infection

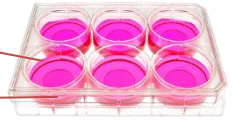


Sansonetti *et al.*, 2004

S. flexneri infection of:



epithelial cells (Caco-2)

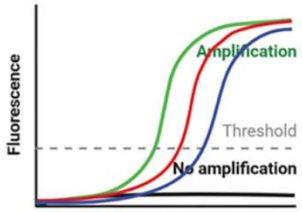


macrophage (U937)

RNA extraction from intracellular bacteria

qRT-PCR analysis

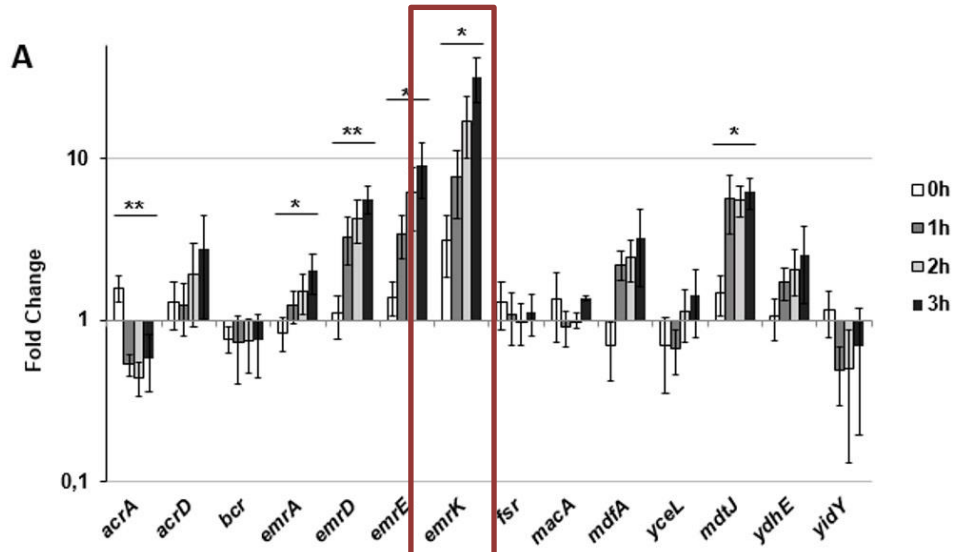
Real-time RT-PCR



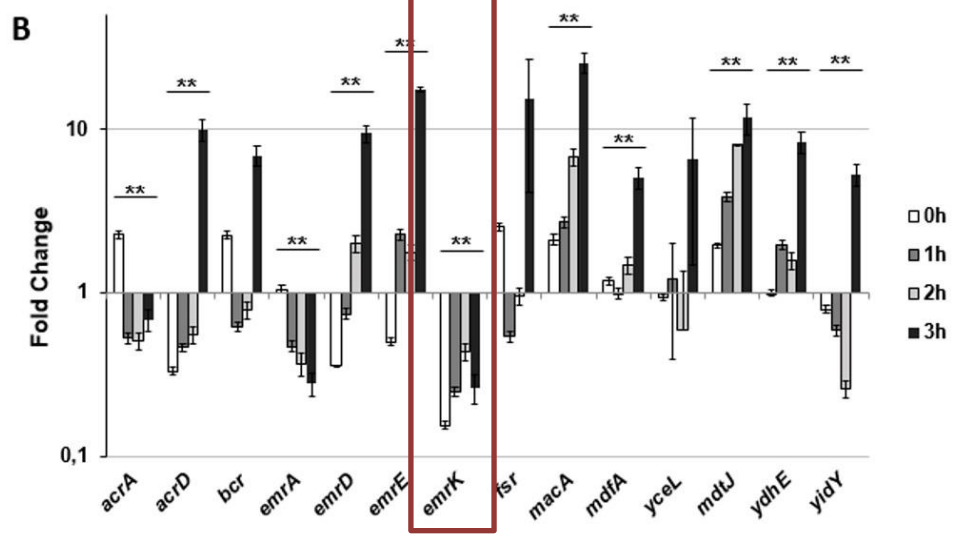
Amplification curve

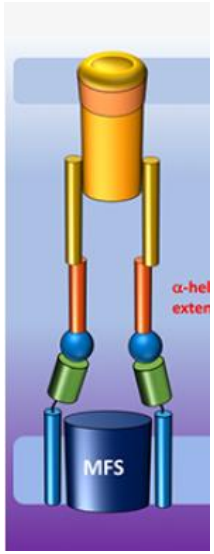
Differential expression of efflux pumps during the infection of macrophages and epithelial cells

macrophages



Caco-2 cells

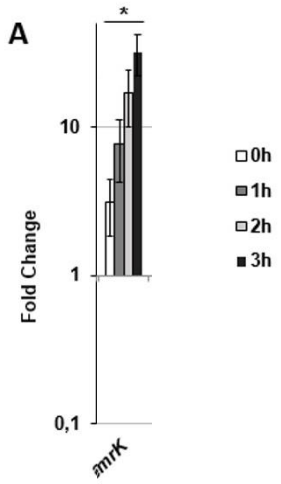




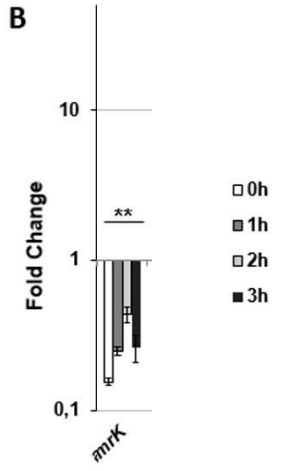
Peculiar expression profile of ***emrKY***: might it be a promising candidate?

Peculiar expression profile of *emrKY*: might it be a promising candidate?

