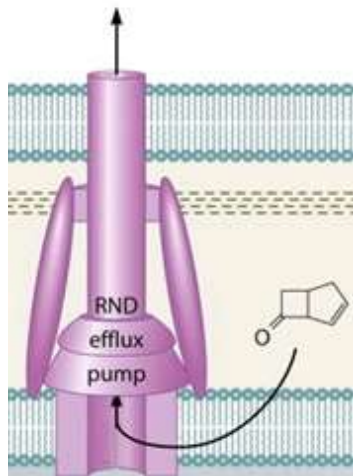


Impact of **efflux mechanisms** on susceptibility and resistance to
beta-lactam antibiotics in *Pseudomonas aeruginosa*:
beyond the usual concepts



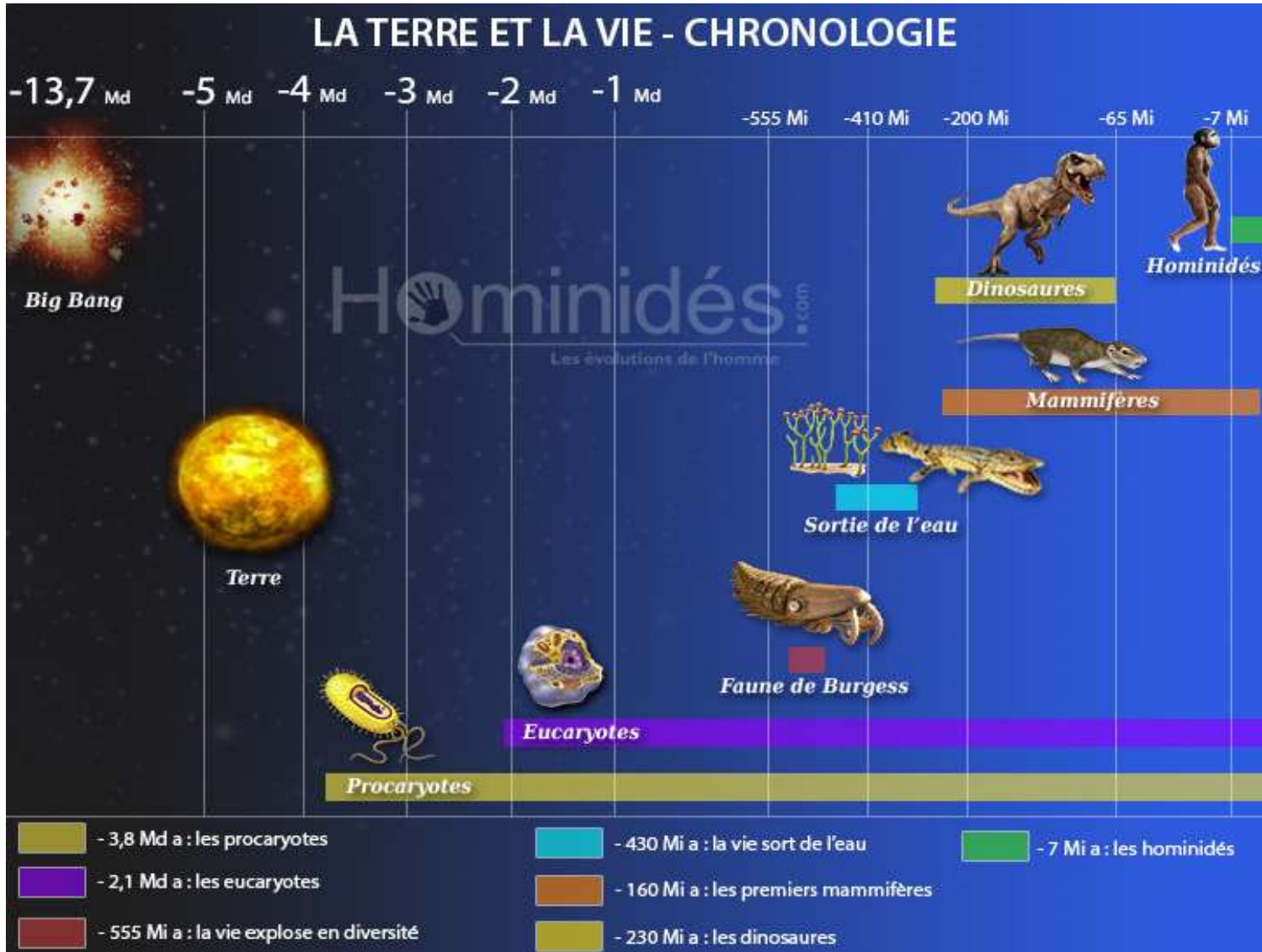
Houssein Abdel Aziz Chalhoub

Promoter: Prof. Françoise Van Bambeke

Cellular and Molecular Pharmacology
Louvain Drug Research Institute
**Université catholique de Louvain, Brussels,
Belgium**

22 December 2016

Bacteria & Humans



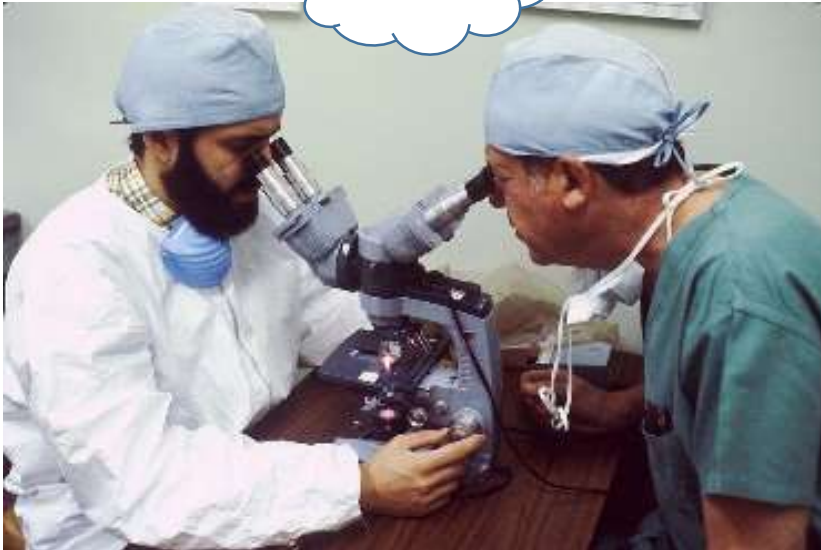
Bacteria: spread widely in the environment...



Bacteria: spread widely in the environment...



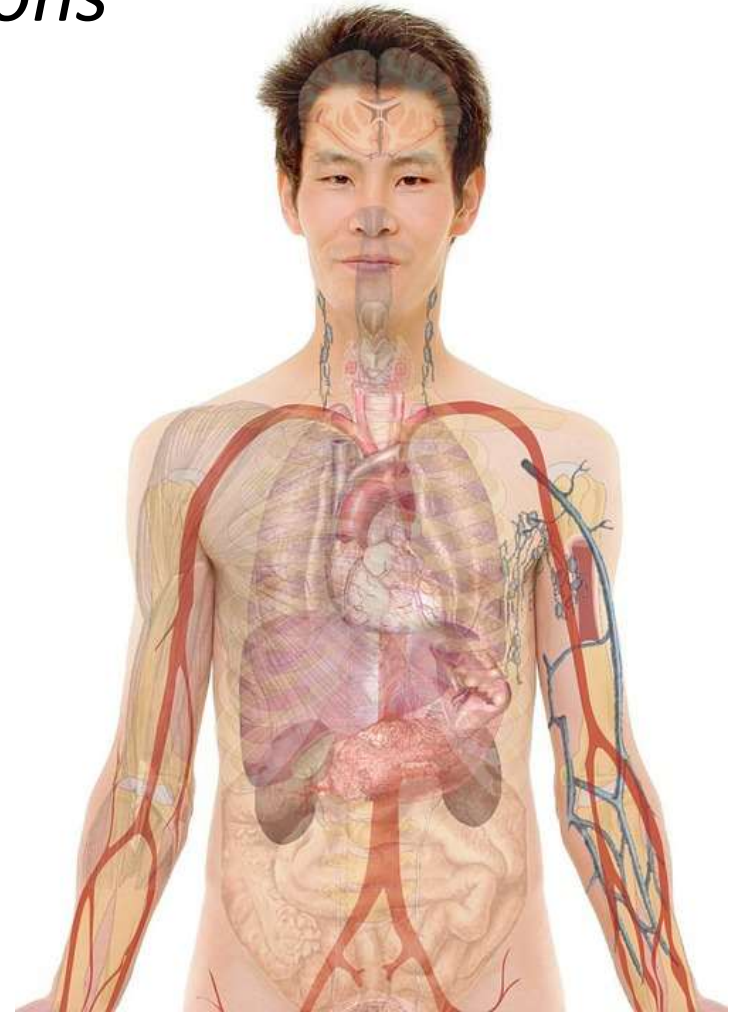
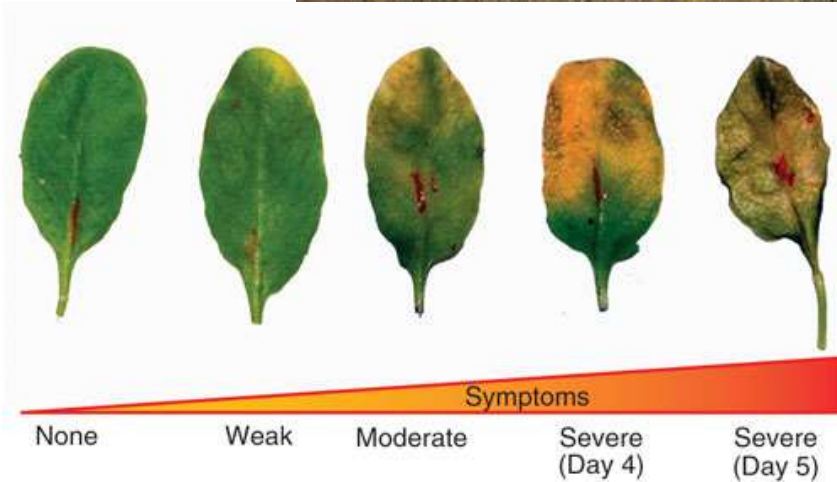
“They are so small that you can’t see them without a microscope, but they are there...”



Bacteria



Bacterial infections



Modeling *Pseudomonas aeruginosa* pathogenesis in plant hosts : doi:10.1038/nprot.2008.224.

Antibiotic resistance: post-antibiotic era ...



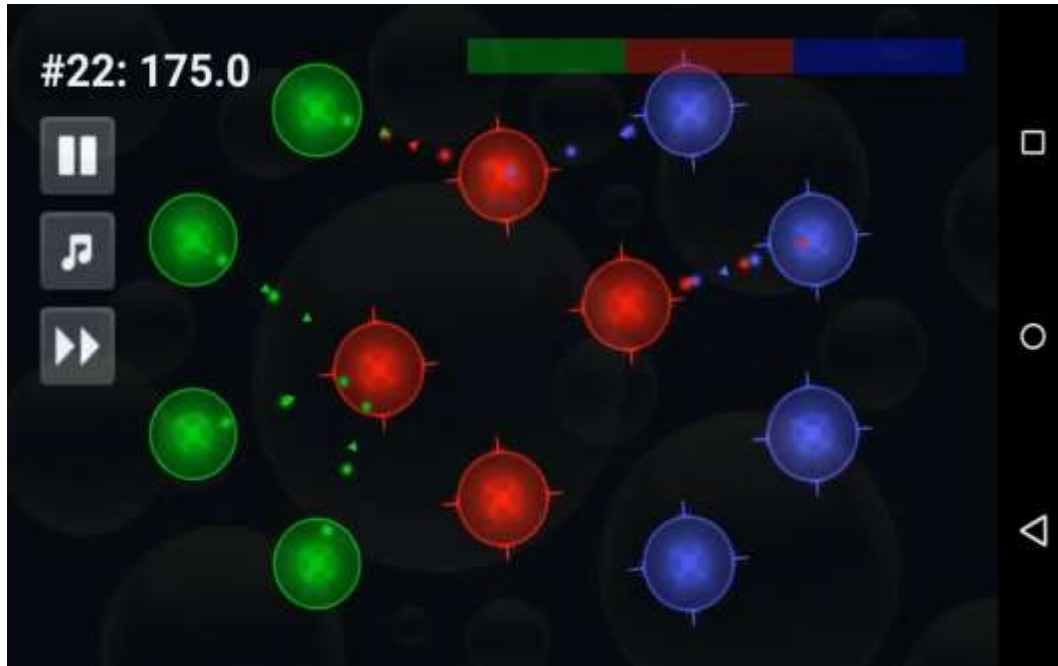
WHO News Release

“Without urgent, coordinated action by many stakeholders, the world is headed for a post-antibiotic era, in which common infections and minor injuries which have been treatable for decades can once again kill.”



- Dr. Keiji Fukuda, WHO Assistant Director-General for Health Security
April 30, 2014

Antibiotic resistance: microbial war...



Antibiotic resistance: revenge of the microbes

Clinical use	
Penicillin 1943	

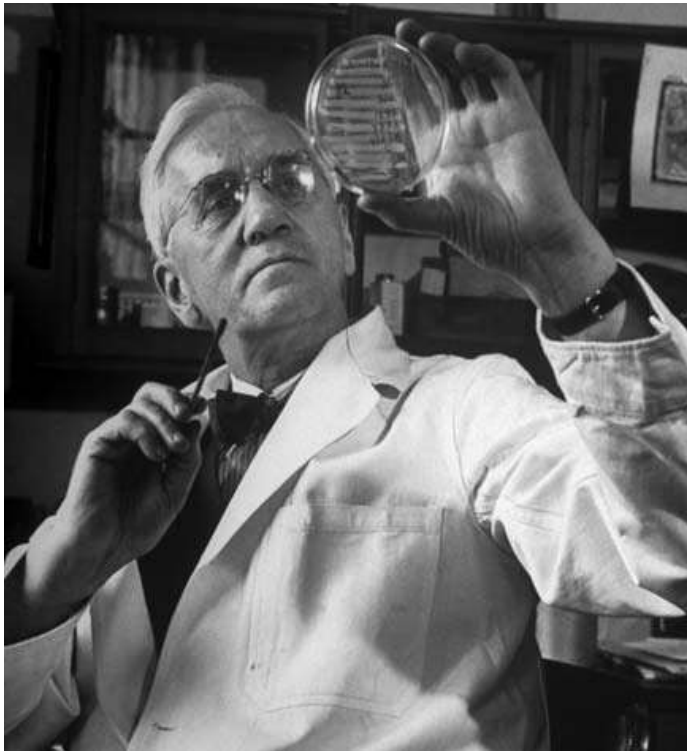


Sir Alexander Fleming

Alfred Eisenstaedt—Time & Life Pictures/Getty Images

Antibiotic resistance: revenge of the microbes

Clinical use	RESISTANCE
Penicillin 1943	1945 (2 years)



Sir Alexander Fleming

Alfred Eisenstaedt—Time & Life Pictures/Getty Images



Antibiotic resistance: revenge of the microbes

Clinical use	RESISTANCE
Penicillin 1943	1945 (2 years)
Vancomycin 1972	1988 (16 years)

Antibiotic resistance: revenge of the microbes

Clinical use	RESISTANCE
Penicillin 1943	1945 (2 years)
Vancomycin 1972	1988 (16 years)
Imipenem 1985	1998 (13 years)

Antibiotic resistance: revenge of the microbes

Clinical use	RESISTANCE
Penicillin 1943	1945 (2 years)
Vancomycin 1972	1988 (16 years)
Imipenem 1985	1998 (13 years)
Daptomycin 2003	2004 (1 year)

Antibiotic resistance: revenge of the microbes

Clinical use	RESISTANCE
Penicillin 1943	1945 (2 years)
Vancomycin 1972	1988 (16 years)
Imipenem 1985	1998 (13 years)
Daptomycin 2003	2004 (1 year)
Ceftaroline 2010	2011 (1 year)