macrophages

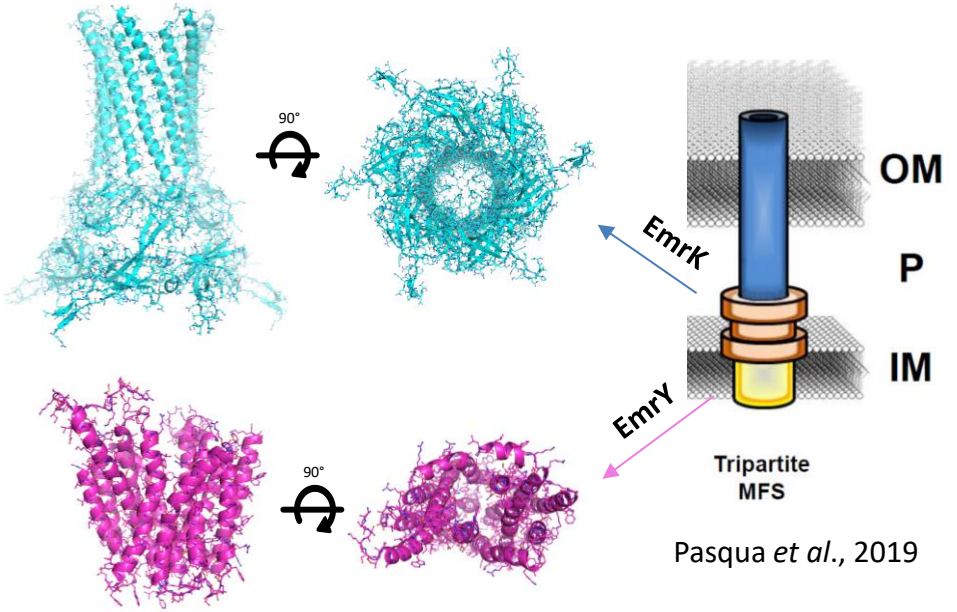


Caco-2 cells



EmrK
periplasmic adaptor protein

EmrY
inner membrane protein

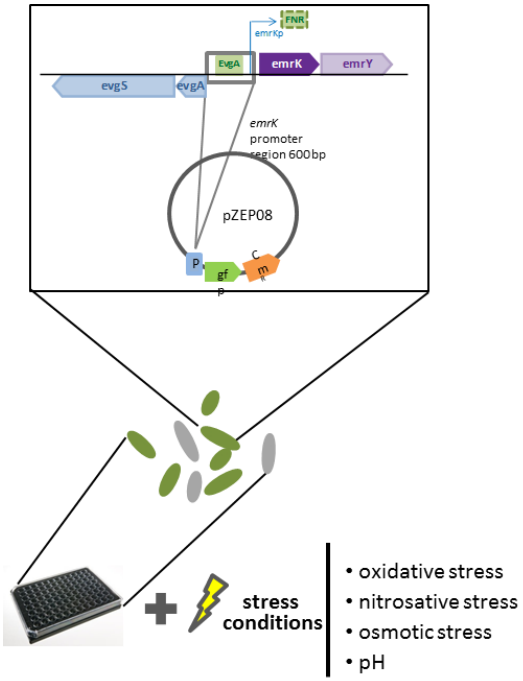


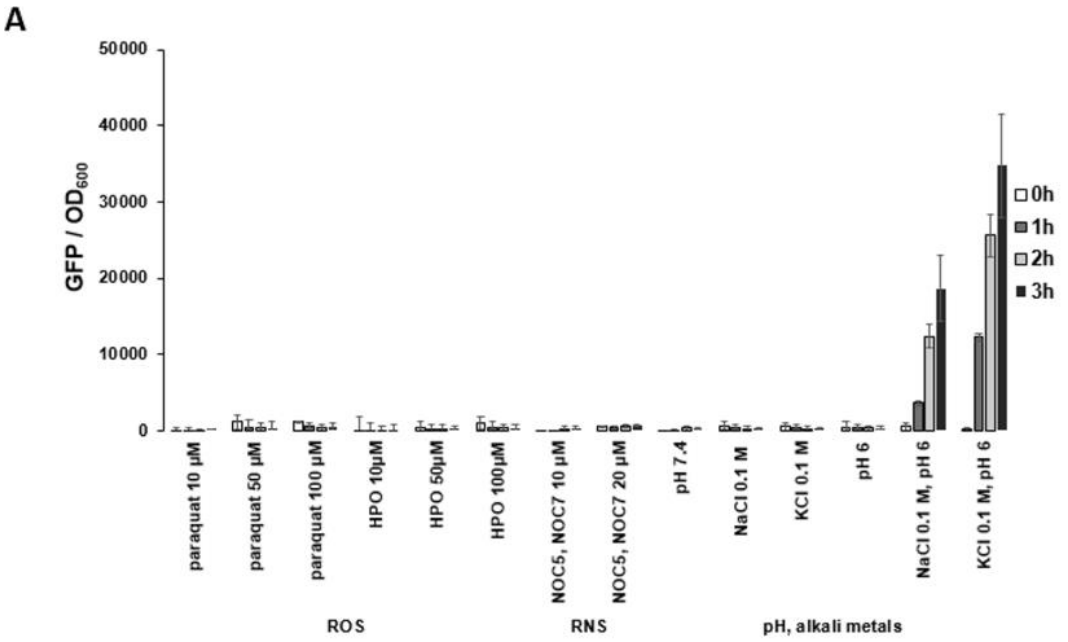
Pasqua *et al.*, 2019

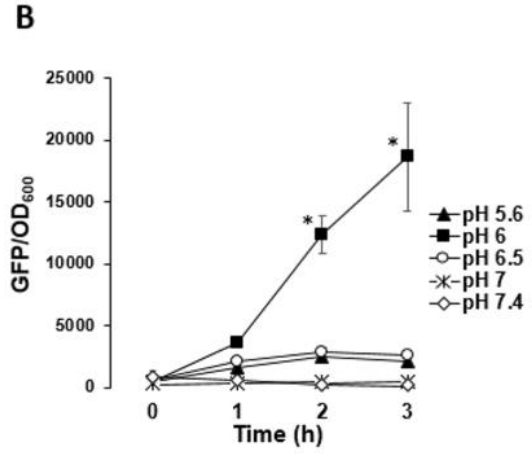
- cell specific expression of ***emrK***
- Weak expression in laboratory conditions
- EmrKY belongs to the Major Facilitator Superfamily (**MFS**)
- Its role is associated with drug resistance in *E. coli* (**MDR**)

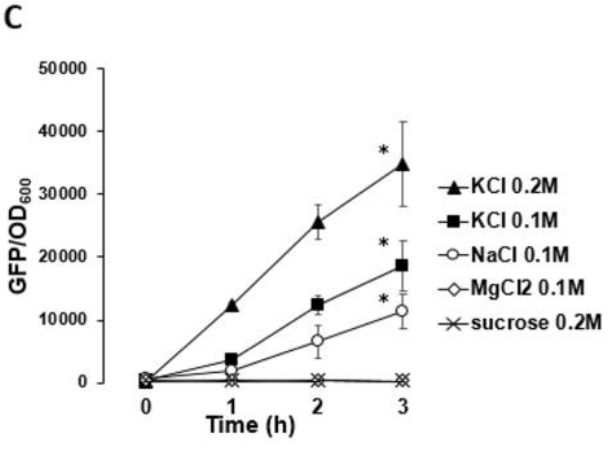
emrKY is notably induced by KCl and pH6

In which conditions could EmrKY be expressed outside the macrophage environment?







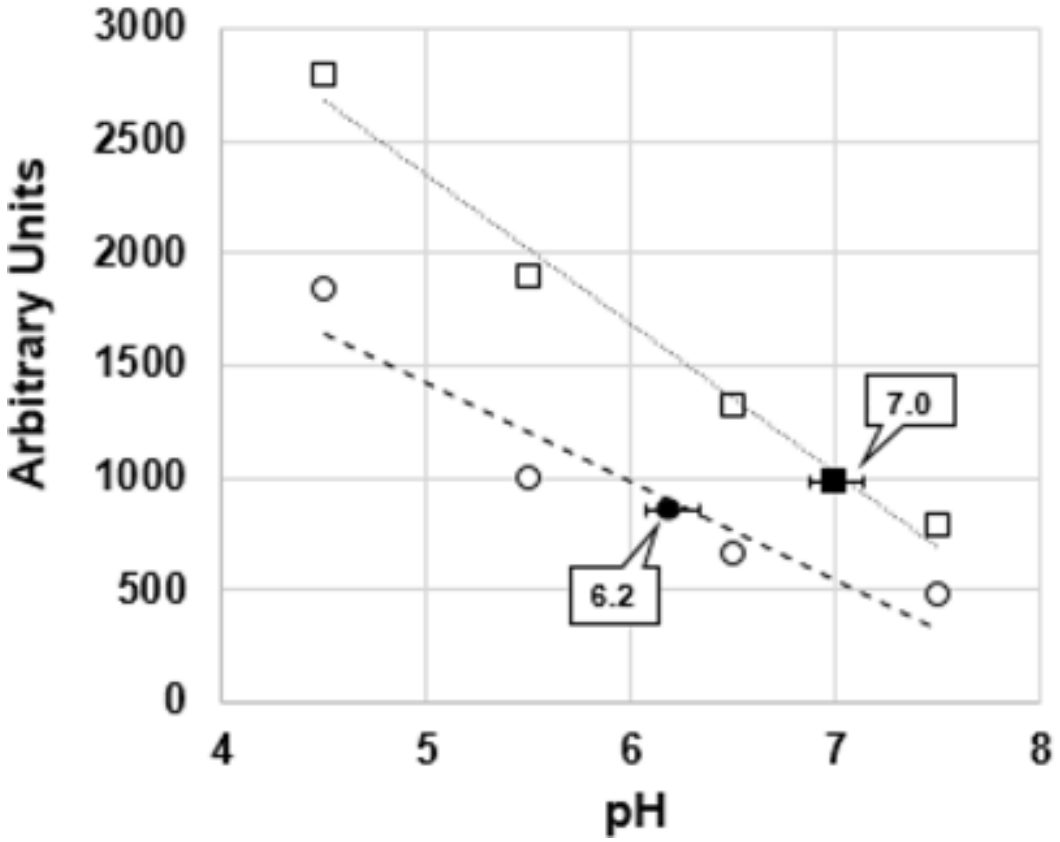


In *Shigella*, **pH6** and high concentration of **alkali metals** are a specific couple of signals that induces expression of *emrK*

Shigella induces mild acidic pH in macrophage cytosol

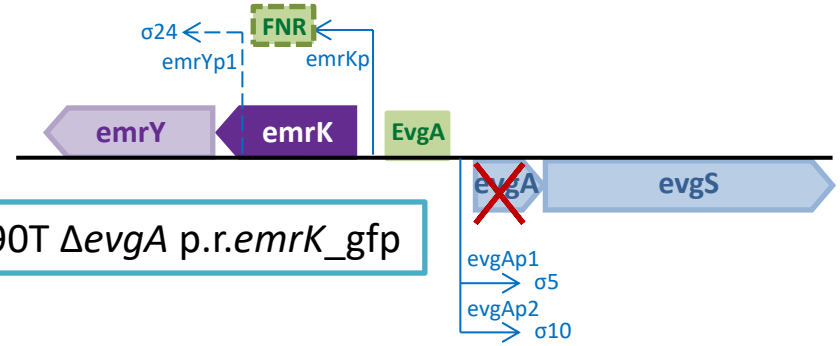
Intracellular pH measurement at 3 hours post infection reveals that:

- pH value of macrophages not infected is ≈ 7;
- pH value of macrophages infected by *Shigella* is ≈ 6.

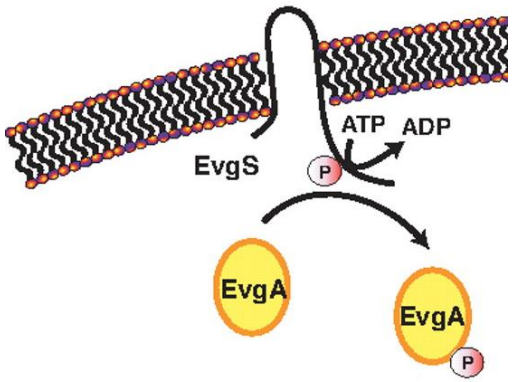


A moderate low pH, together with the presence of K+, are signals that *Shigella* encounters within macrophages

EvgA is responsible for the *emrK* induction in presence of KCl and pH6 and…



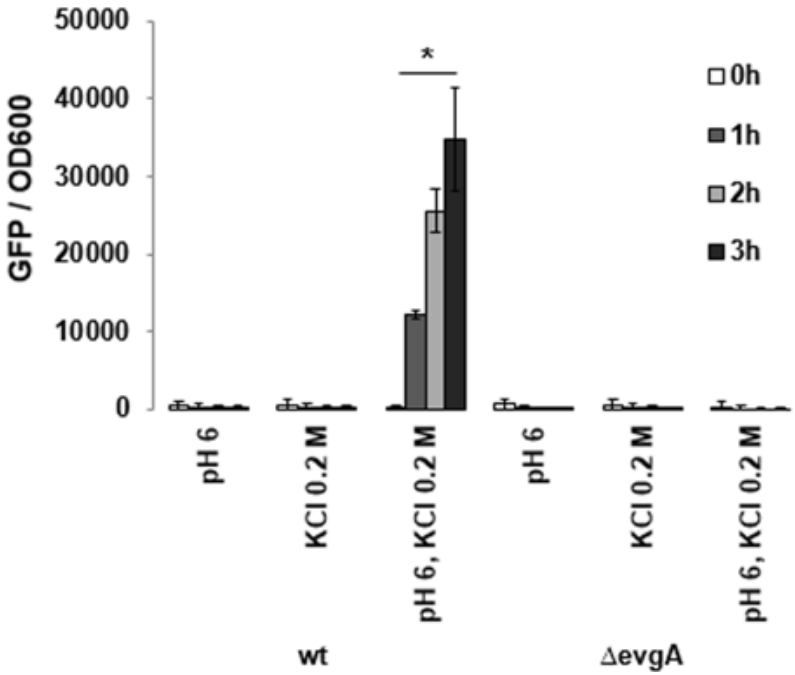
M90T Δ*evgA* p.r.*emrK*_gfp



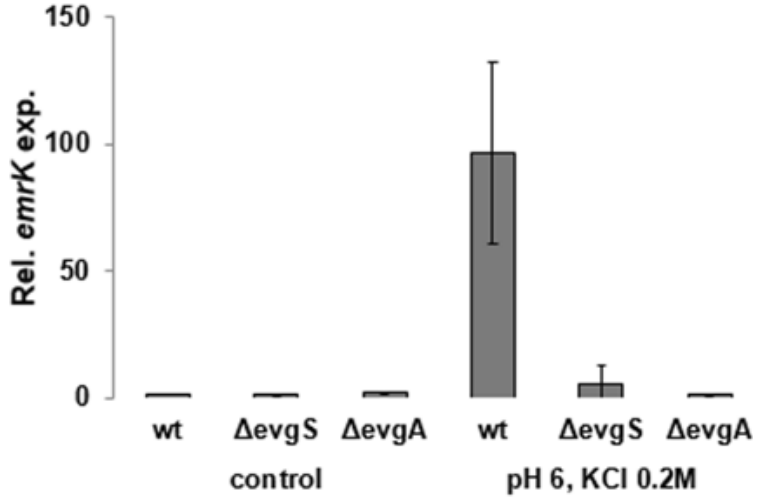
Modified from Eguchi *et al*., 2007

The two-component system **EvgAS** regulates *emrKY* expression in presence of KCl and pH6