Antibiotic resistance: revenge of the microbes

Clinical use



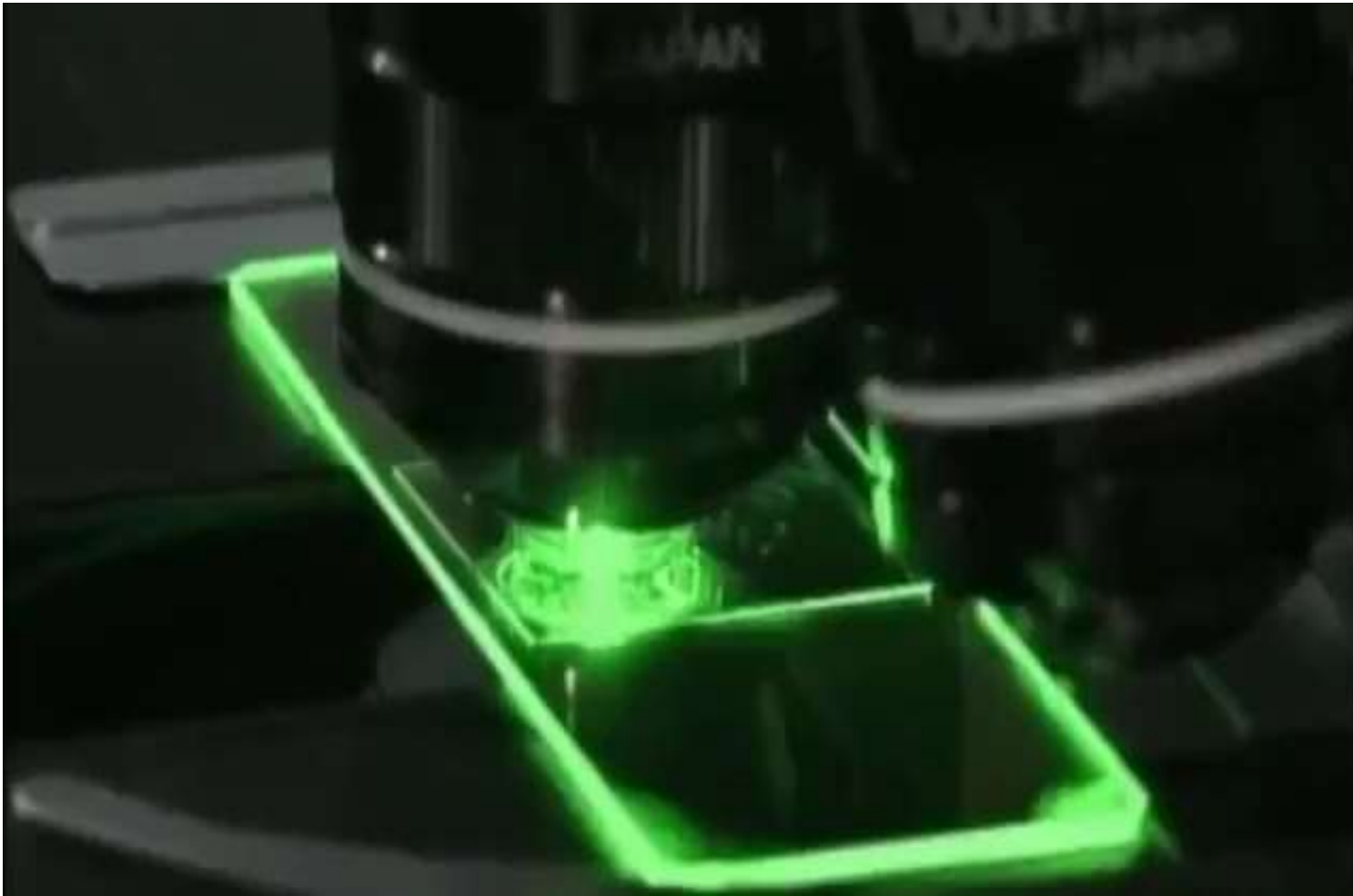
RESISTANCE



Pseudomonas aeruginosa



Pseudomonas aeruginosa: moving by the use of flagella...



From:
https://www.youtube.com/watch?v=a_5FToP_mMY
A clip from the NOVA production, "Judgment Day."

Pseudomonas aeruginosa: takes advantage of a sick person...



Low immune function

HIV



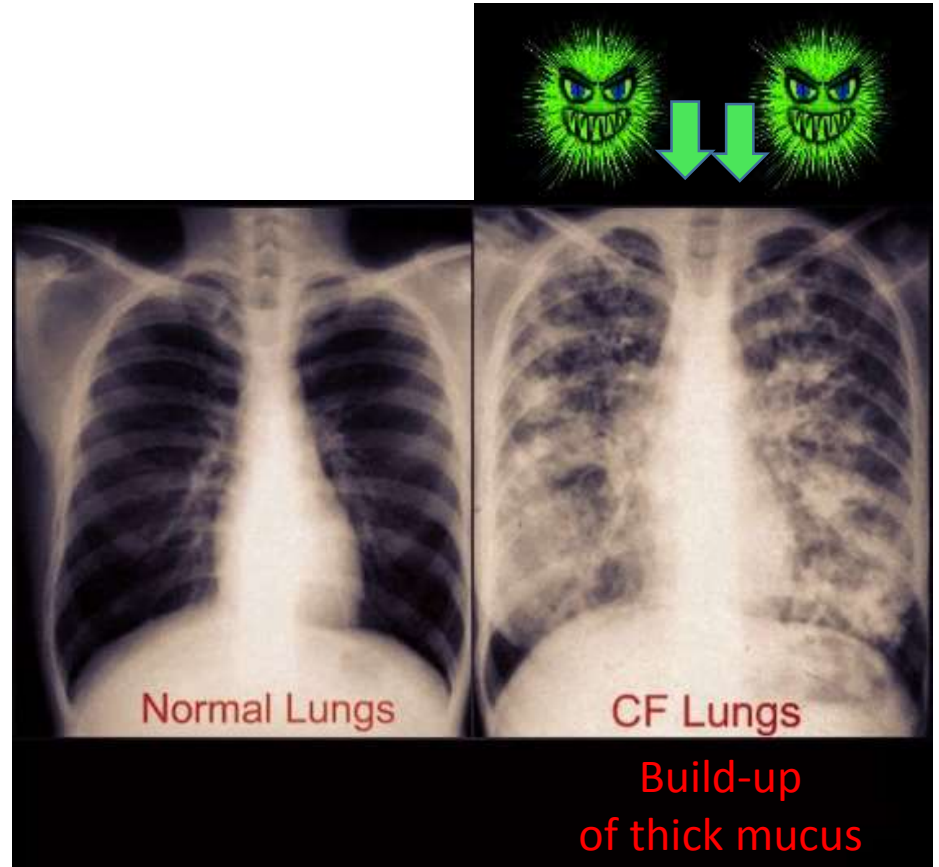
**Cancer patients
receiving
chemotherapy**

**Severe
burns**

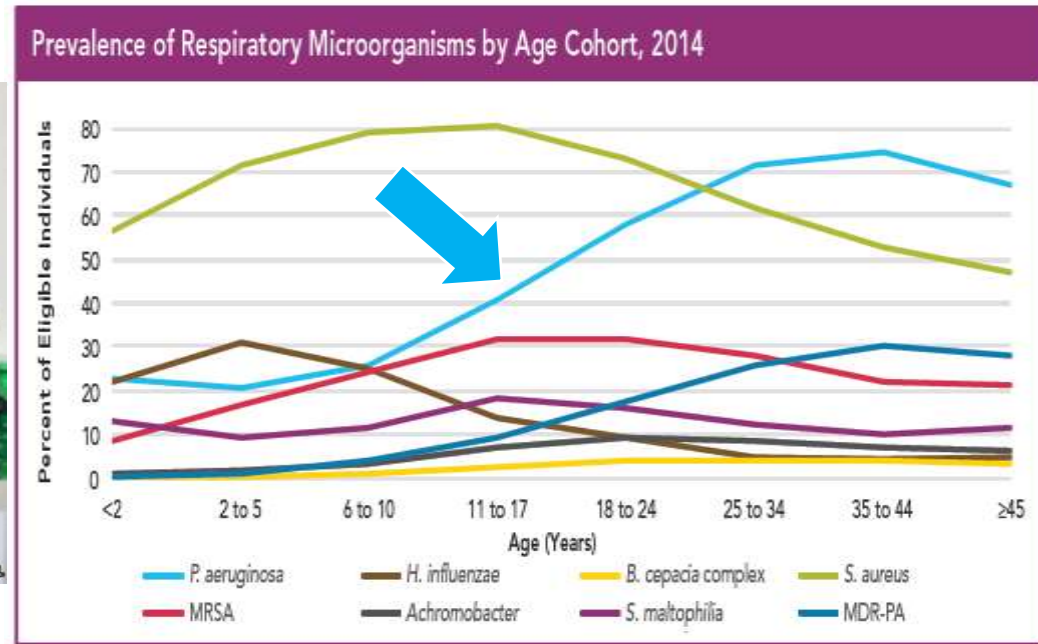
**Invasive
surgery**

Cystic Fibrosis

Opportunistic pathogens: Lung infections in **Cystic Fibrosis** patients



Pseudomonas aeruginosa: Lung infections in **Cystic Fibrosis** patients



Annual Data Report 2014 *Cystic Fibrosis Foundation Patient Registry*

Classes of antipseudomonal antibiotics

β -lactams

Aminoglycosides

Quinolones

Polymyxins



Classes of antipseudomonal antibiotics

Aztreonam



Aminoglycosides
(Tobramycin)

Quinolones
(Levofloxacin)



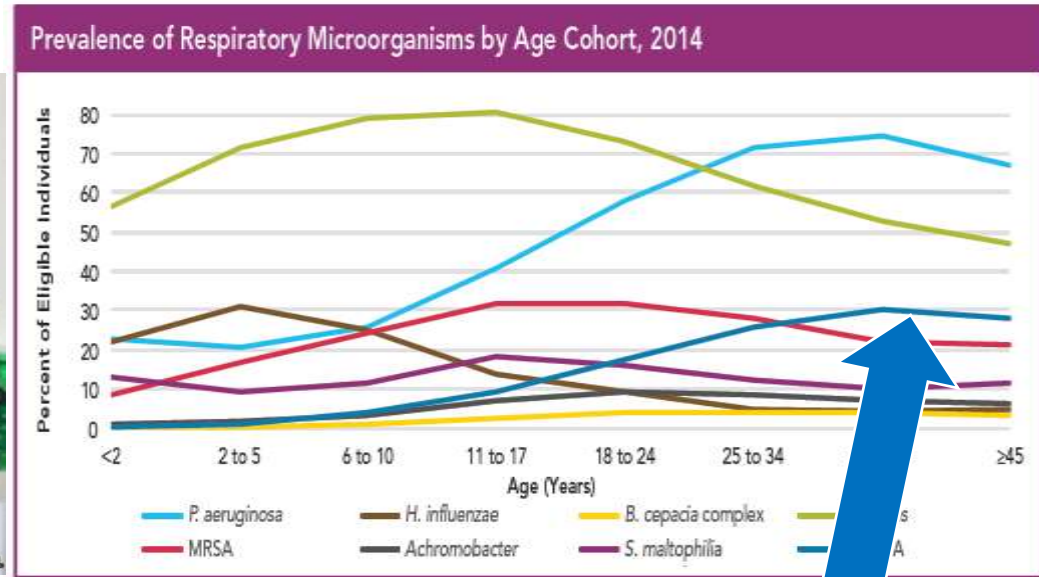
Polymyxins
(colistin)

Classes of antipseudomonal antibiotics



Quinolones

Pseudomonas aeruginosa: Lung infections in **Cystic Fibrosis** patients



Annual Data Report 2014 *Cystic Fibrosis Foundation Patient Registry*

33



**Multidrug-Resistant
*Pseudomonas aeruginosa***

<http://www.bbc.com/news/health-32755065>

Classes of antipseudomonal antibiotics

β -lactams

Penicillins:

Ticarcillin / clavulanic acid
Piperacillin / Tazobactam

Carbapenems:

Meropenem
Imipenem

Cephalosporins:

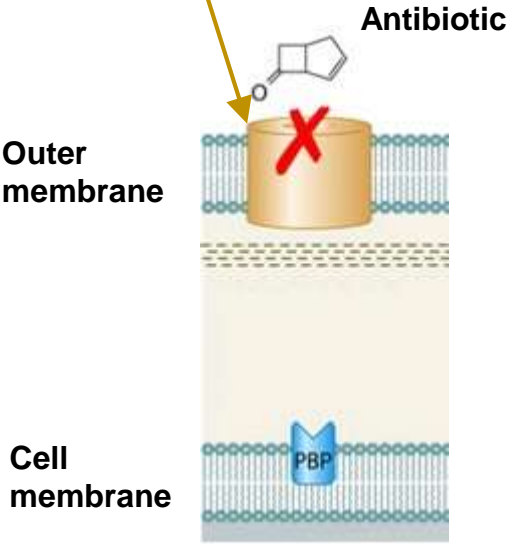
Ceftazidime, cefepime

Monobactam: Aztreonam



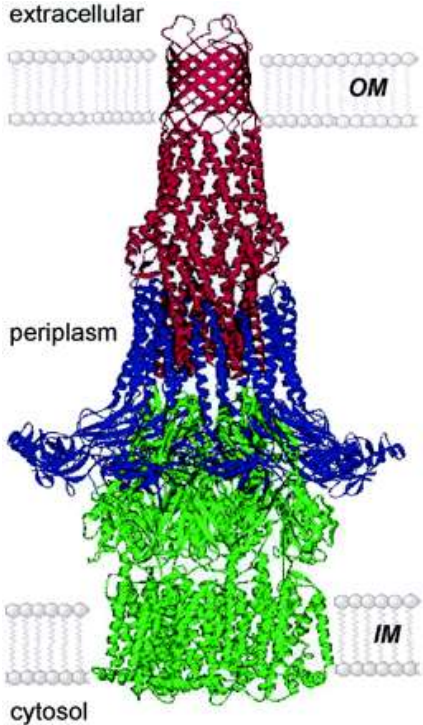
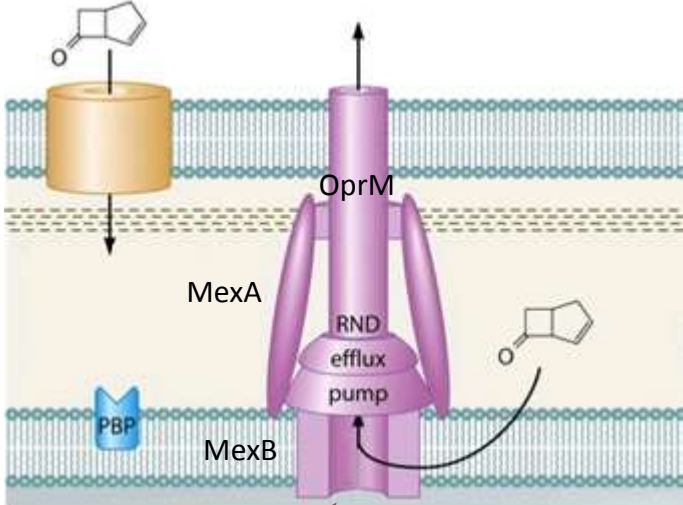
P. aeruginosa and resistance mechanisms to β -lactam antibiotics

Downregulating / mutating porins [OprD]
 β -lactams (imipenem, meropenem...)



Adapted from Lister et al. Clin. Microbiol. Rev. 2009; 22: 582-610
<http://www.bellybelly.com.au/baby/baby-hunger-cues/>
http://thestir.cafemom.com/big_kid/181864/expired_medicine_dates_still_safe

P. aeruginosa and resistance mechanisms to β -lactam antibiotics



Active efflux [MexAB-OprM, MexXY-OprM, MexEF-OprN, MexCD-OprJ]
 β -lactams (ticarcillin, meropenem...)

Adapted from: Lister et al. Clin. Microbiol. Rev. 2009; 22: 582-610.
Clin Microbiol Rev. 2006 Apr;19(2):382-402.