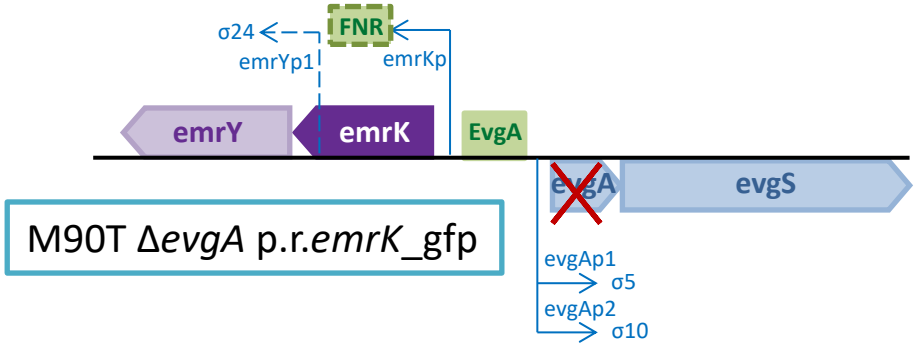
GFP monitoring



qRT-PCR



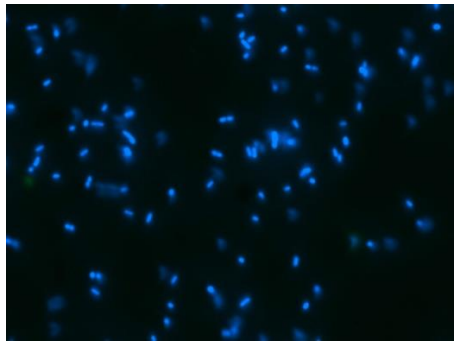
…within macrophages

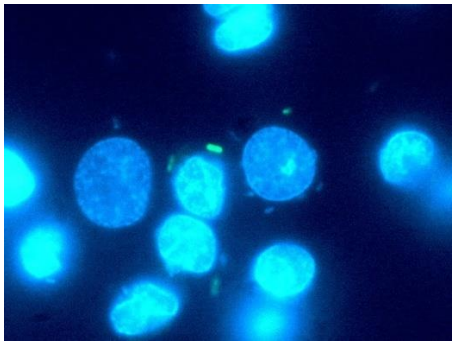


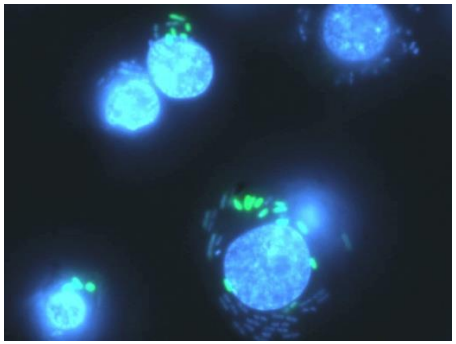
M90T Δ*evgA* p.r.*emrK*_gfp

EvgA promotes the induction of *emrK* during the infection of macrophages

M90T p.r.*emrK*_gfp

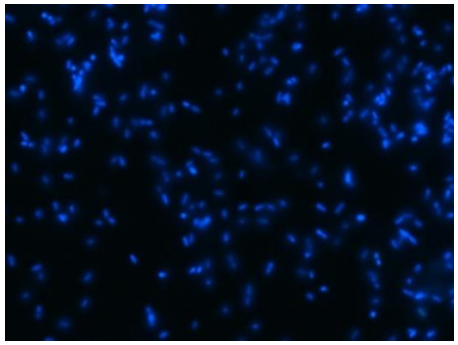


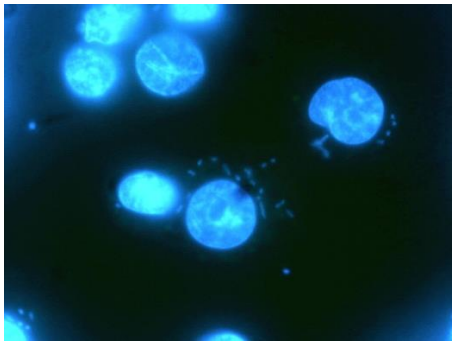


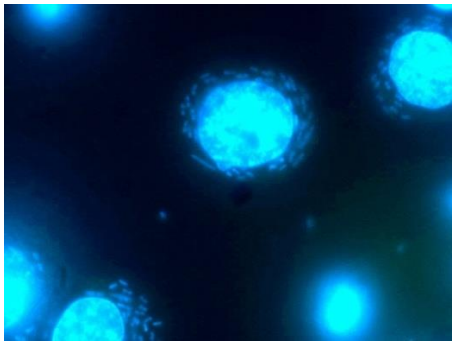


RPMI — 0h — 3h

M90T Δ*evgA* p.r.*emrK*_gfp

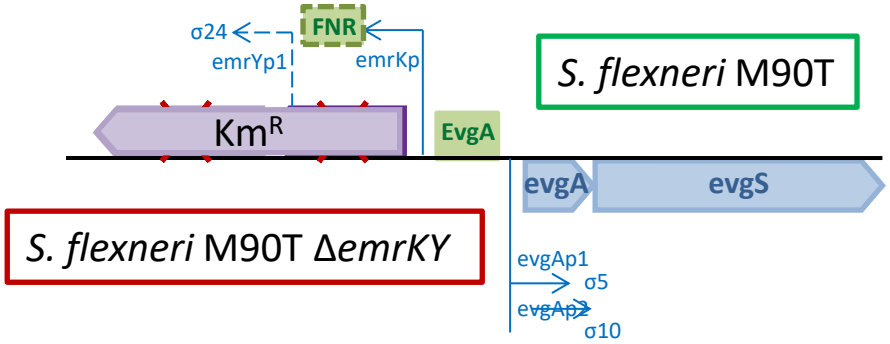




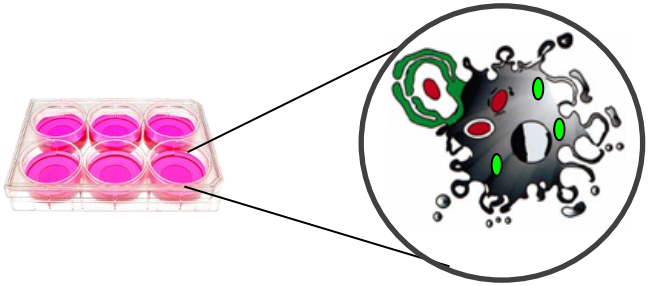


Does the EmrKY EP provide an advantage to *Shigella* survival within macrophages? Experiment workflow

Costruction of *S. flexneri* M90T mutant by deletion of *emrKY* operon



Macrophage (U937) co-infection with mutant strain (Km^R) mixed to wild type strain



Lysis of cells infected and release of intracellular bacteria

Evaluation of intracellular survival of mutant strain vs wild type strain (CFU/ml)

plating of intracellular bacteria on LB agar plates

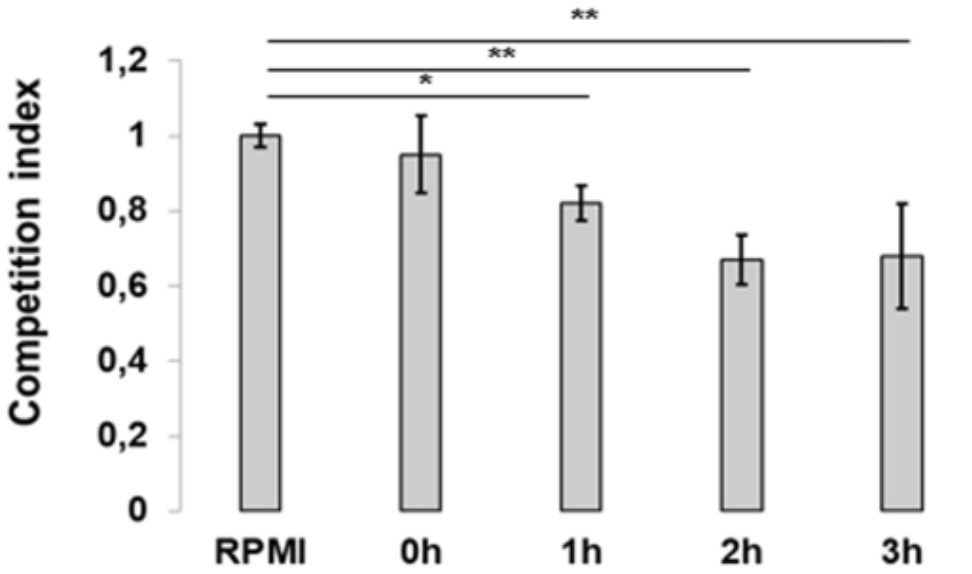
replica plating on LB agar and LB agar Km plates

$$\text{C.I.} = \frac{\text{M90T } \Delta emrKY}{\text{M90T}}$$

Competition Index

Does the EmrKY EP provide an advantage to *Shigella* survival within macrophages?

$$\text{C.I.}_{\text{Competition Index}} = \frac{\text{M90T } \Delta emrKY}{\text{M90T}}$$



Pasqua *et al.*, 2019

EmrKY is important for *Shigella* fitness whitin the host

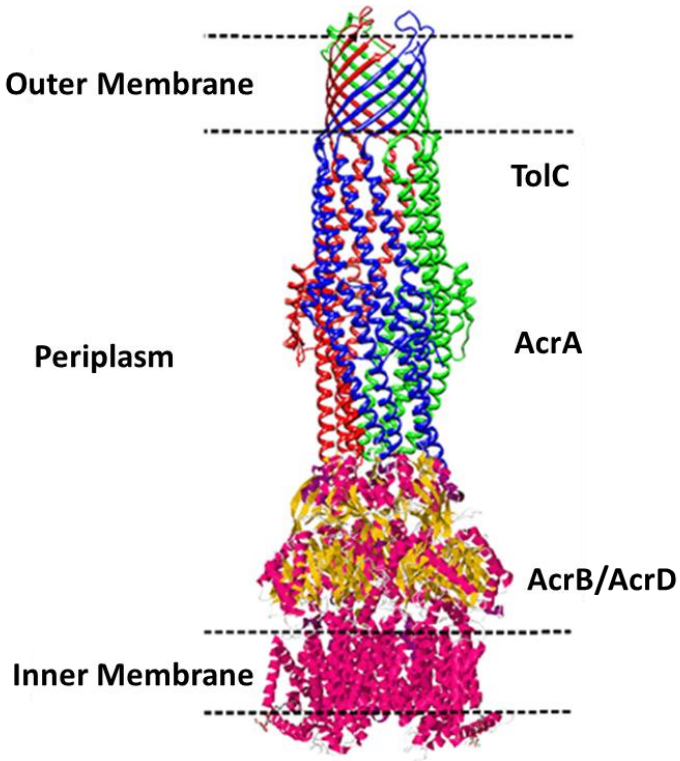
MDR Efflux Pumps, more than antibiotic resistance: the case of AcrAB

AcrAB is one of the most relevant MDR EPs:

- Wide substrate profile
- High abundance
- Contribution to virulence in various pathogens

AcrD is a homologue of AcrB and needs AcrA to make a functional efflux pump.

Work by Nickerson et al., 2017 shows that AcrAB is required in *Shigella* for biofilm formation and is involved in resistance to bile salts.



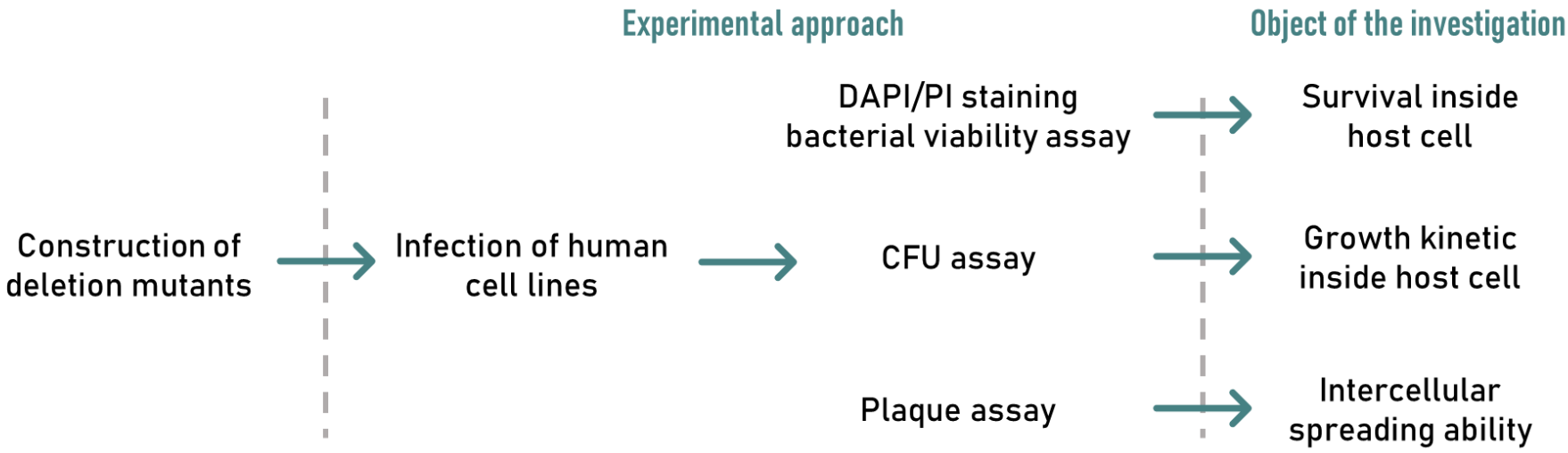
Puzari and Chetia, 2017

Genes on *Shigella* chromosome

Aim of this work:

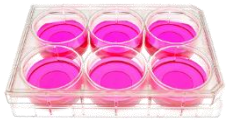
Investigate the role of AcrAB and AcrD on the *Shigella* pathogenesis. Monitor how the loss of the pumps affects the capability of *Shigella* to invade and survive inside macrophages and epithelial cells.