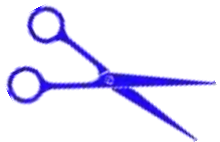
β -lactamases: antibiotic degradation



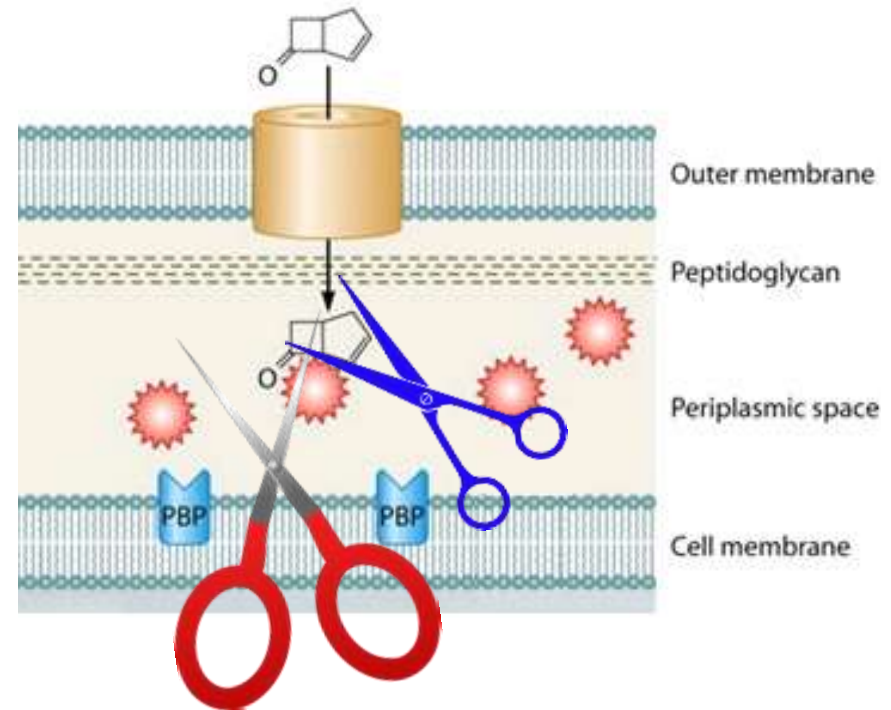
Extended-Spectrum- β -Lactamases:
ceftazidime



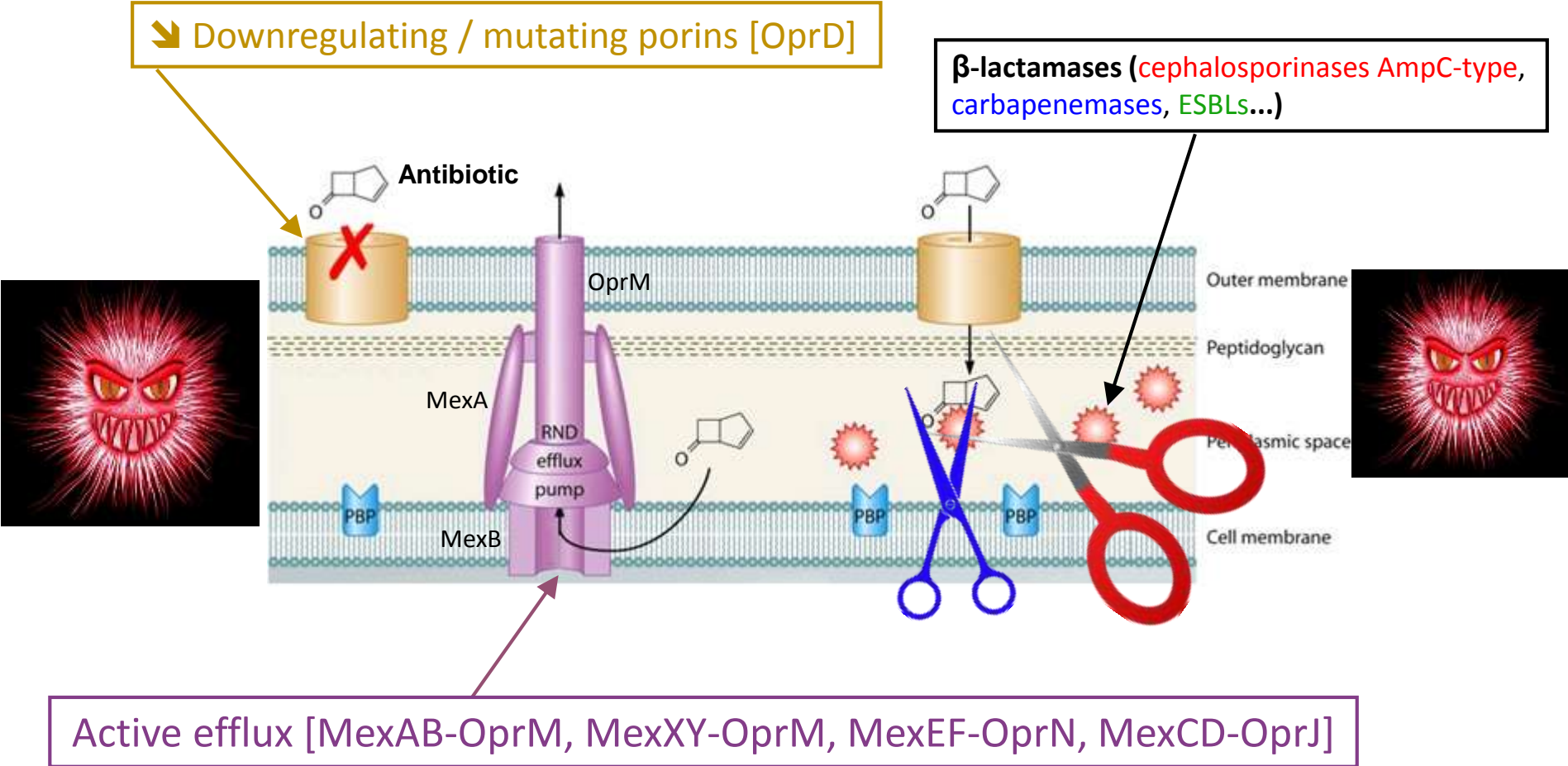
Cephalosporinase AmpC:
ceftazidime



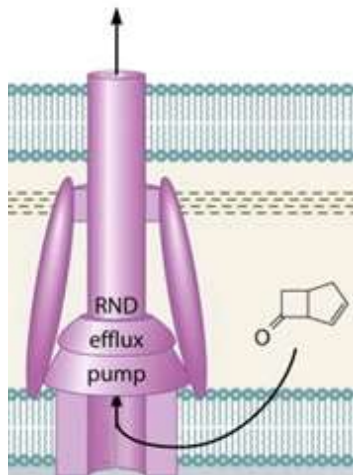
Carbapenemase:
meropenem, ceftazidime



P. aeruginosa and resistance mechanisms to β -lactam antibiotics



Impact of **efflux mechanisms** on susceptibility and resistance to
beta-lactam antibiotics in *Pseudomonas aeruginosa*:
beyond the usual concepts



Hussein Abdel Aziz Chalhoub

Promoter: Prof. Françoise Van Bambeke

Cellular and Molecular Pharmacology
Louvain Drug Research Institute

**Université catholique de Louvain, Brussels,
Belgium**

22 December 2016

Main objectives of the thesis

1. Activity of beta-lactam antibiotics against *Pseudomonas aeruginosa* from patients with cystic fibrosis

2. Role of **efflux** versus other resistance mechanisms against:
 - a. Meropenem (carbapenems)
 - b. Ceftazidime / avibactam (new β -lactamases inhibitor)
 - c. Temocillin (old beta-lactam antibiotic)



Source of isolates



- International collection of *P. aeruginosa* from patients with cystic fibrosis chronically exposed to different antipseudomonal agents.

→ Bacterial isolates: **333 clinically relevant isolates** were obtained from:

- ❖ Dr Michael Tunney (Queen's University of Belfast, **UK**): n=99;
- ❖ Drs Anne Vergison / Olivier Denis (Hôpital Erasme, Brussels, **Belgium**): n=88;
- ❖ Prof. Patrick Plésiat (CHRU Besançon, Besançon, **France**): n=80;
- ❖ Prof. Barbara Kahl (University of Münster, Münster, **Germany**): n=68.

Antibacterial activity of beta-lactams against *P. aeruginosa* from cystic fibrosis patients

	Ticarcillin	Piperacillin + Tazobactam	Ceftazidime	Imipenem	Meropenem
Breakpoint (mg/L)	16	16	8	8	8

Antibacterial activity of beta-lactams against *P. aeruginosa* from cystic fibrosis patients

	Ticarcillin	Piperacillin + Tazobactam	Ceftazidime	Imipenem	Meropenem
Breakpoint (mg/L)	16	16	8	8	8



EUCAST

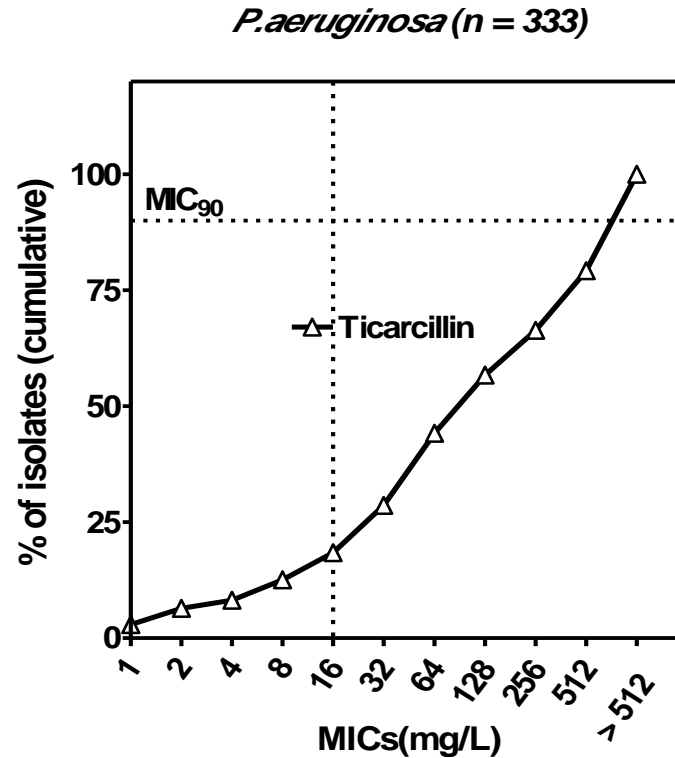
EUROPEAN COMMITTEE
ON ANTIMICROBIAL
SUSCEPTIBILITY TESTING

European Society of Clinical Microbiology and Infectious Diseases

- A microorganism is defined as susceptible or resistant by a level of antimicrobial activity associated with a high likelihood of therapeutic success or therapeutic failure, respectively.

Antibacterial activity of beta-lactams against *P. aeruginosa* from cystic fibrosis patients

	Ticarcillin
Breakpoint (mg/L)	16
% Susceptible	18
% Resistant	82
MIC ₉₀ (mg/L)	>512



Antibacterial activity of beta-lactams against *P. aeruginosa* from cystic fibrosis patients

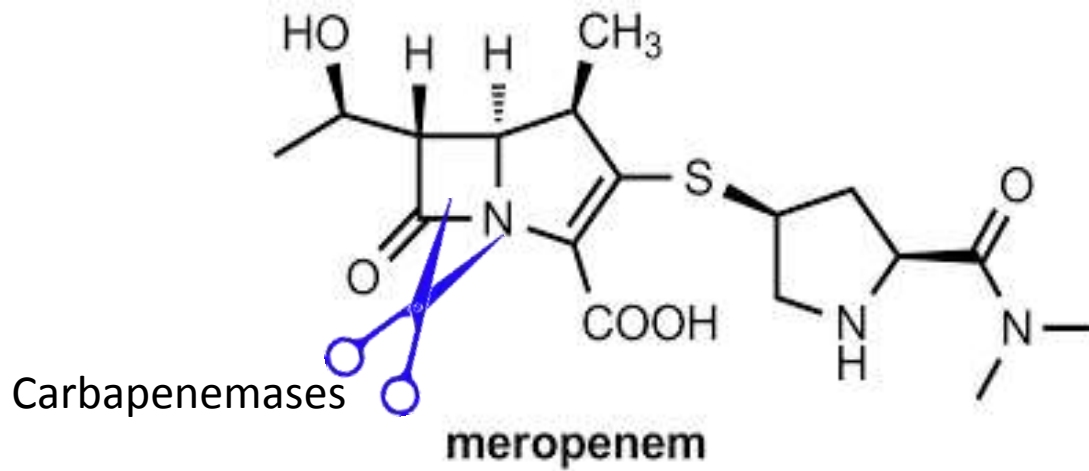
	Ticarcillin	Piperacillin + Tazobactam	Ceftazidime	Imipenem	Meropenem
Breakpoint (mg/L)	16	16	8	8	8
% Susceptible	18	32	36	51	49
% Resistant	82	68	64	49	51
MIC ₉₀ (mg/L)	>512	512	512	32	16

- **Summary.** Imipenem and meropenem were the most active antibiotics...

Meropenem (β -lactam)



- Stable to AmpC and ESBL but not carbapenemases.



High-level resistance to meroperem

Pseudomonas
(cystic fibrosis patients)

Meropenem MIC = 64-128 mg/L

High-level resistance to meroperem

Comparison with *Pseudomonas* from hospital acquired pneumonia

Pseudomonas
(cystic fibrosis patients)

Meropenem MIC = 64-128 mg/L

Pseudomonas
(Hospital acquired pneumonia)

Meropenem MIC = 128 mg/L

High-level resistance to meropenem

Comparison with *Pseudomonas* from hospital acquired pneumonia

Pseudomonas
(cystic fibrosis patients)

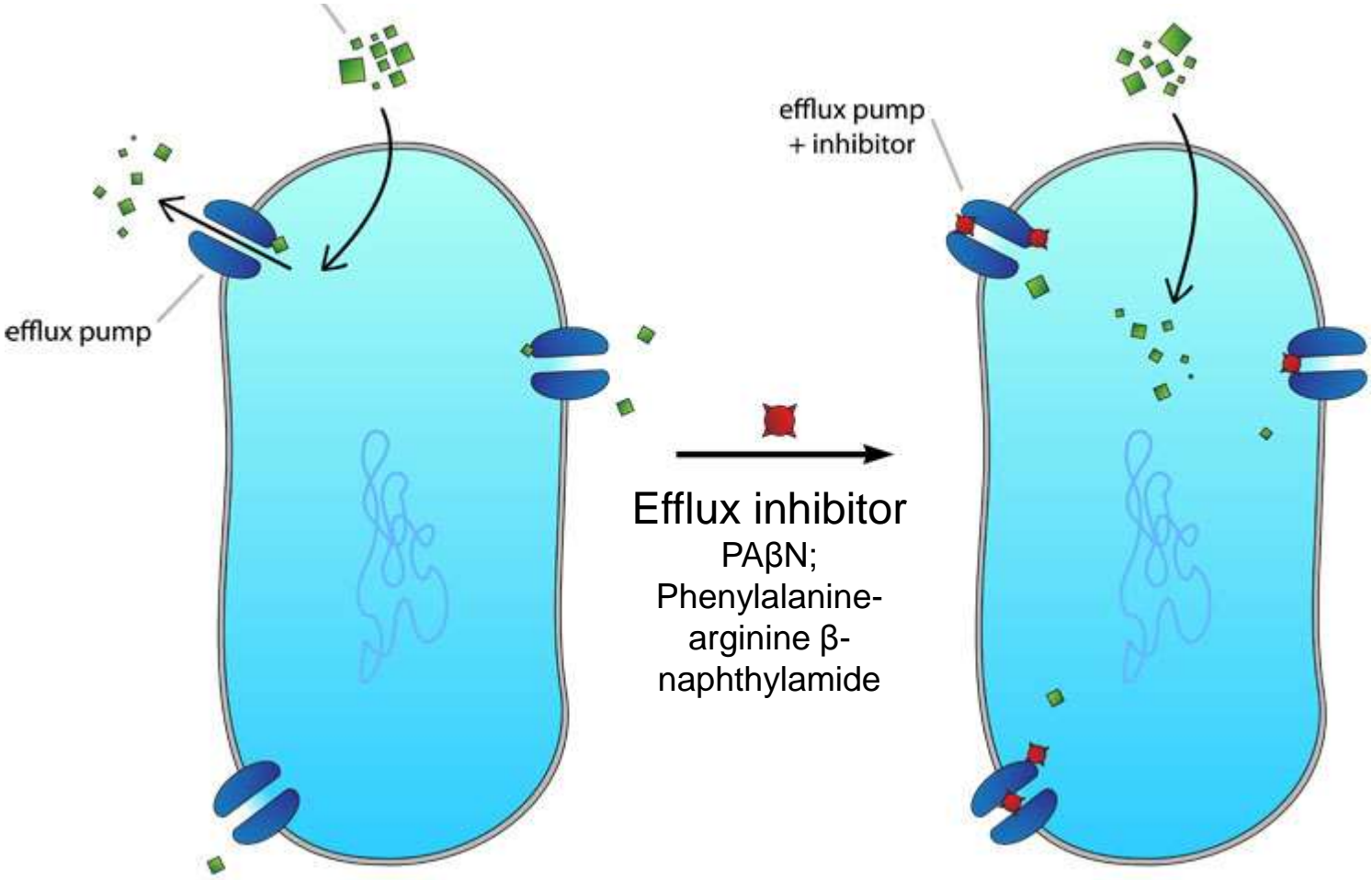
Meropenem MIC = 64-128 mg/L

Pseudomonas
(Hospital acquired pneumonia)

Meropenem MIC = 128 mg/L

	Cystic fibrosis	Hospital acquired pneumonia
AmpC-type cephalosporinases	X	
ESBL(s)		
Carbapenemase(s)		X

Meropenem + efflux inhibitor in *Pseudomonas*



Adapted from Jason Sello, Brown university, 2011

High-level resistance to meropenem

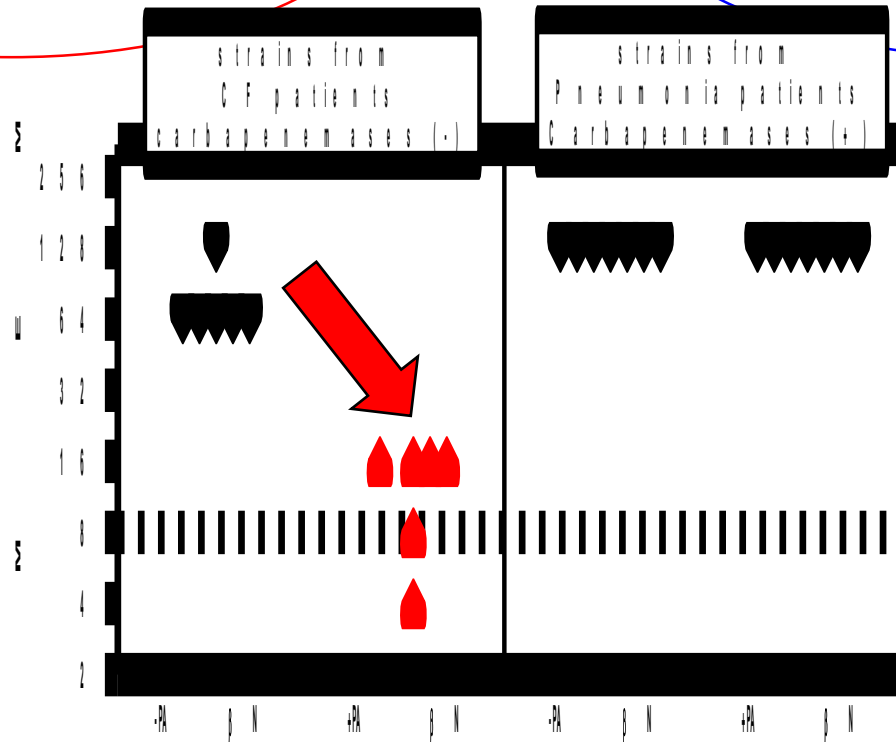
Comparison with *Pseudomonas* from hospital acquired pneumonia

Pseudomonas
(cystic fibrosis patients)

Meropenem MIC = 64-128 mg/L

Pseudomonas
(Hospital acquired pneumonia)

Meropenem MIC = 128 mg/L



High-level resistance to meropenem

Comparison with *Pseudomonas* from hospital acquired pneumonia

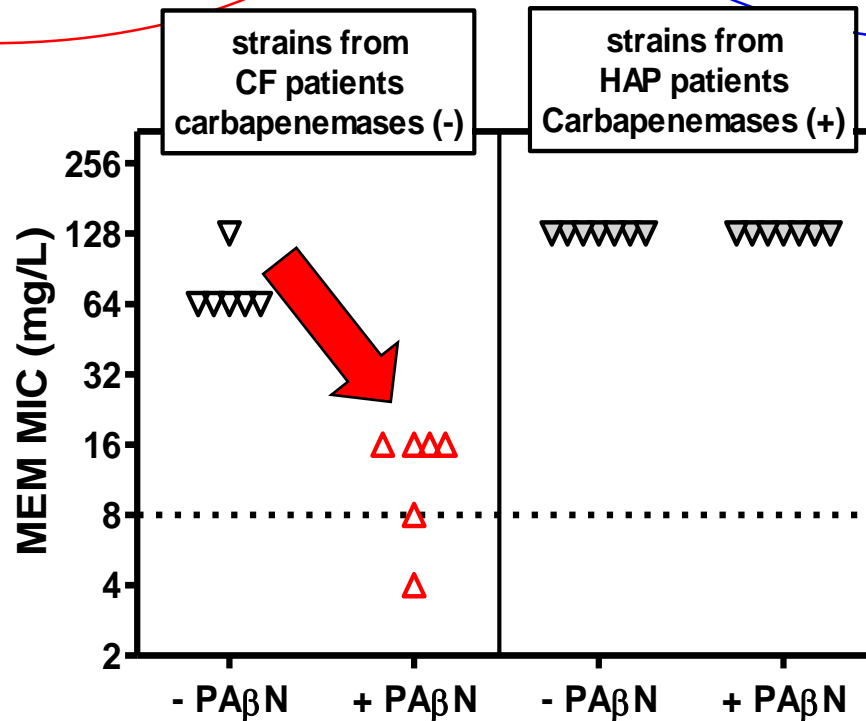
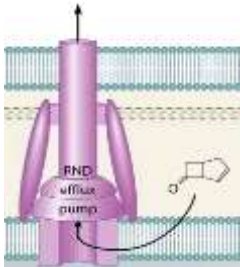
Pseudomonas
(cystic fibrosis patients)

Meropenem MIC = 64-128 mg/L

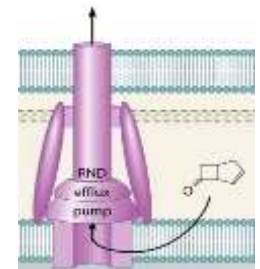
Pseudomonas
(Hospital acquired pneumonia)

Meropenem MIC = 128 mg/L

MexAB-OprM ↑
MexXY-OprM ↑
MexCD-OprJ ↑



MexAB-OprM ↑
MexXY-OprM ↑
MexCD-OprJ ↑



High-level resistance to meroperem

Comparison with *Pseudomonas* from hospital acquired pneumonia

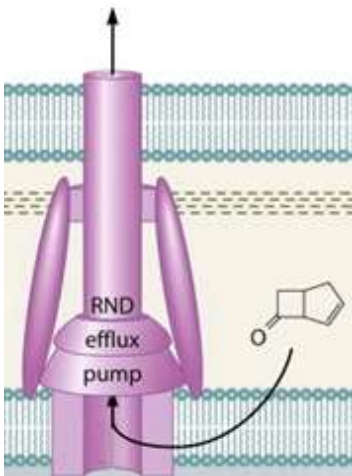
Pseudomonas
(cystic fibrosis patients)

Pseudomonas
(Hospital acquired pneumonia)



- Sequencing *OprD* genes confirmed these results (premature stop mutations)

Impact of **efflux mechanisms** on susceptibility and resistance to
beta-lactam antibiotics in *Pseudomonas aeruginosa*:
beyond the usual concepts



1- [Efflux + / porin -] are enough to confer high-level resistance to meropenem.

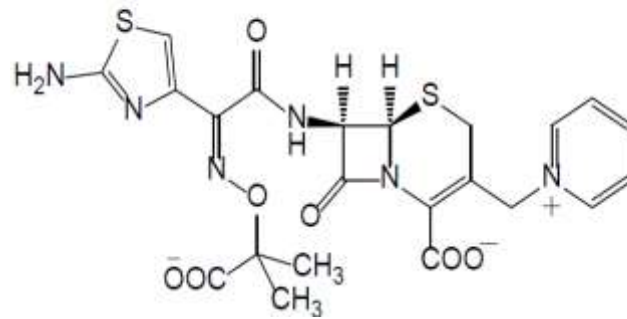
High-level resistance to ceftazidime

	Cystic fibrosis	Hospital acquired pneumonia
AmpC-type cephalosporinases	X	
ESBL(s)		
Carbapenemase(s)		X

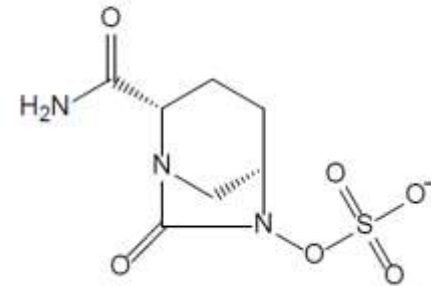
AmpC cephalosporinases inhibitor: avibactam



Ceftazidime



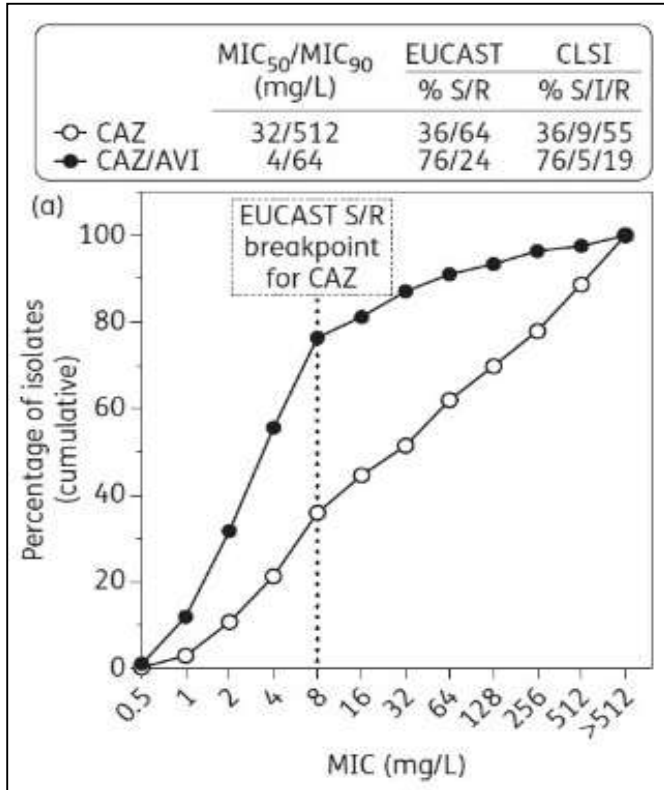
Avibactam



Ceftazidime/avibactam activity in the whole collection of isolates (n = 333)

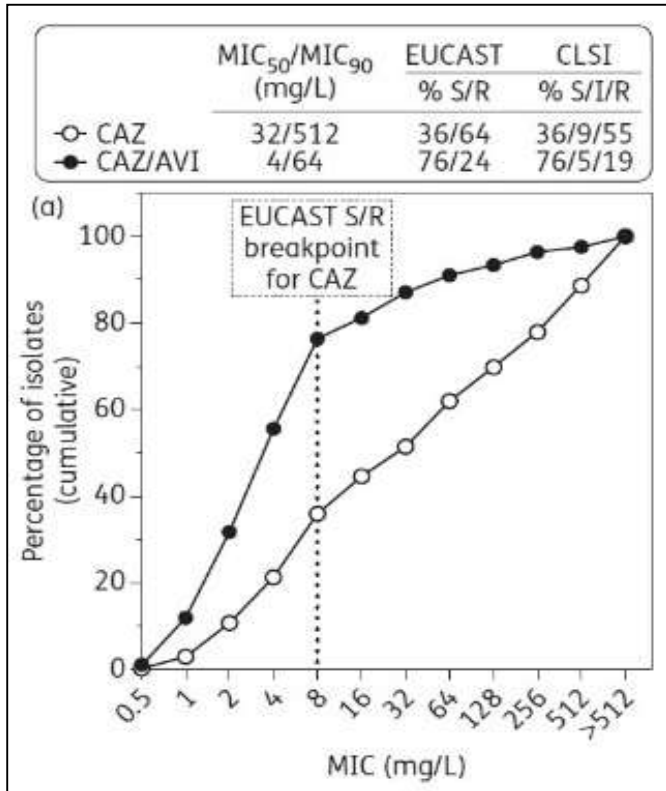


Promising results
for cystic fibrosis patients



- Susceptible isolates to ceftazidime increased from **36% to 76 %** after addition of avibactam [4mg/L].

Ceftazidime/avibactam activity in the whole collection of isolates (n = 333)



**24 % resistance
to this new combination**

!?

The mechanism of resistance to ceftazidime/avibactam combination

Control strains (induced with sub-MIC imipenem)	MIC (mg/L)
	Ceftazidime
Reference strain, PAO1	32
PAO1 overproducing MexXY-OprM	32
PAO1 overproducing MexCD-OprJ	32

(Prepared for submission as a correspondence to *the Journal of Antimicrobial Chemotherapy*)

Chalhoub *et al.*, Poor membrane permeability impedes avibactam activity in *Pseudomonas aeruginosa*

The mechanism of resistance to ceftazidime/avibactam combination

Control strains (induced with sub-MIC imipenem)	MIC (mg/L)	
	Ceftazidime	Ceftazidime+Avibactam
Reference strain, PAO1	32	0.125
PAO1 overproducing MexXY-OprM	32	0.125
PAO1 overproducing MexCD-OprJ	32	0.125

(Prepared for submission as a correspondence to *the Journal of Antimicrobial Chemotherapy*)

Chalhoub *et al.*, Poor membrane permeability impedes avibactam activity in *Pseudomonas aeruginosa*

The mechanism of resistance to ceftazidime/avibactam combination

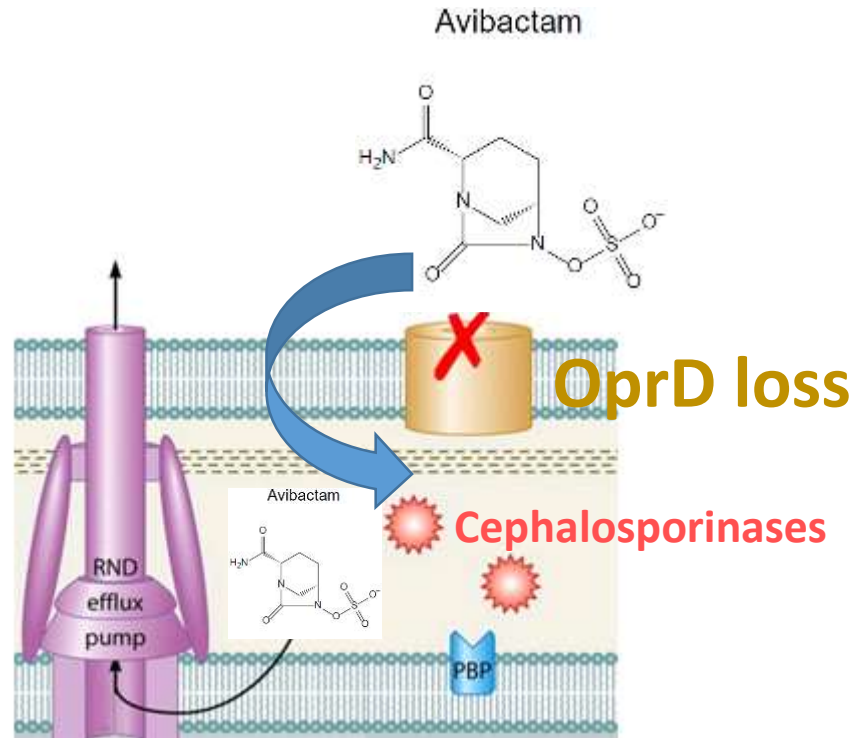
Control strains (induced with sub-MIC imipenem)	MIC (mg/L)	
	Ceftazidime	Ceftazidime+Avibactam
Reference strain, PAO1	32	0.125
PAO1 overproducing MexXY-OprM	32	0.125
PAO1 overproducing MexCD-OprJ	32	0.125
PAO1 overproducing MexAB-OprM	32	4
PAO1 overproducing MexEF-OprN	32	2
PAOD1 OprD-mutant	32	4

(Prepared for submission as a correspondence to *the Journal of Antimicrobial Chemotherapy*)

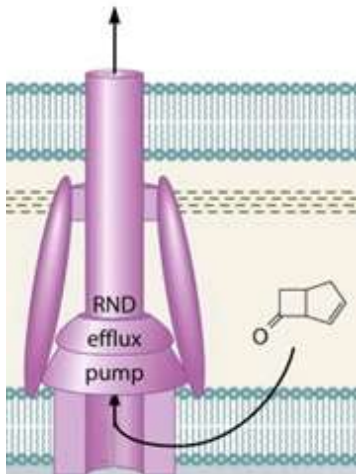
Chalhoub et al., Poor membrane permeability impedes avibactam activity in *Pseudomonas aeruginosa*

The mechanism of resistance to ceftazidime/avibactam combination

MexAB-OprM,
MexEF-OprN
efflux systems
overexpression

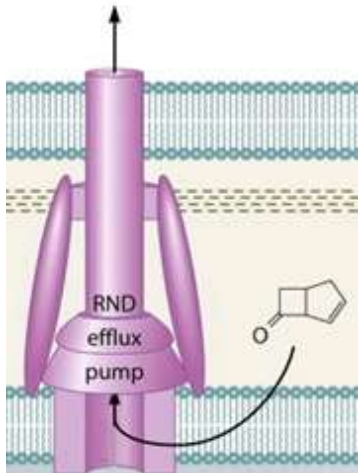


Impact of **efflux mechanisms** on susceptibility and resistance to
beta-lactam antibiotics in *Pseudomonas aeruginosa*:
beyond the usual concepts



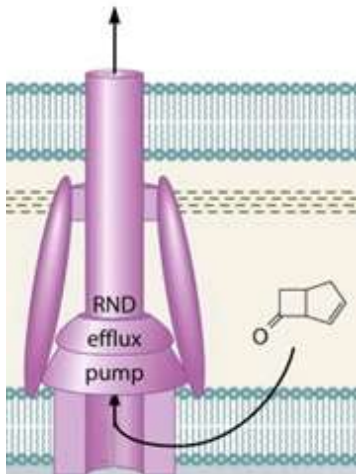
- 1- [Efflux + / porin -] are enough to confer high-level resistance to meropenem
- 2- [Efflux + / porin -] are pre-existing as resistance mechanisms to avibactam, which is not used yet in CF patients !!