Our approach:



Lack of AcrAB affects *Shigella* survival inside epithelial cells

The contribution of AcrAB to *Shigella* survival inside macrophages and epithelial cells is different, as we measured through DAPI/PI double staining of intracellular bacteria recovered at different time points during infection.



Shigella infection of epithelial cells up to 4h

Recovery of intracellular bacteria after cell lysis

Staining with DAPI / PI (Propidium Iodide)

- DAPI → entire population
- PI → dead cells

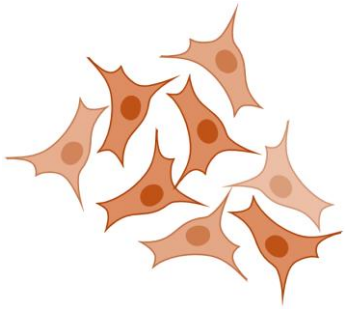
Analysis of flurescence

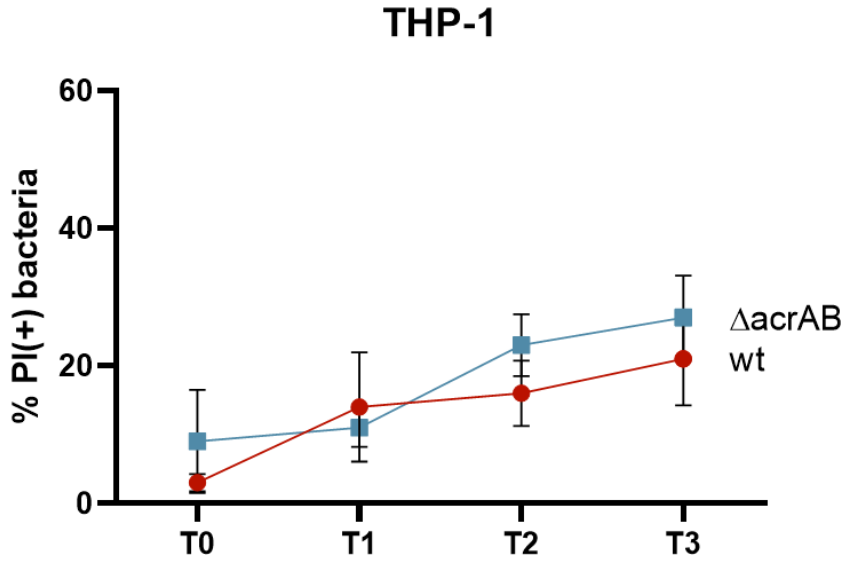
% of PI positive bacteria for each time of infection

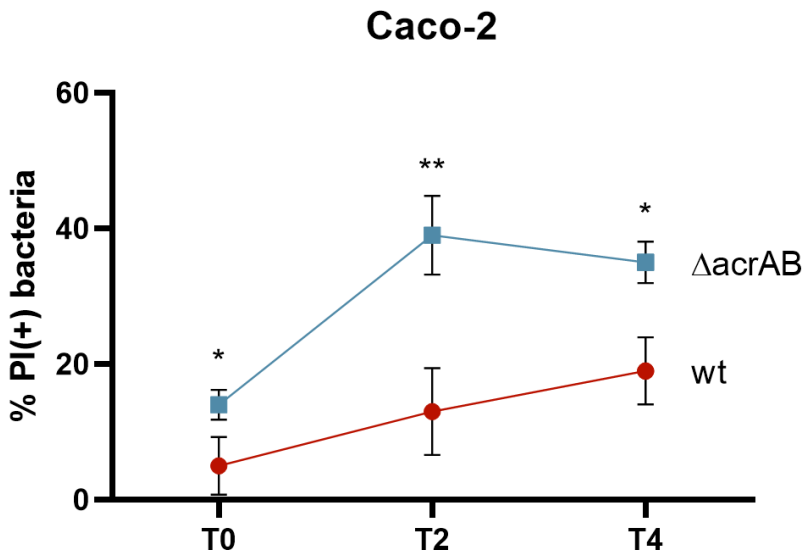
Lack of AcrAB affects

Shigella survival inside epithelial cells

The contribution of AcrAB to *Shigella* survival inside macrophages and epithelial cells is different, as we measured through DAPI/PI double staining of intracellular bacteria recovered at different time points during infection.

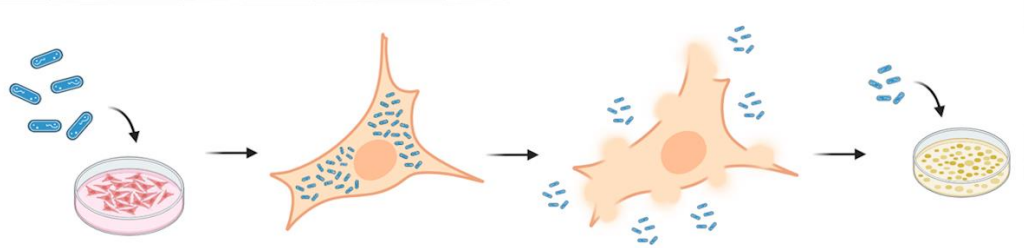




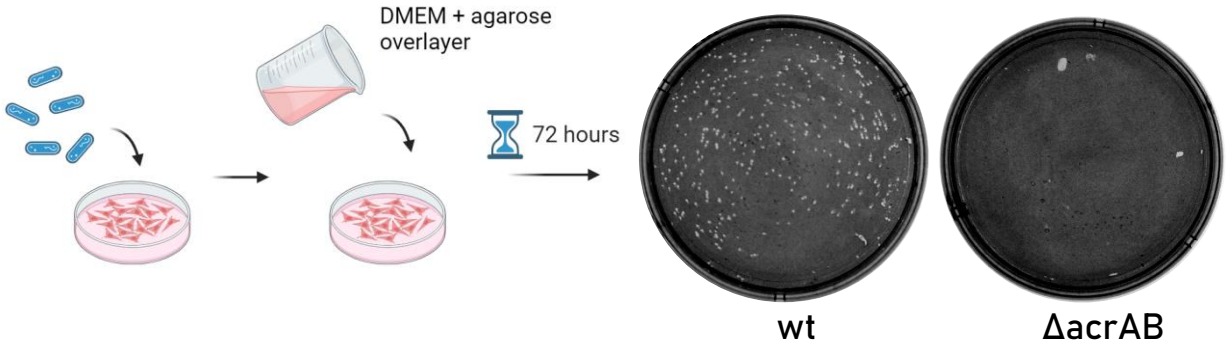


Focus on *Shigella* ability to successfully invade epithelial cells without the AcrAB pump.

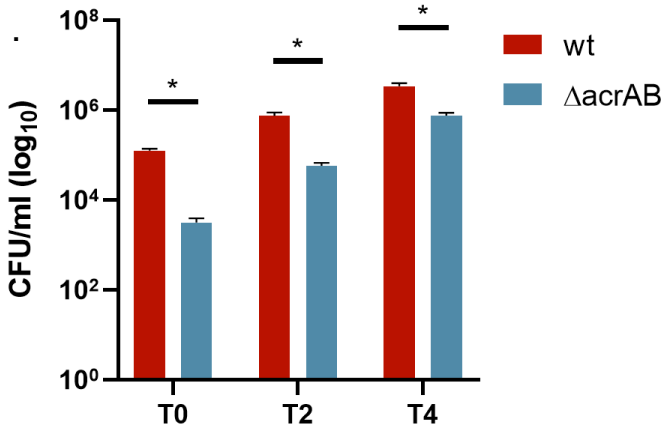
A. CFU assay. Δ*acrAB* survive inside the epithelial cells, but the ones who survive exhibit a growth kinetic like the parental wt strain.