Impact of **efflux mechanisms** on susceptibility and resistance to
beta-lactam antibiotics in *Pseudomonas aeruginosa*:
beyond the usual concepts



Is there any antibiotic resistant to beta-lactamases, and not affected by efflux systems and OprD porin downregulation ??

Impact of **efflux mechanisms** on susceptibility and resistance to
beta-lactam antibiotics in *Pseudomonas aeruginosa*:
beyond the usual concepts



Temocillin (β -lactam)

- Derived from the class of carboxypenicillins (ticarcillin, carbenicillin)



by Beecham Pharmaceuticals in the **1989s**

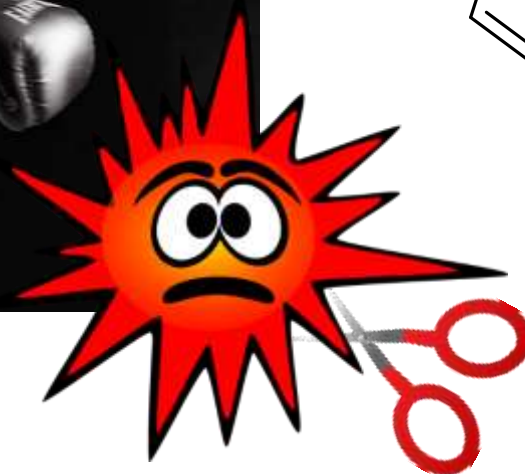


- **Withdrawn due to lack of activity** against wild-type strains of *Pseudomonas aeruginosa*, Gram-positive organisms and anaerobes.
- Not used against *Pseudomonas*.

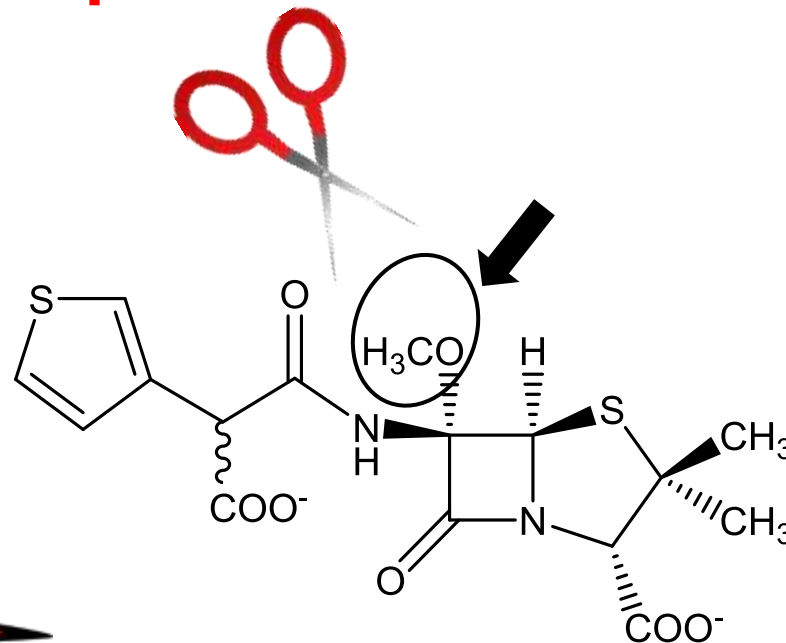
Temocillin: stable against β -lactamases



Temocillin

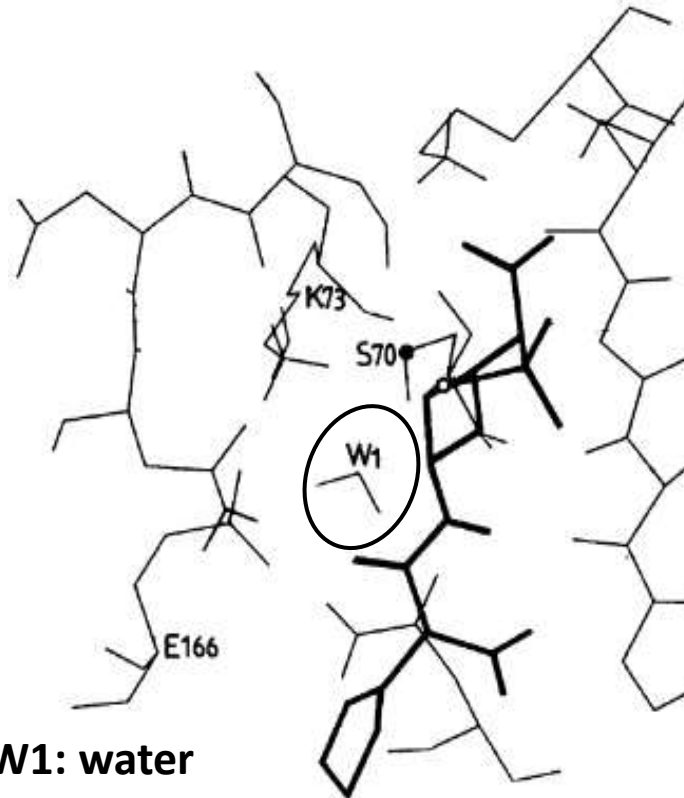
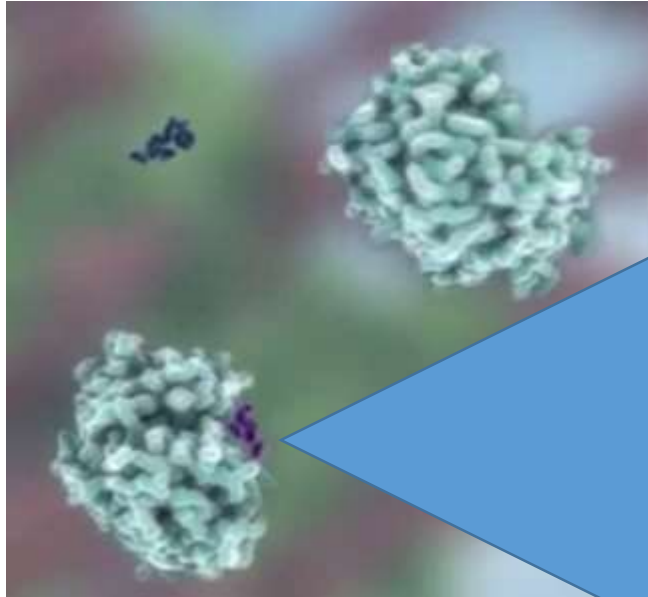


H₂O + β -lactamases



Temocillin

Temocillin versus ticarcillin in the active site of β -lactamases

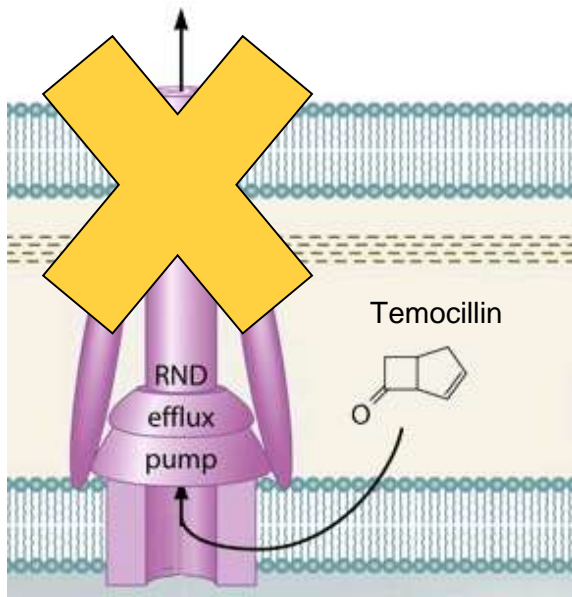


**W1: water
molecule**

Adapted from:

- Cubist pharmaceuticals
- A Matagne *et al.* - [Biochem J.](#) 1993 Aug 1; 293(Pt 3): 607–611.

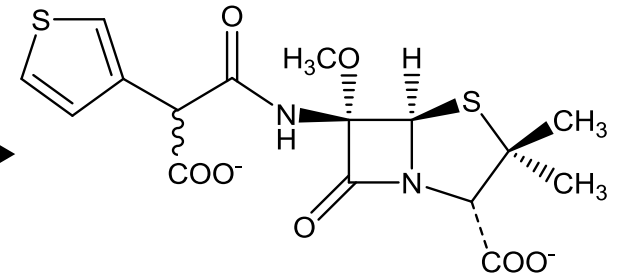
Temocillin & Efflux in *P. aeruginosa*



$\Delta mexAB$

Susceptible

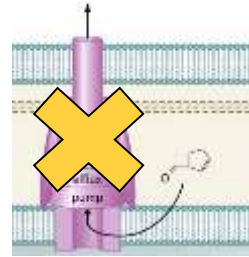
Temocillin



In 2012
FACM Group,
LDRI, Belgium

Isogenic strains of <i>PA</i>	MIC (mg/L)	
	temocillin	ticarcillin
Wild type PAO1	512	64
PAO1 $\Delta mexAB$	2	1

Temocillin & Efflux in *P. aeruginosa*

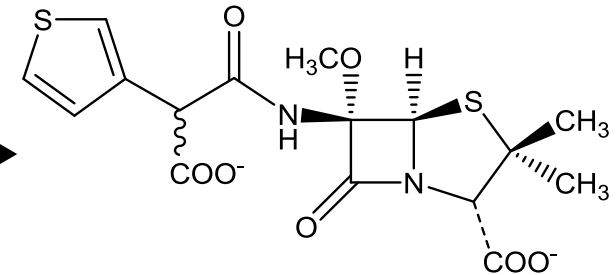


$\Delta mexAB$

Susceptible



Temocillin



In 2012
FACM Group,
LDRI, Belgium

Natural mutations of these efflux pumps can be observed in isolates from patients with cystic fibrosis !!!

Related publication : Buyck *et al.* J. Antimicrob. Chemother. 2012; 67: 771-5.

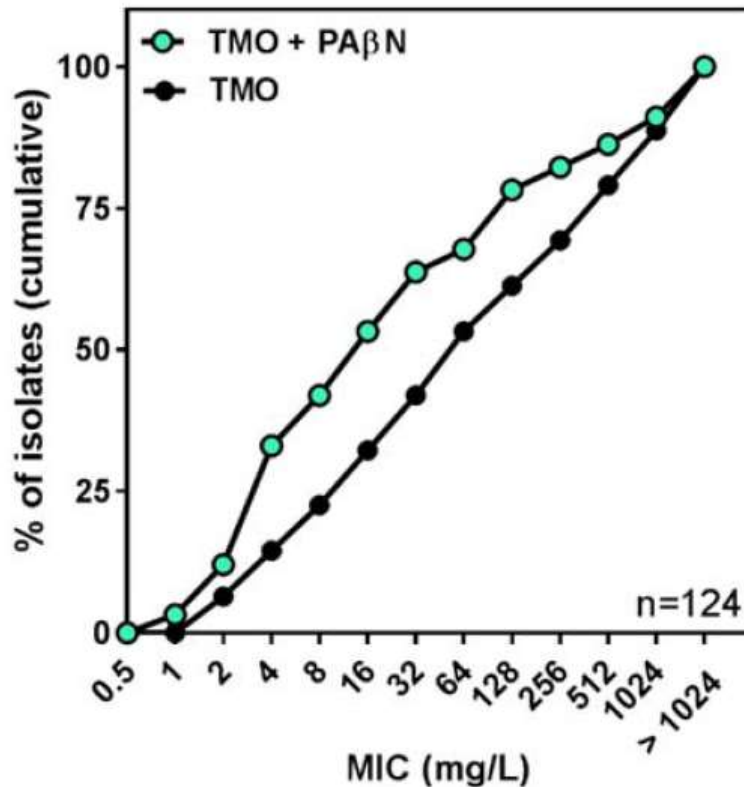
Temocillin considered as inactive against *Pseudomonas* showed surprising activity !!!

	TIC	Temocillin	TZP	CAZ	Meropenem	Ceftazidime +avibactam
Breakpoint	16	16	16	8	8	8
MIC₉₀	>512	>512	512	512	16	64
% Susceptible	18	29[#]	32	36	49	76
% Resistant	82	71	68	64	51	24

proposed breakpoint for temocillin in Belgium: 16 mg/L

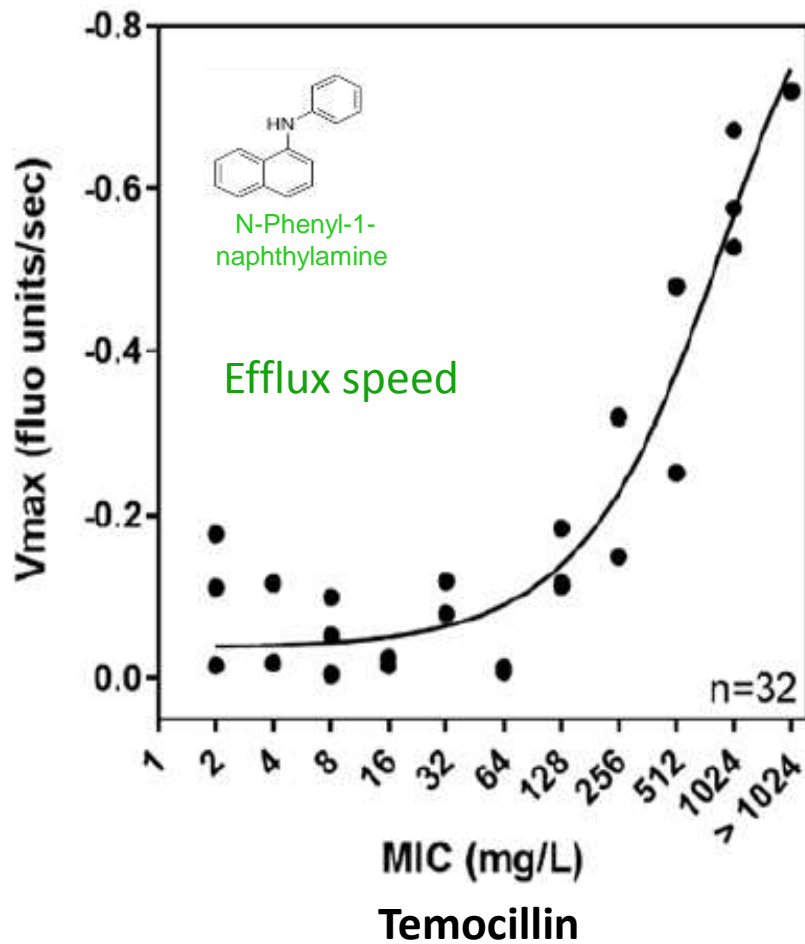
Temocillin + efflux inhibitor in *Pseudomonas*

(a) MIC distribution of temocillin



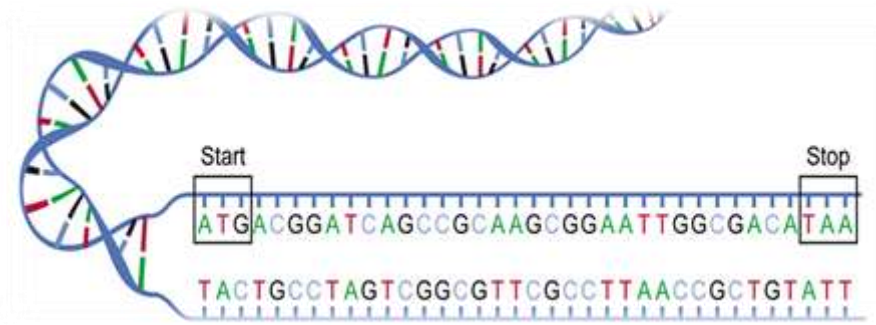
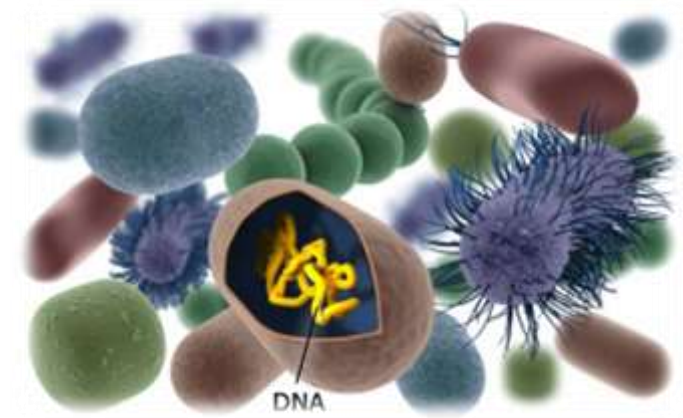
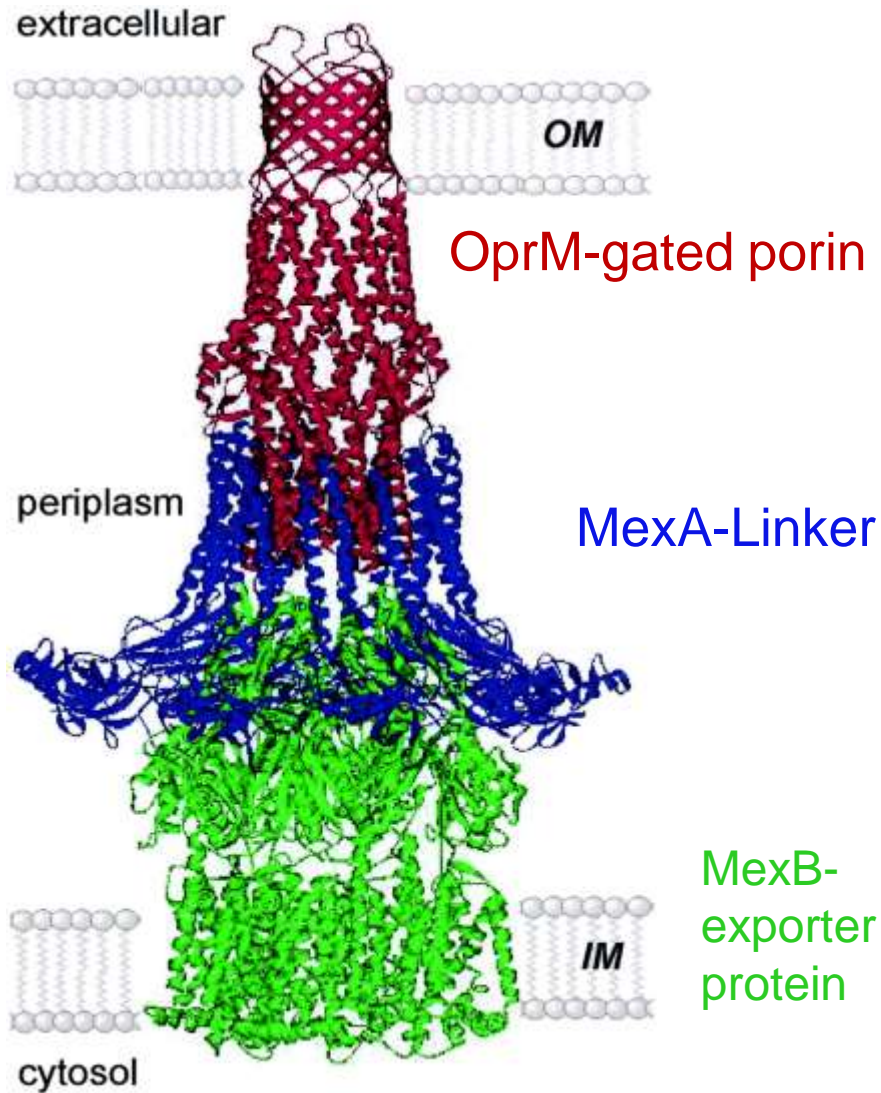
- Activity of temocillin was improved in the presence of the efflux inhibitor
- % susceptibility increased from ~30 to 55%

Speed of efflux by MexAB-OprM pumps



- Activity of temocillin was improved in the presence of the efflux pump inhibitor.
- **Lowest efflux speed for isolates with temocillin MICs < 128mg/L.**

Sequencing *mexA/B* genes in *P. aeruginosa* from cystic fibrosis patients

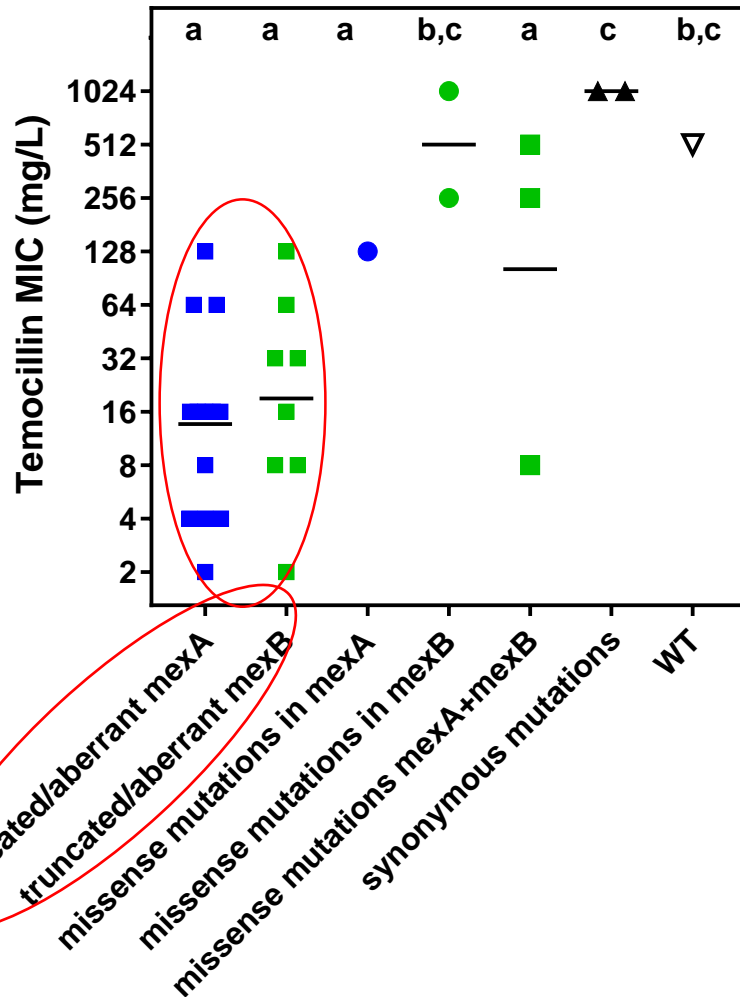


Sequencing *mexA/B* genes in *P. aeruginosa* from cystic fibrosis patients

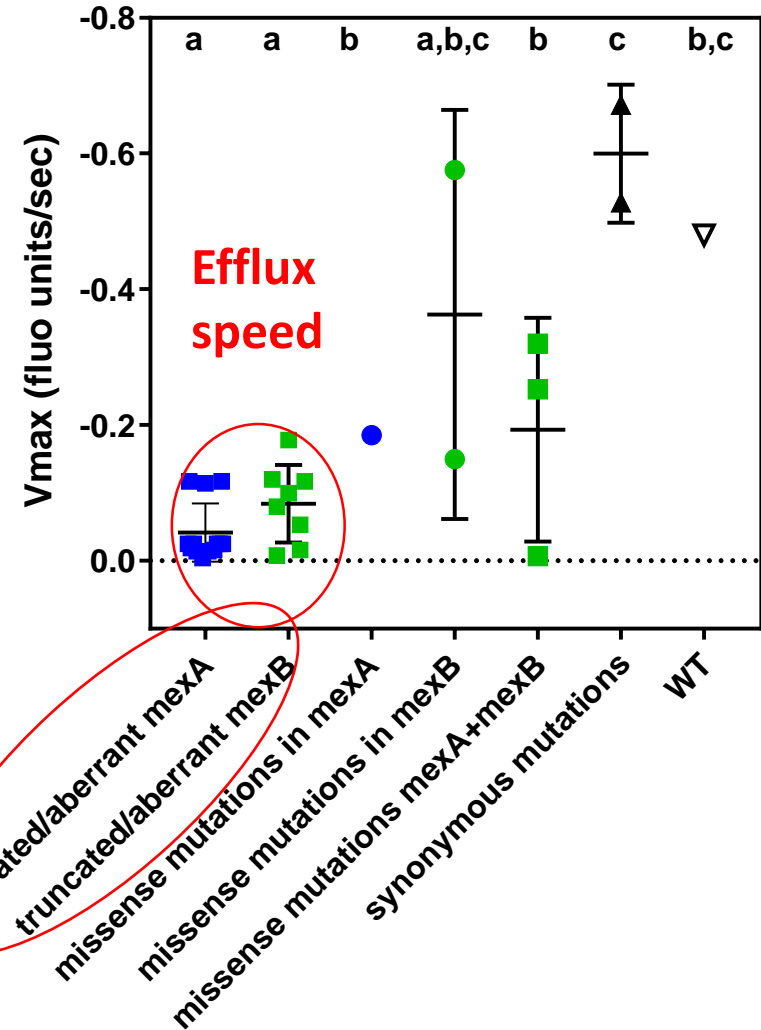
Major mutations	Minor mutations
<ul style="list-style-type: none">-DNA Fragment Deletions-Stop mutations	<ul style="list-style-type: none">-Synonymous mutations-Missense mutations

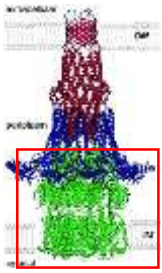
Mutations in *mexA/B* genes in *P. aeruginosa* as a function of temocillin activity

(e) MIC vs. mutations



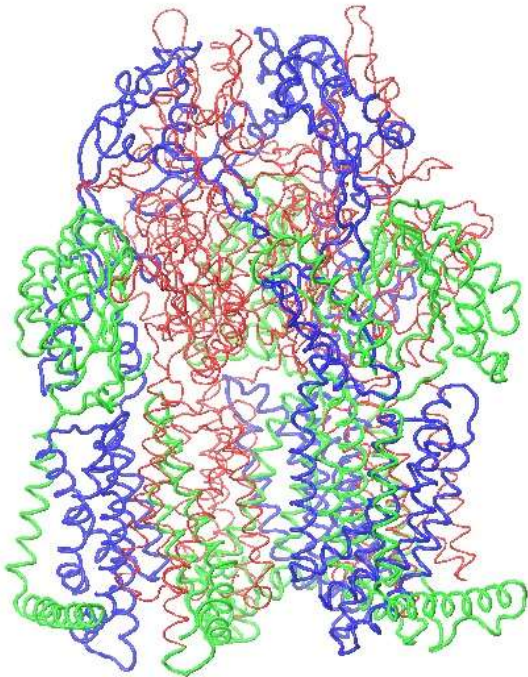
(f) NPN efflux vs. mutations





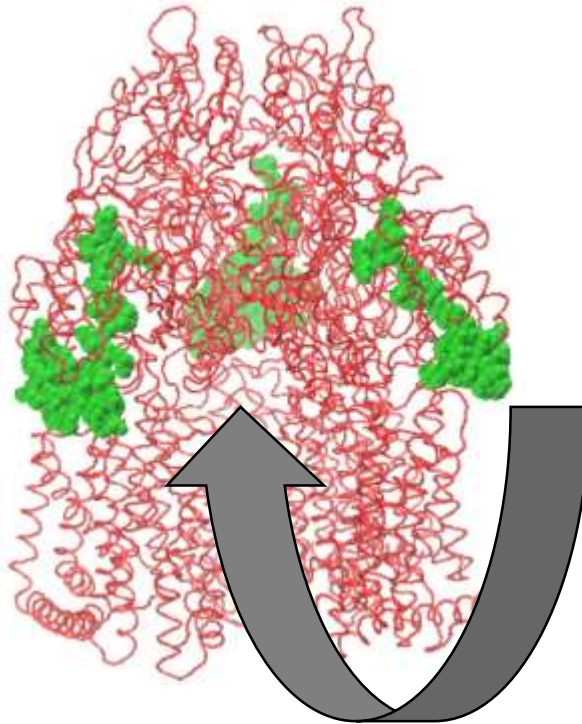
Modelisation *mexB* mutations in the same clone isolated from 3 German patients

MexB mutant
(truncated + aberrant protein)
Temocillin MIC = 8 mg/L



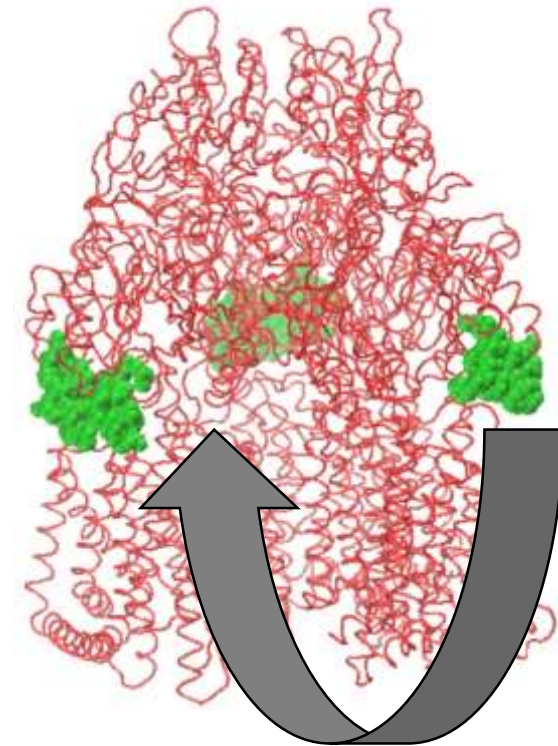
Front view

MexB mutant
(AA substitutions)
Temocillin MIC = 256 mg/L



Front view

MexB mutant
(AA substitutions)
Temocillin MIC = 512 mg/L



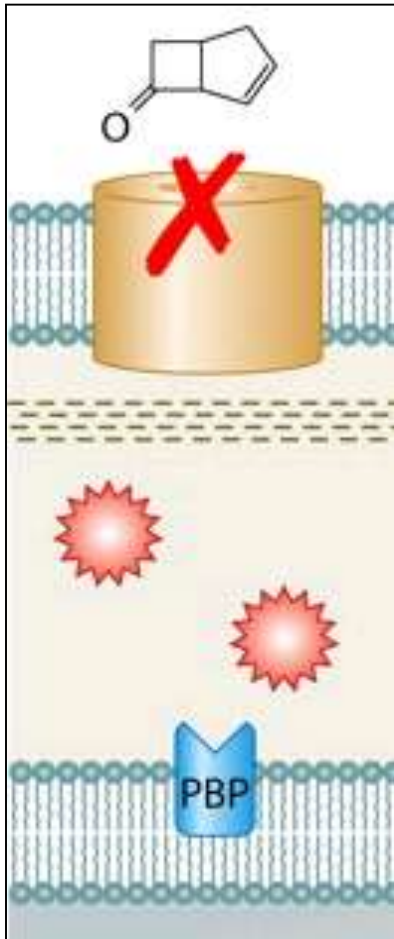
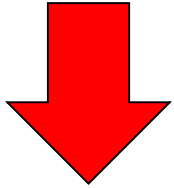
Front view

Truncated MexB and low efflux speed but high temocillin MIC !