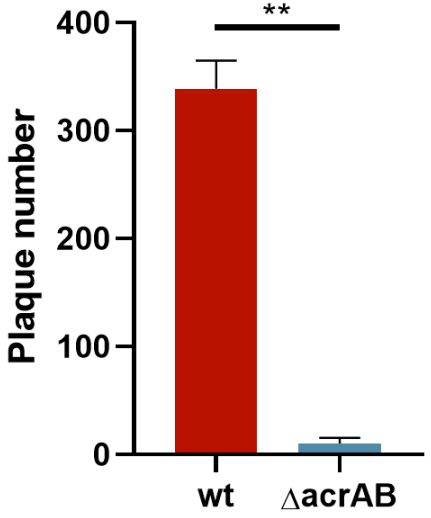
B. Plaque assay. The mutants mostly failed to form plaques.



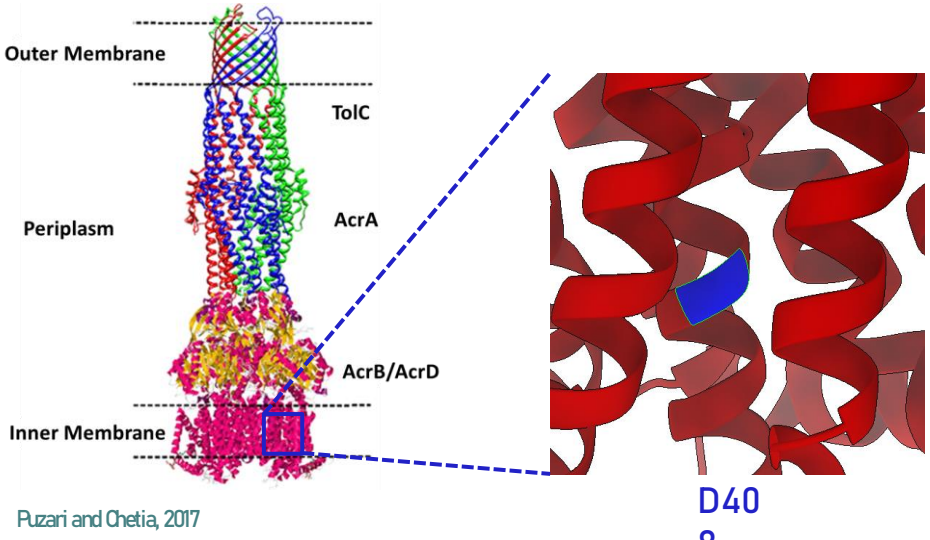
A.



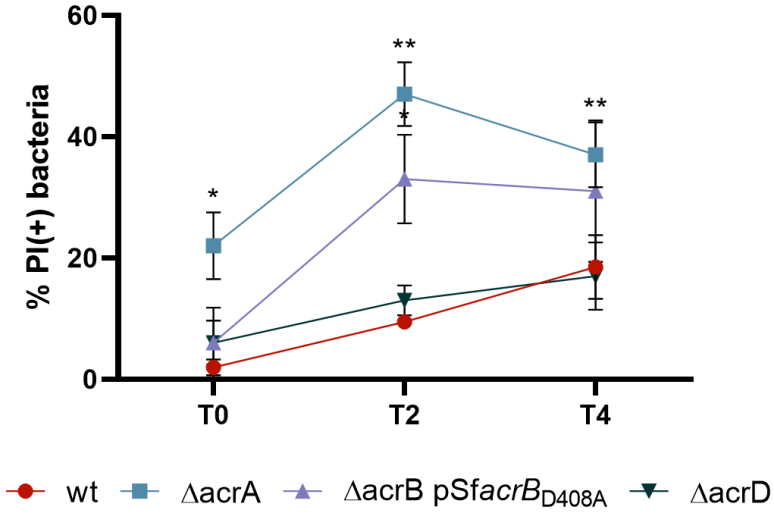
B.



AcrA and AcrB contribute to intracellular viability



Puzari and Chetia, 2017



Each component of the MDR EP has specific functions. We investigated their role in intracellular viability of *Shigella*.

Parallel epithelial cells infection was carried out with single mutants lacking *acrA*, *acrB* or *acrD*. DAPI/PI staining of intracellular bacteria at different time points.

AcrA and AcrB play a main role in the infection, but the role of AcrD is dispensable.

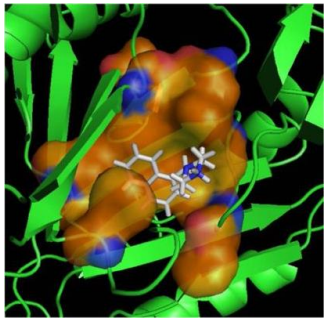
Inhibition of EPs activity

New strategies in the post-antibiotic era

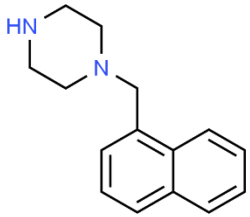
NMP belongs to the family of arylpiperazines and is a specific inhibitor of AcrB by acting as its substrate.

100 µM NMP added to the bacteria just before the infection.

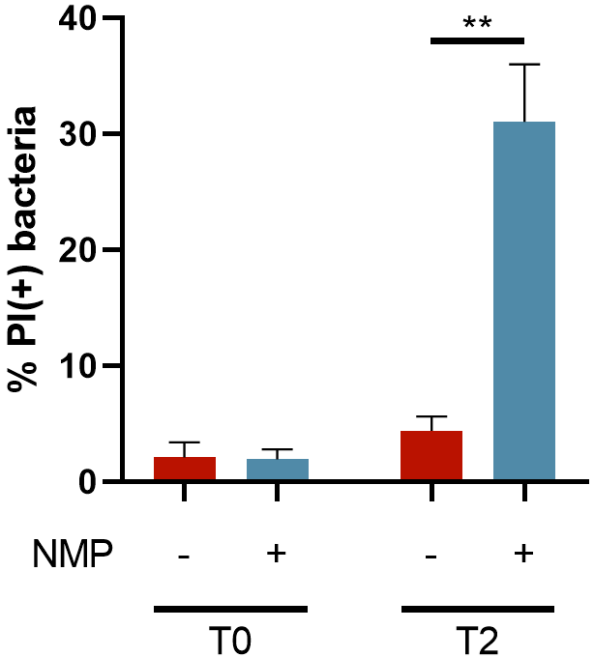
Shigella wt phenotype after NMP treatment is like the ΔacrB mutant phenotype.