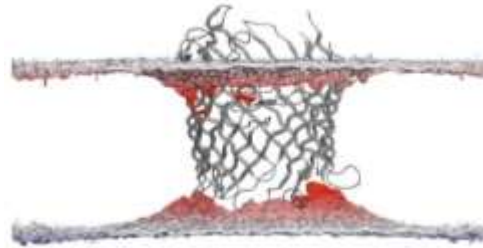
➤ Truncated MexB (M720_Q1046del): temocillin MIC = 128 mg/L

Strains	MIC (mg/L)	
	Temocillin	Ticarcillin
Wild type <i>delta mexB</i>	2	1
Clinical isolate <i>delta mexB</i>	128	32
Wild type	512	128

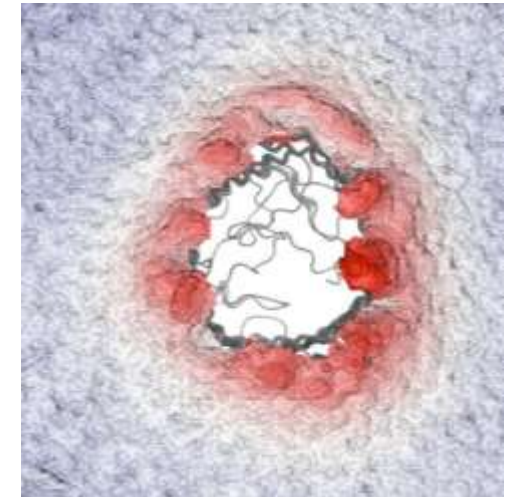
Influx of temocillin inside *Pseudomonas* ??



OprD family, 19 members of carboxylate channels



Porin
(Front view)



Porin
(Extracellular side)

Temocillin & the 19 Gates (OprD porin family) of *P. aeruginosa*



Related publication: Chalhoub *et al*, Nature Sci. Reports. in press.

Outer membrane carboxylate channels OccD/K in *P. aeruginosa*

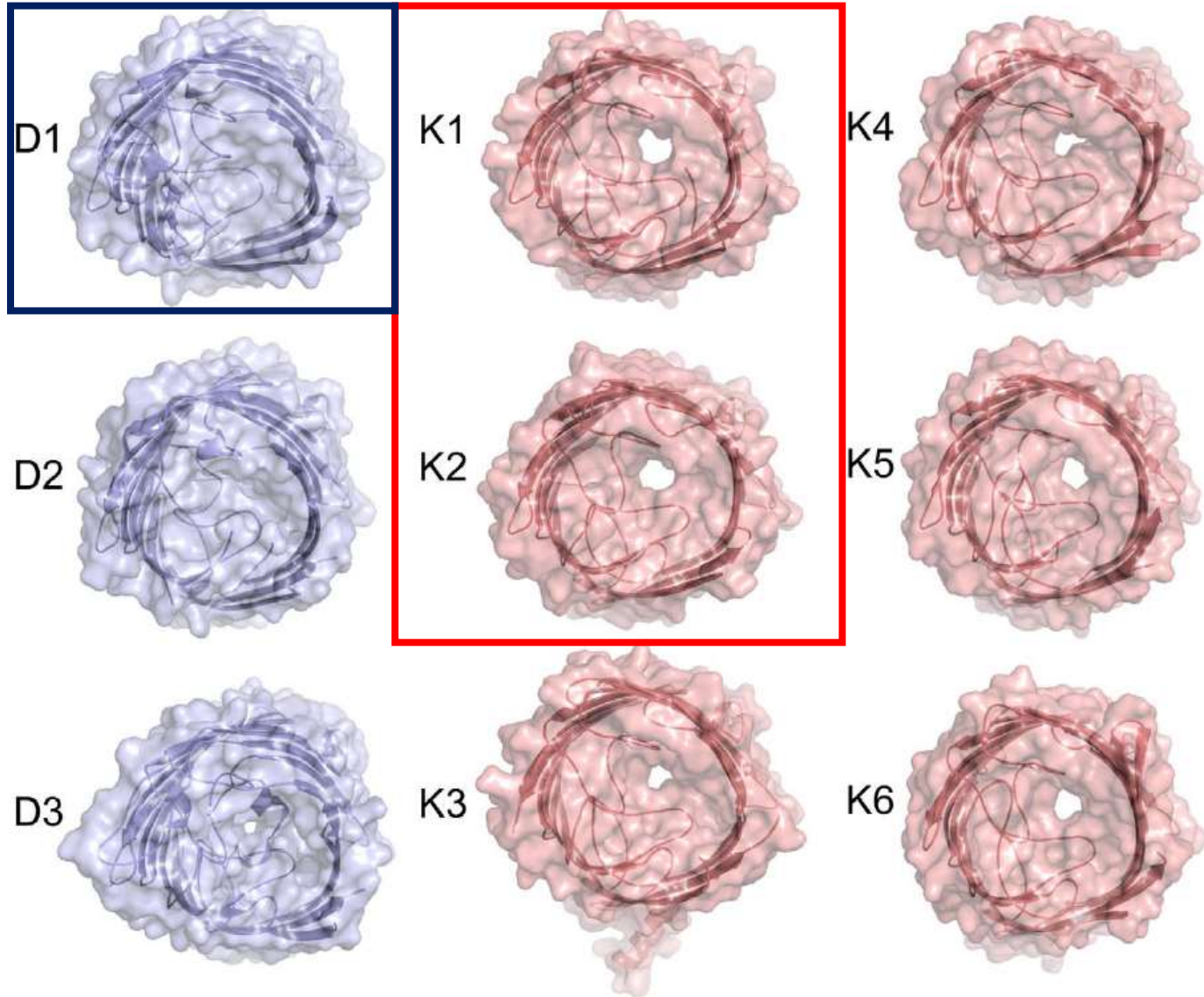
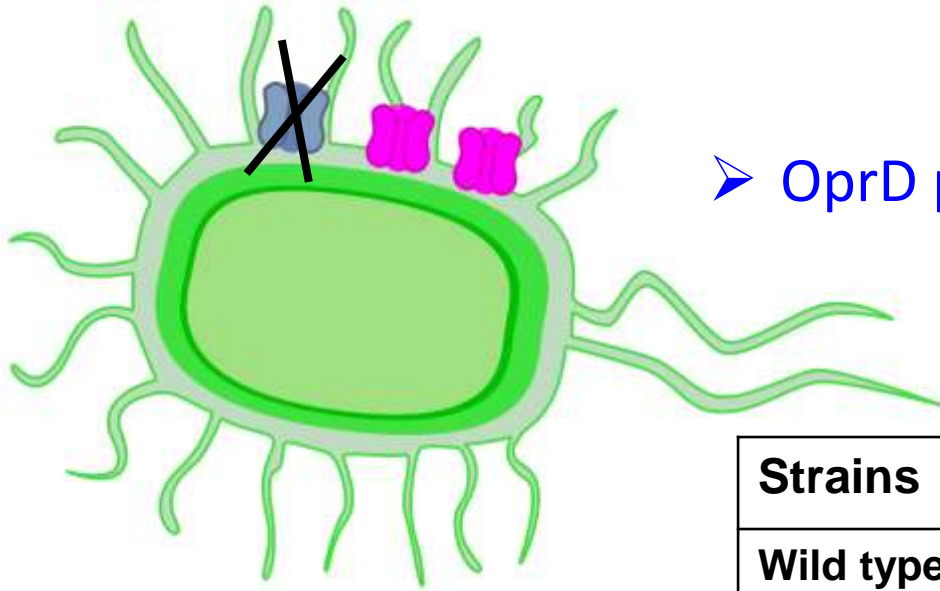


Figure 1. Crystal structures of Occ channels show a wide variety in pore sizes. Transparent surface representations from the extracellular side for OccD1–D3 (blue) and OccK1–K6 (salmon). The channels are shown in identical orientations. This and other structure figures were made with PYMOL (The PyMOL Molecular Graphics System, Schrödinger, LLC).
doi:10.1371/journal.pbio.1001242.g001

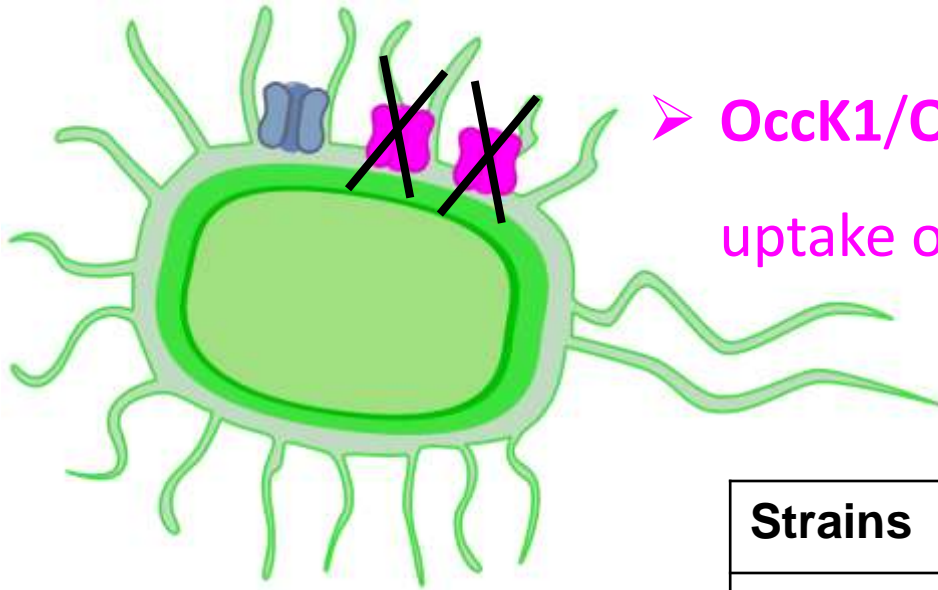
oprD-single mutant of *P. aeruginosa*



➤ *OprD* porin is not utilized by temocillin !

Strains	MICs (mg/L)		
	Temocillin	MEM	IPM
Wild type PA	256	0.37	1
PA:: <i>oprD</i>	256	3	4

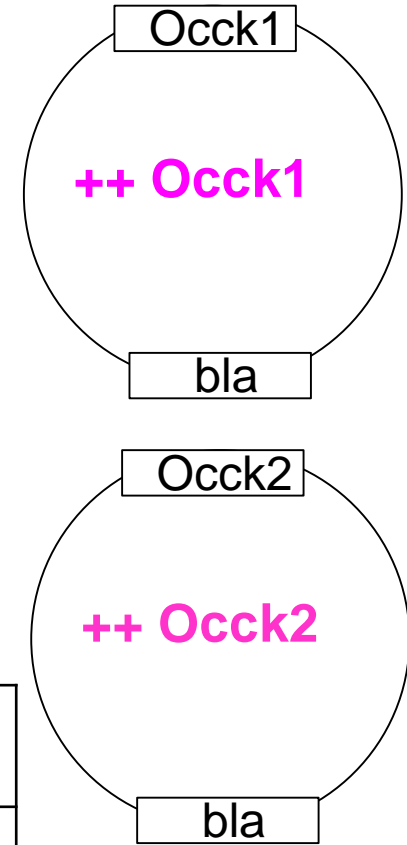
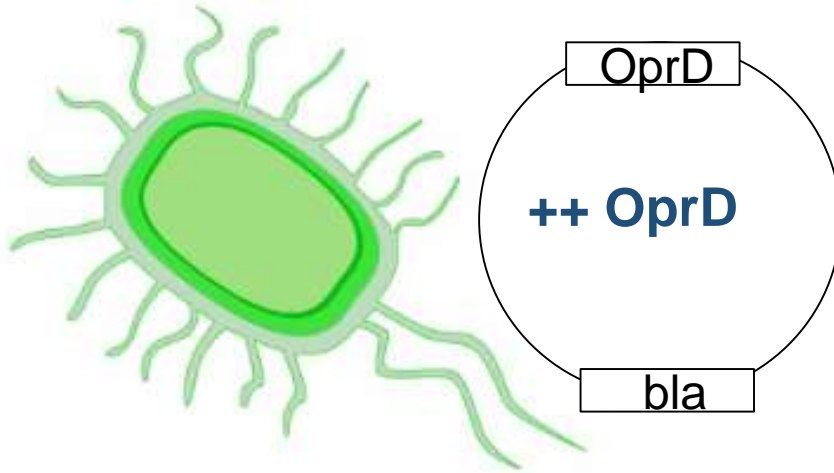
Blocking **OccK1** in **occK2**-single mutant of *P. aeruginosa*



➤ **OccK1/OccK2** porins are involved in the uptake of temocillin

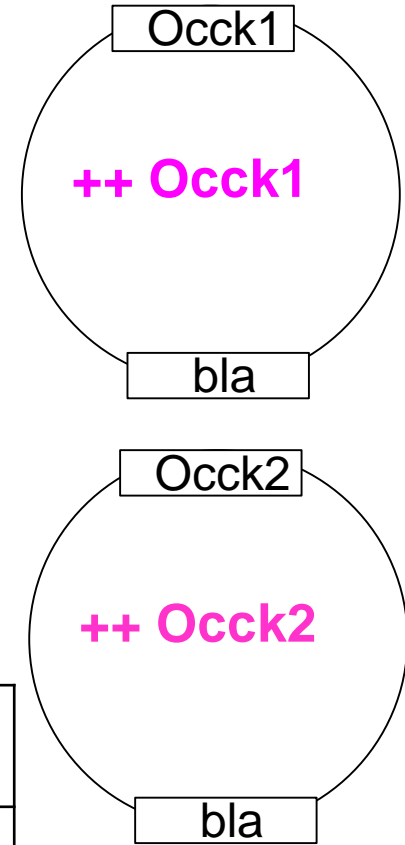
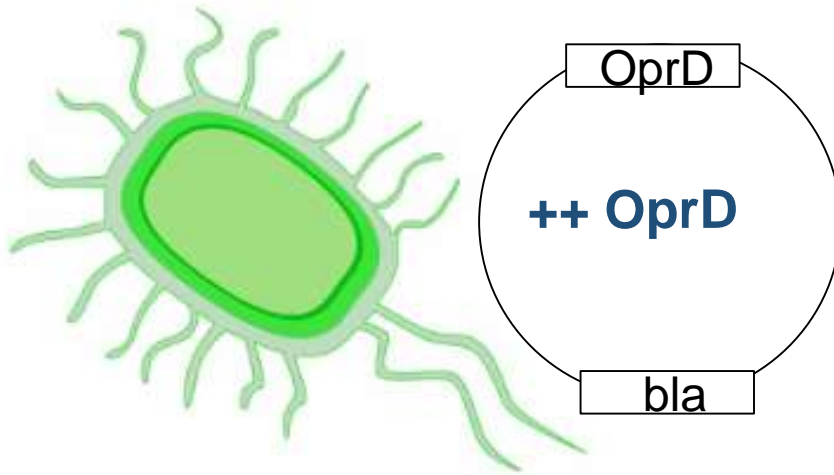
Strains	MICs (mg/L)		
	Temocillin	MEM	IPM
Wild type PA	256	0.37	1
PA:: occK2/1	786	0.37	1

Overexpression of **OccK1** or **OccK2** in porin-deficient *E.coli*



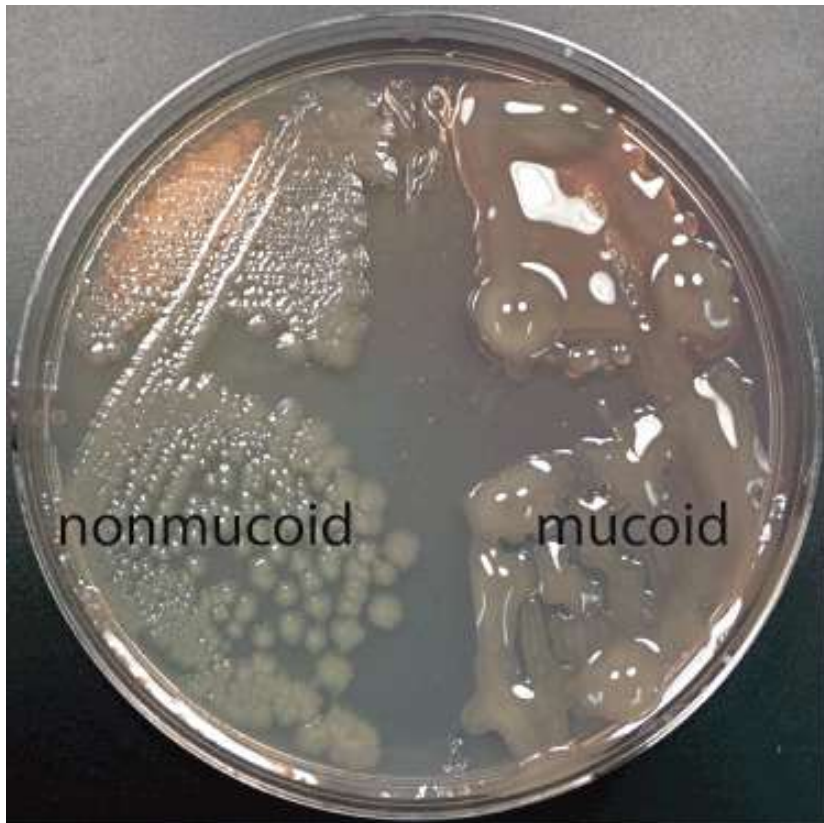
MICs (mg/L)	MEM	IPM	TMO
Empty vector	1	1.5	40
+OprD	0.225	0.225	40
+OccK1	1	1.5	20
+OccK2	1	1.5	22

Overexpression of **OccK1** or **OccK2** in porin-deficient *E.coli*



MICs (mg/L)	MEM	IPM	TMO
Empty vector	1	1.5	40
+OprD	0.225	0.225	40
+OccK1	1	1.5	20
+OccK2	1	1.5	22

Exopolysaccharides (alginate) from *P. aeruginosa*

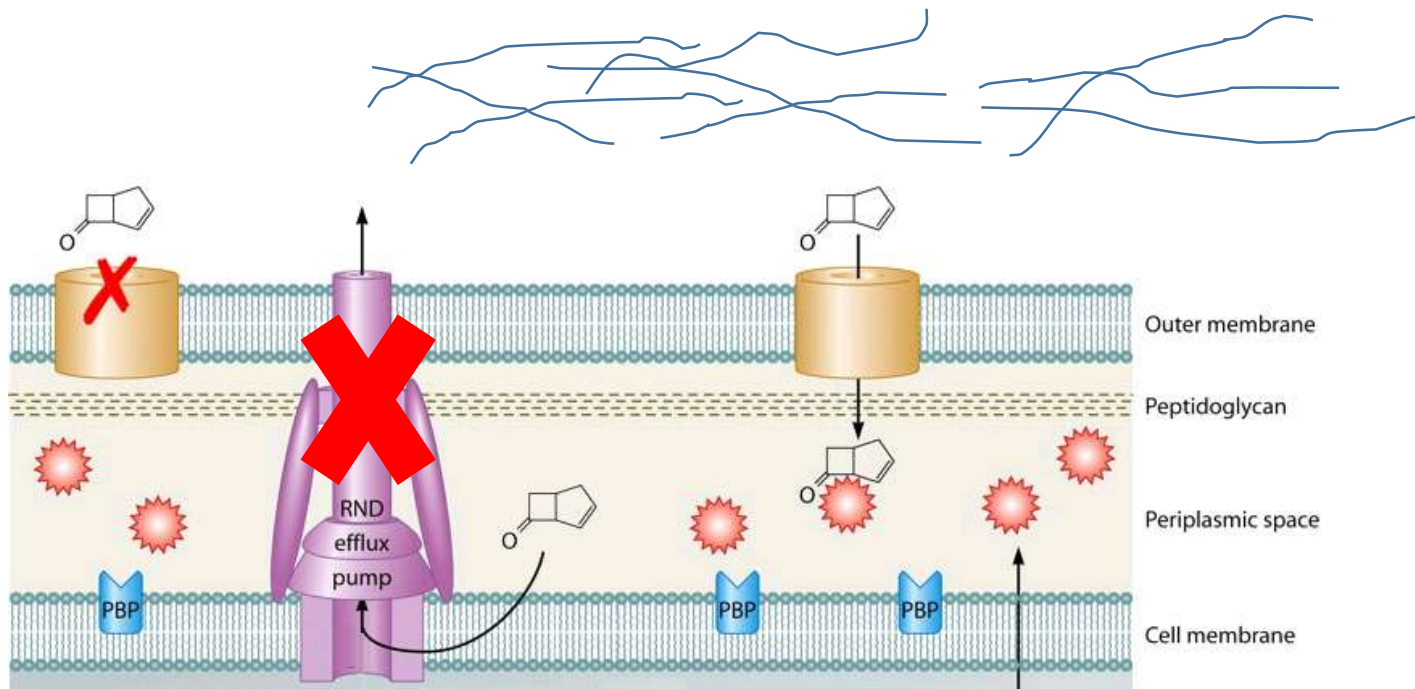


Mol Microbiol. 2012 May;84(4):595-607.



Niels Bagge et al. Antimicrob. Agents Chemother. 2004;48:1175-1187

Exopolysaccharides (alginate-like) ??



The diffusion of beta-lactam antibiotics through mixed gels of cystic fibrosis-derived mucin and *Pseudomonas aeruginosa* alginate.

[Bolister N¹](#), [Basker M](#), [Hodges NA](#), [Marriott C](#).

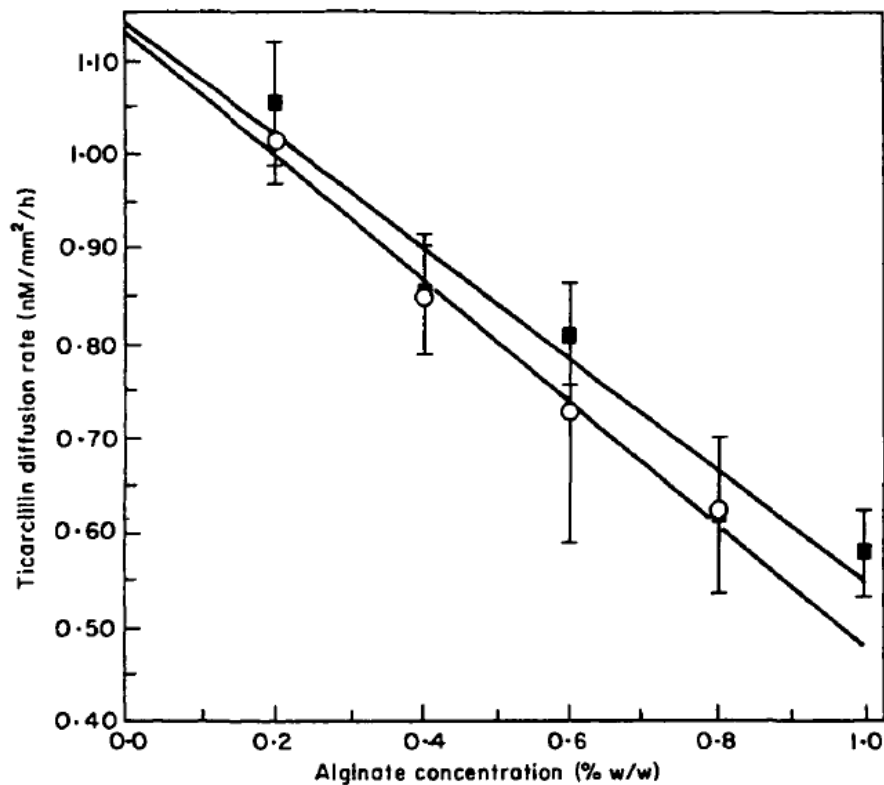
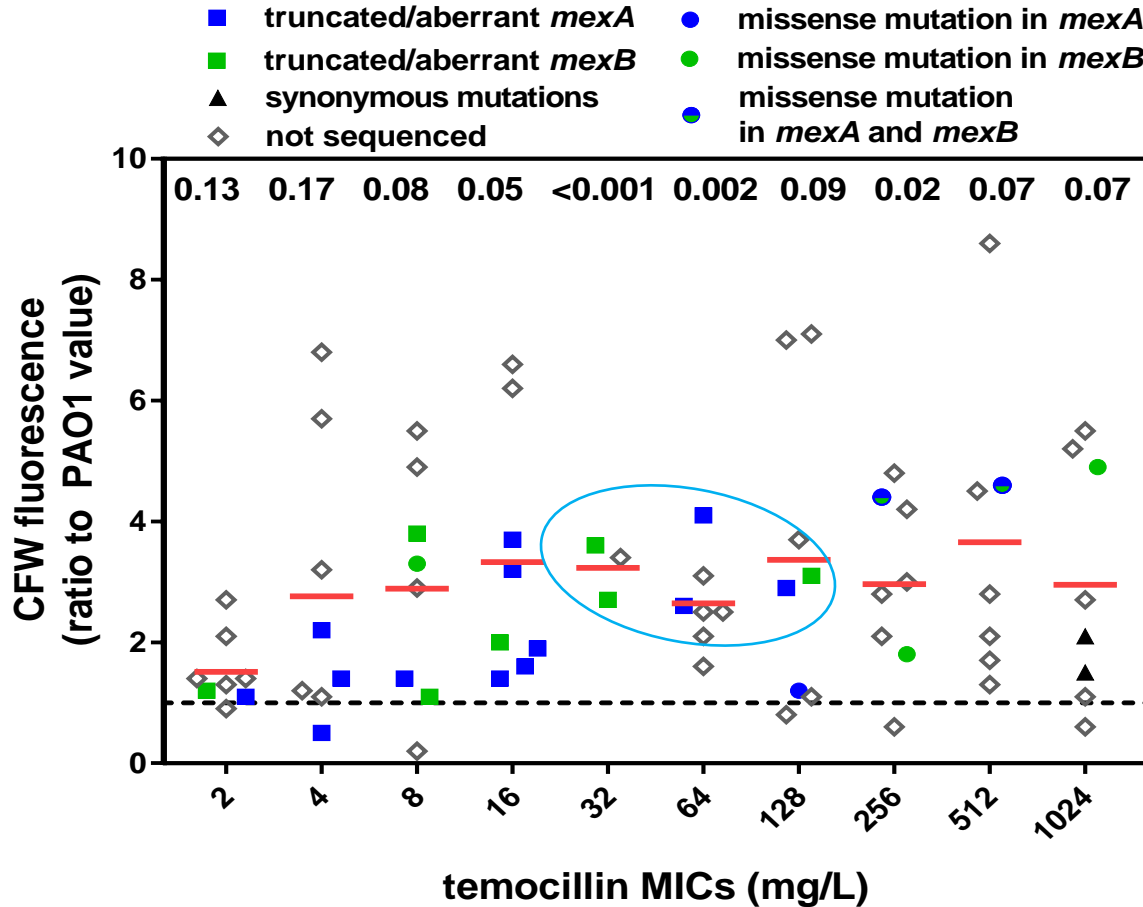


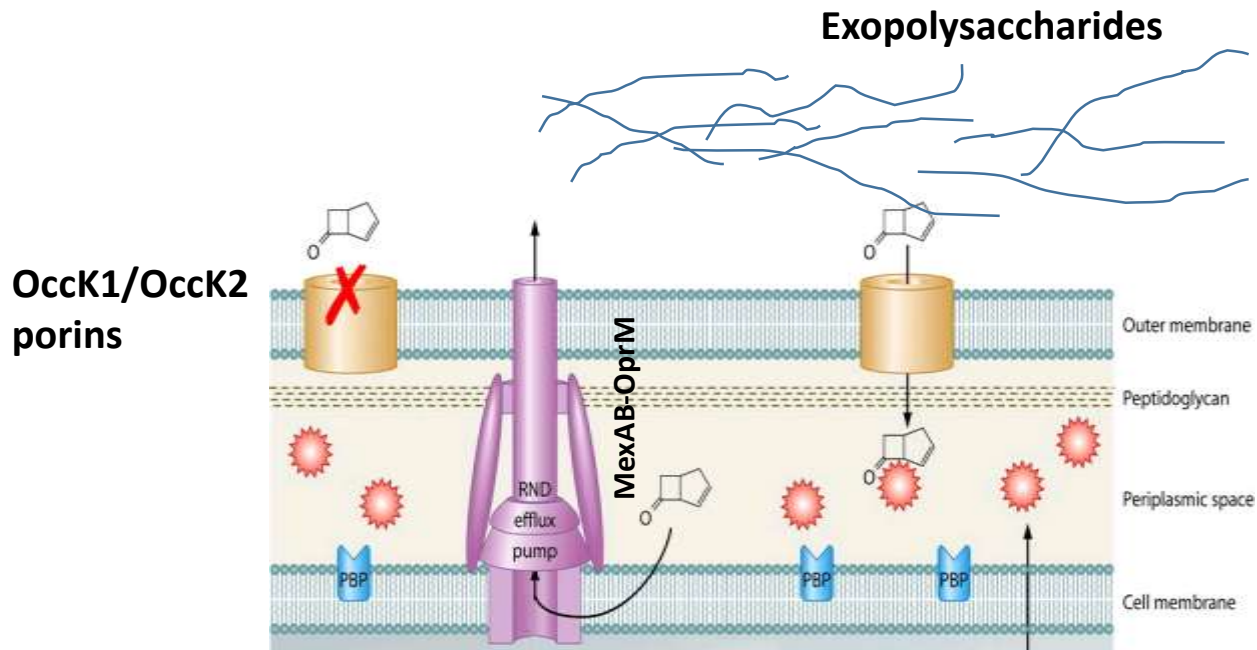
Figure 2. Rate of diffusion of ticarcillin through gels containing alginate alone (■) and alginate/mucin mixtures (○), mean \pm s.d. shown.

Exopolysaccharide abundance in cultures of clinical isolates as a function of temocillin MICs

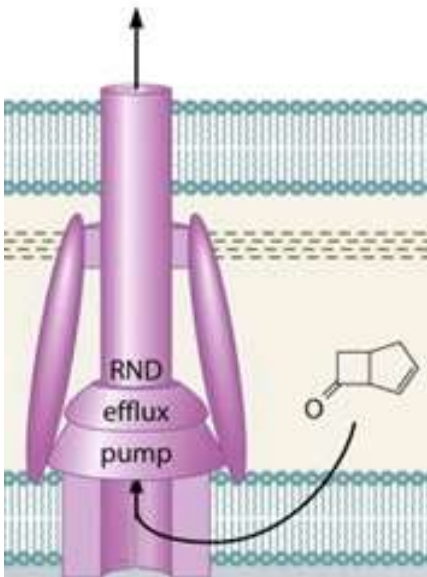


Summary (temocillin in cystic fibrosis)

- ✓ Temocillin resistance is mainly due to active MexAB efflux system with participation of exopolysaccharides production and/or OccK1/OccK2-porin loss !



Impact of **efflux mechanisms** on susceptibility and resistance to
beta-lactam antibiotics in *Pseudomonas aeruginosa*:
beyond the usual concepts



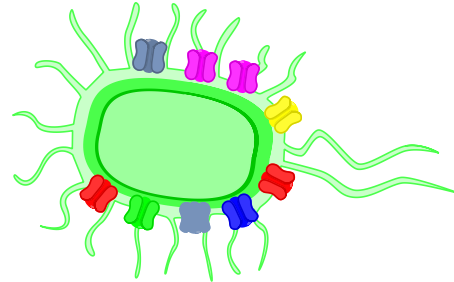
- 1- [Efflux + / porin -] are enough to confer high-level resistance to meropenem
- 2- [Efflux + / porin -] are pre-existing as resistance mechanisms to avibactam, which is not used yet in CF patients !!
- 3- **Mutations in MexAB-OprM efflux pumps appear as a mechanism of restored susceptibility to temocillin in *Pseudomonas* !!!**

Conclusion

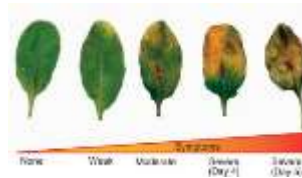
- ❑ Efflux is a key mechanism of resistance for *Pseudomonas* from CF patients.
- ❑ Porins are important players if associated with efflux.
- ❑ Bacterial outer membrane is sufficiently strong barrier against β -lactams.

Conclusion

- ❑ Several paralogous efflux and porin genes to accommodate and extrude different substrates.



- ❑ *Pseudomonas* invades different territories with enough fitness and adaptability.



Conclusion

- ❑ Temocillin should be included in CF antimicrobial susceptibility testing.
- ❑ Temocillin could be also used as carbapenem sparing agent against *Pseudomonas* with OprD porin mutations !



<http://www.bbc.com/news/health-32755065>

General perspectives (research)

- ❑ Check the prevalence of mutations in *mexA/mexB* genes in *Pseudomonas* from a recent CF population (2016/2017).
- ❑ Determine whether *mexA/mexB* mutations in CF are beneficial for the activity of temocillin only or could also affect other substrates.

Perspectives (management of CF infections)

- ❑ Develop fast techniques to screen epidemic and risky clones.
- ❑ Prevent intra- and inter-hospital dissemination of epidemic *Pseudomonas* clones.
- ❑ Establishment of early appropriate and targeted antimicrobial therapies.

Acknowledgments



Cystic Fibrosis centres



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Professor Mathias Winterhalter



JACOBS
UNIVERSITY

Dr Hector Rodriguez-Villalobos



Cliniques universitaires
SAINT-LUC
UCL BRUXELLES

*Un hôpital
pour la Vie*

Acknowledgments



Welcome



Acknowledgments

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