Li et al., 2015

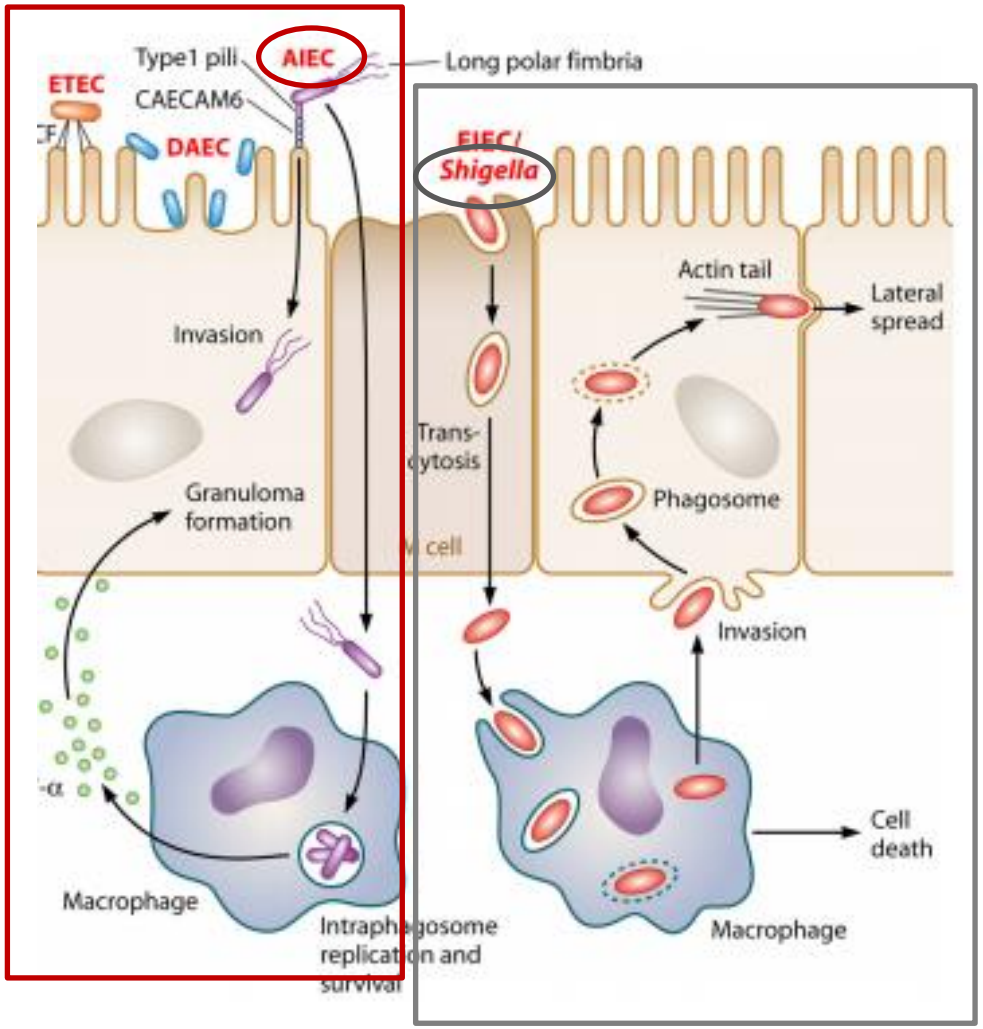


1-(1-naphthyl-methyl)-piperazine (NMP)



Role of multidrug efflux pumps during intracellular life of AIEC

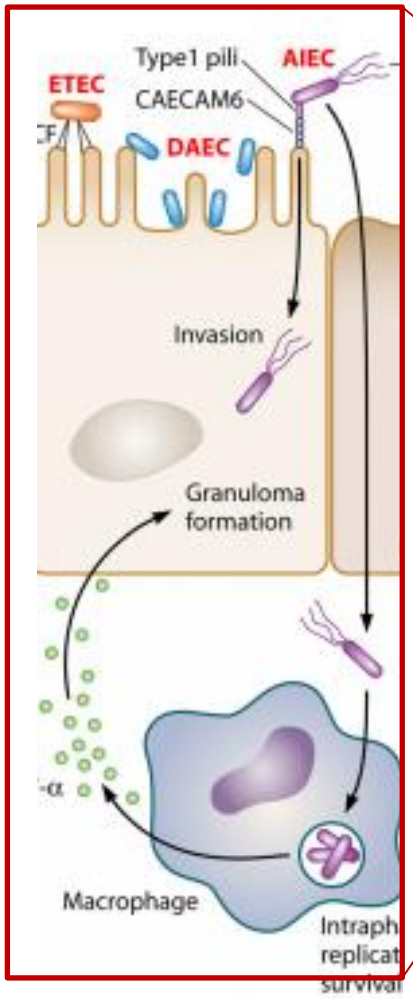
Adherent Invasive *Escherichia coli*

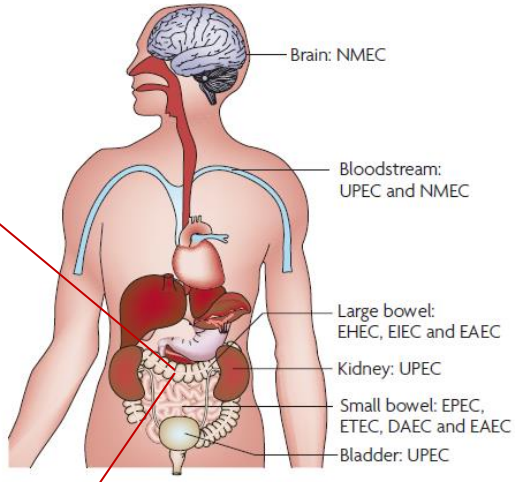


Shigella flexneri

Role of multidrug efflux pumps during intracellular life of AIEC

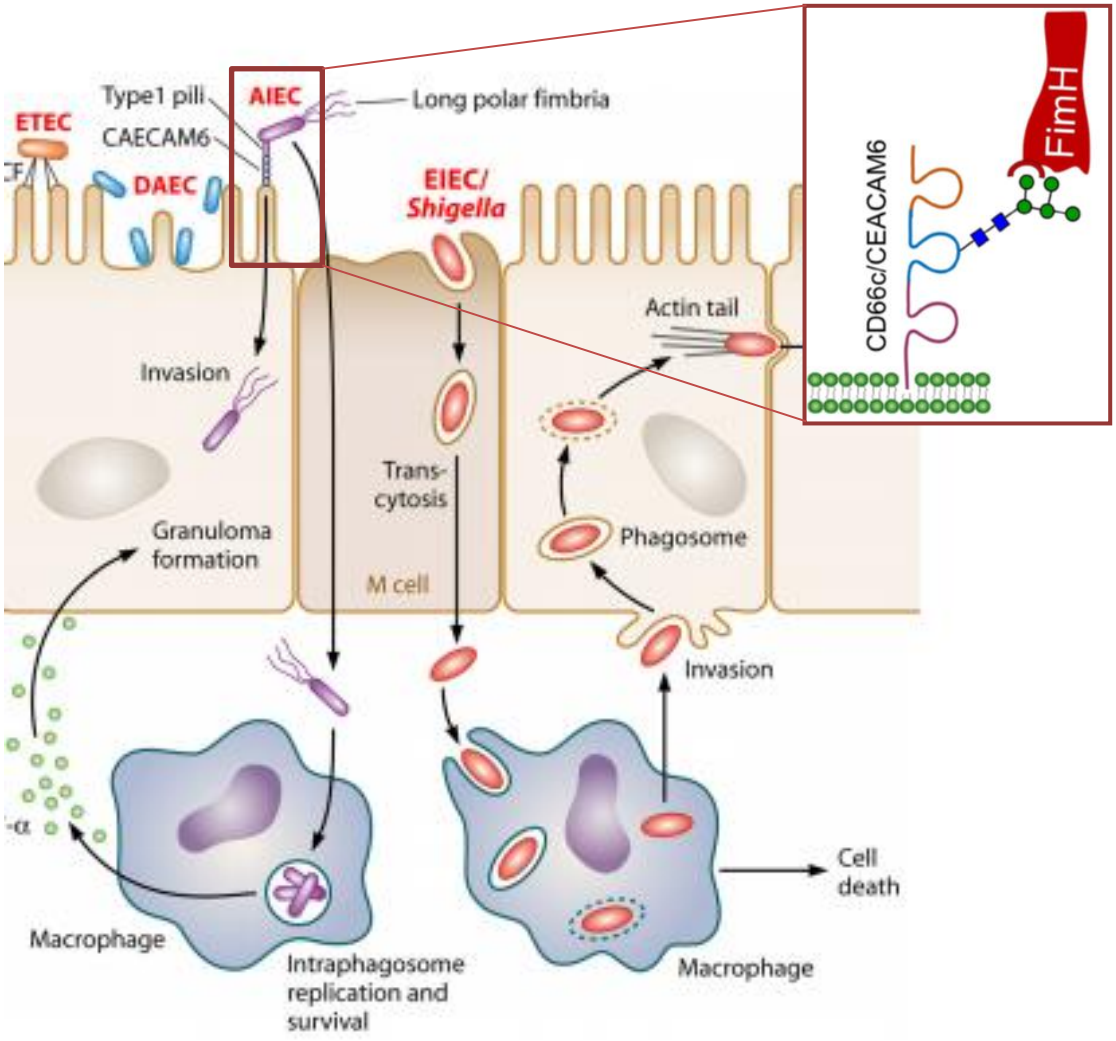
Adherent Invasive *Escherichia coli*





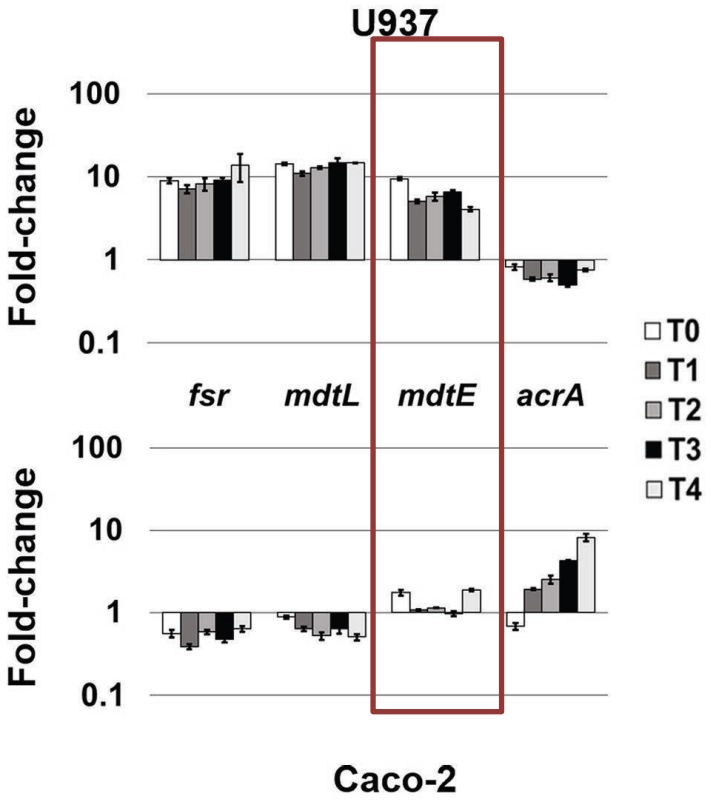
- Belong to the diarrhoeagenic *E. coli*
- Gram-negative intracellular pathogens
- Associated with Crohn's disease (CD)
- Reference strain LF82

Main characteristics of AIEC infection process



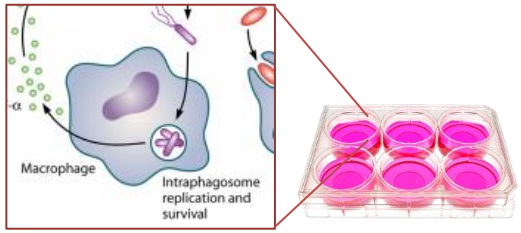
- AIEC use **type I pili** with oligomannose-specific lectin FimH at its tips to bind the CEACAM6 receptor
- AIEC persist and multiply intracellularly in **epithelial cells** in late endosomes
- AIEC survive and replicate inside maturing phagolysosomes in **macrophage** without induce cell death

MDR EPs specifically respond to different cellular environment



Modulation of *fsr*, *mdtL*, *mdtEF*, and *acrA* EP genes appears to be driven by specific cell environment → we focus our attention on ***mdtEF***, highly expressed in LF82 infecting macrophages.

Does MdtEF contribute to AIEC survival inside macrophages? Experiment workflow



LF82 infection of macrophages up to 5h

Recovery of intracellular bacteria after macrophage lysis

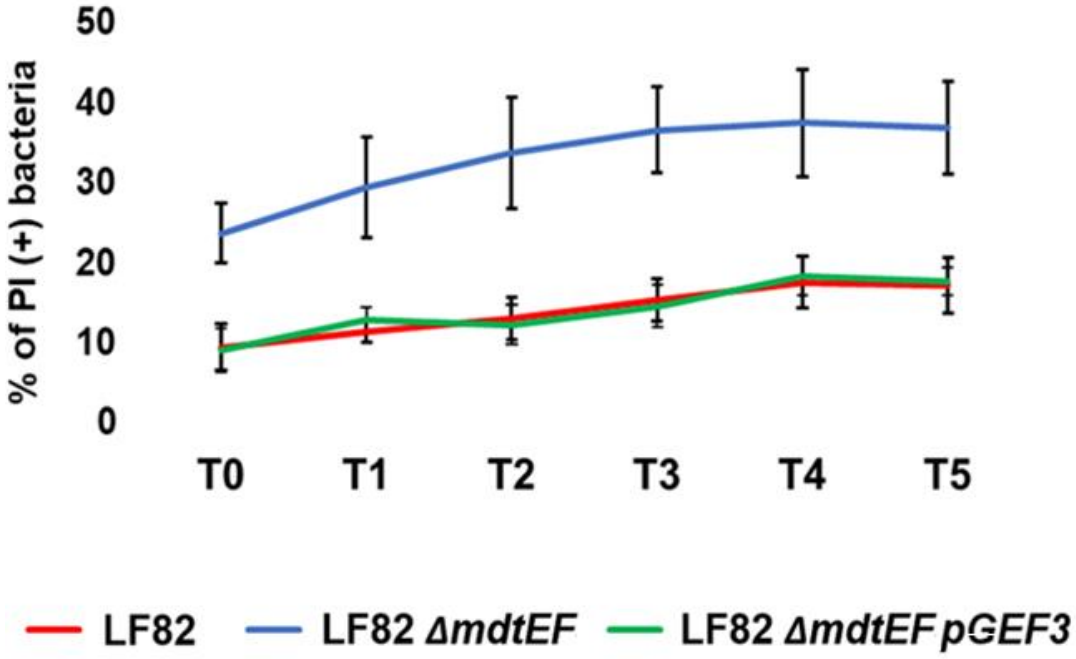
Staining with DAPI / PI (Propidium Iodide)

- DAPI → entire population
- PI (Propidium Iodide) → dead cells

Analysis of flurescence

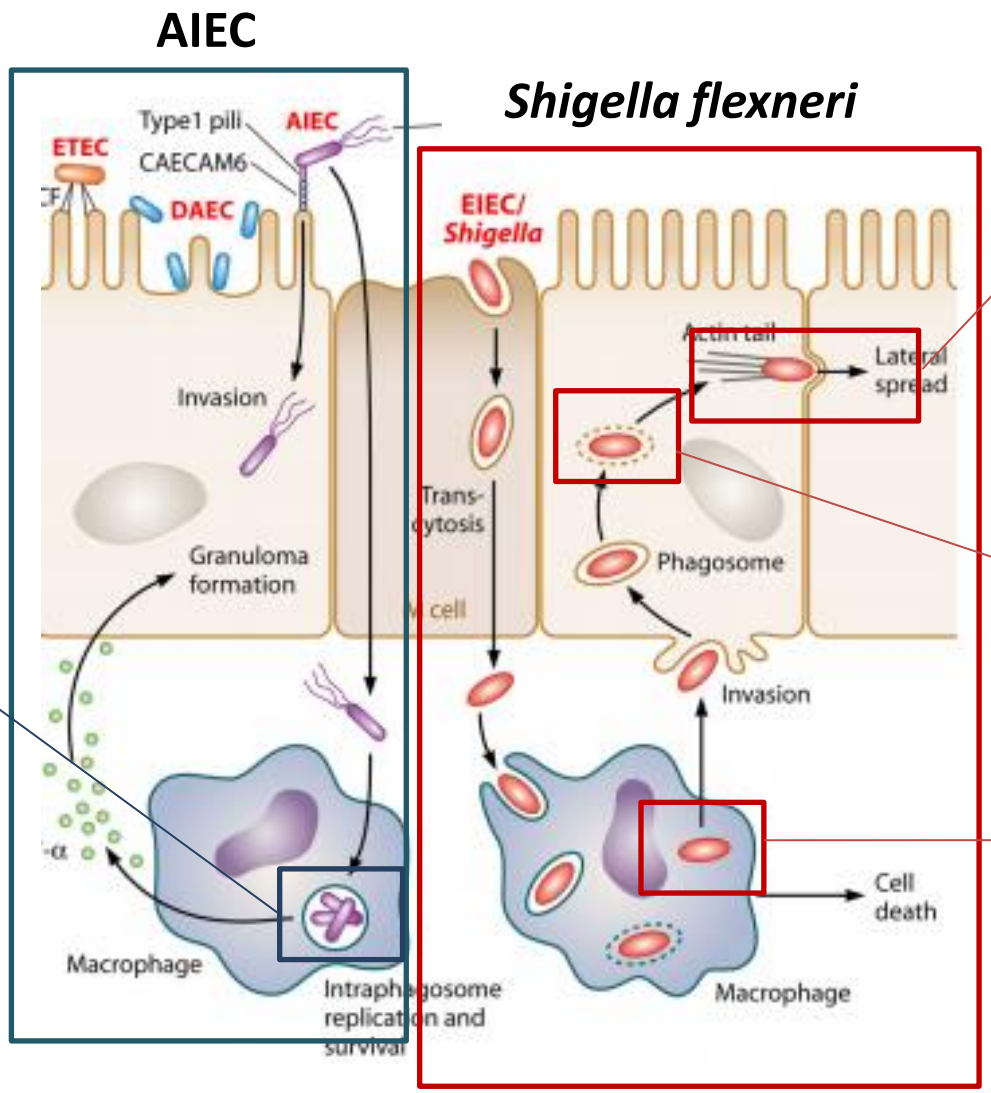
% of PI positive bacteria for each time of infection

Does MdtEF contribute to AIEC survival inside macrophages?



Deletion of *mdtEF* genes significantly impairs survival of LF82 in macrophages

Efflux pumps in the *Shigella*/AIEC – host interaction



Survival in macrophages:
- MdtEF
- AcrAB

Intercellular spreading:
- AcrAB

Survival in epithelial cells:
- AcrAB

Survival in macrophages:
- EmrKY