Genetic characterization of lipopolysaccharide-modifying genes involved in polymyxin resistance in *E. coli* and *K. pneumoniae* carrying MCR-1 by sequential time-kill experiments approach

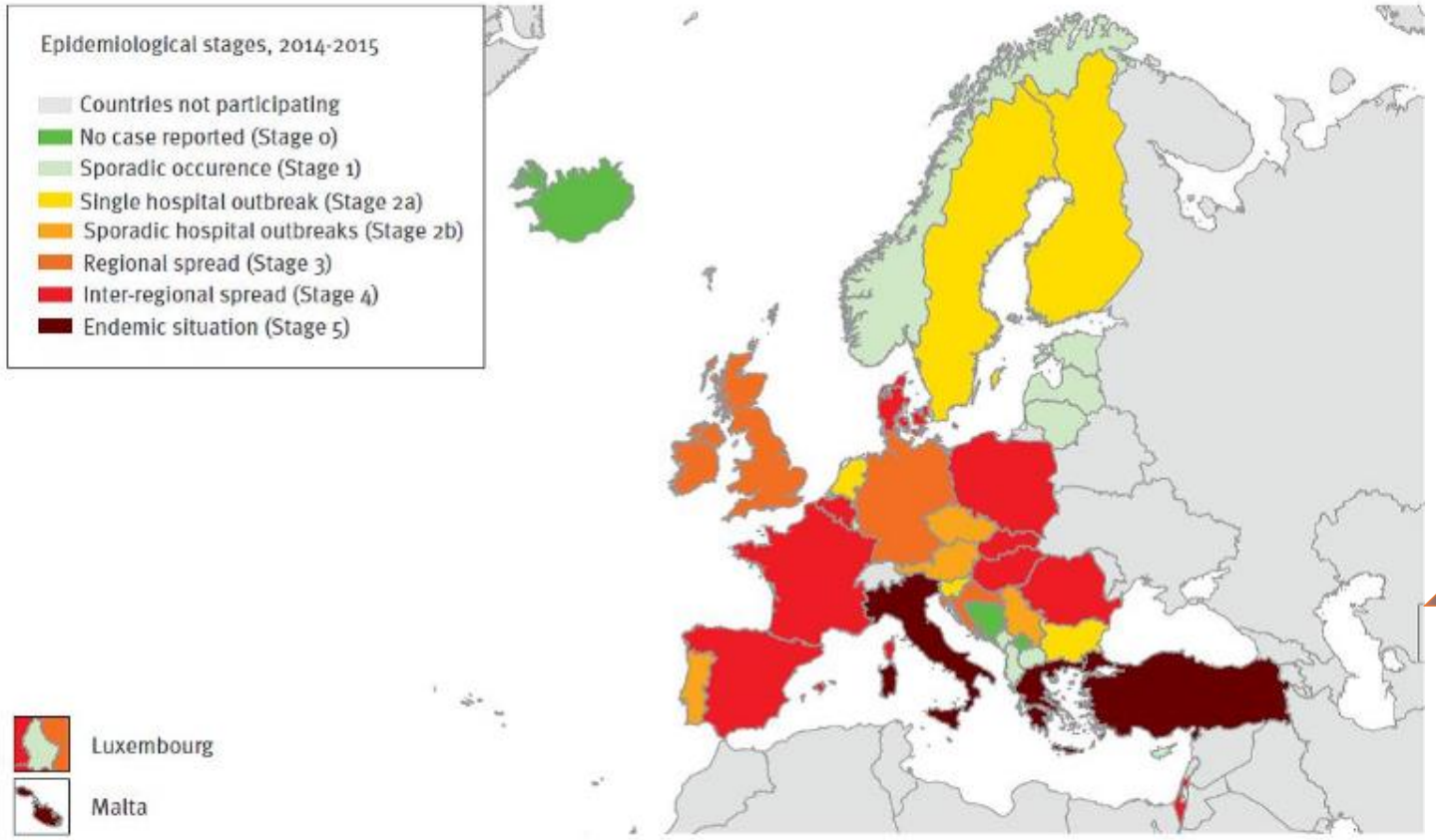
Hariyanto IH

INSERM U1070 – Pharmacology of Antimicrobial Agents

POITIERS

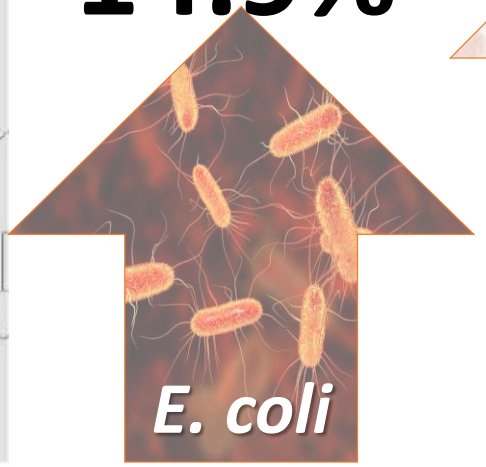
15e congrès national de la SFM, 30 septembre - 2 octobre 2019, Paris

Occurrence of carbapenemase-producing Enterobacteriaceae (*K.pneumoniae* and *E. coli*) in 38 European countries

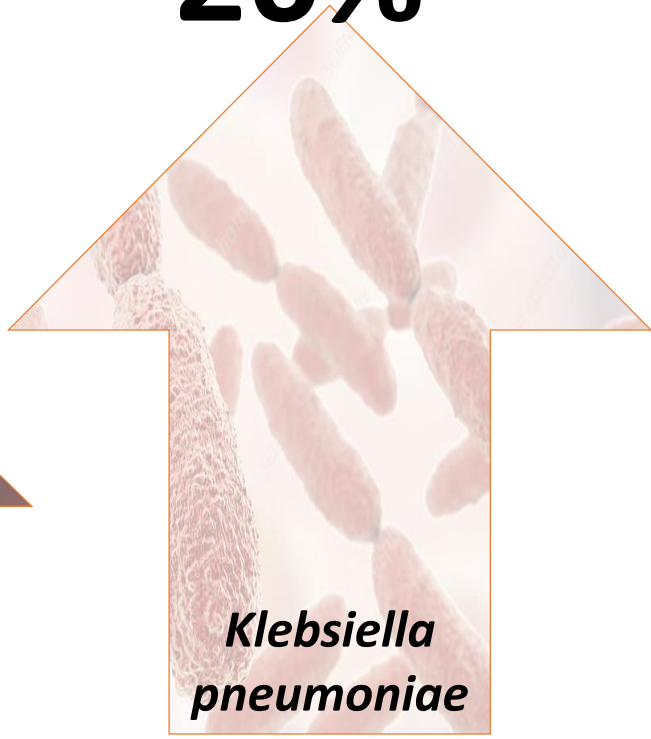


European Centre for Disease Prevention and Control, Stockholm, 2016

14.9%

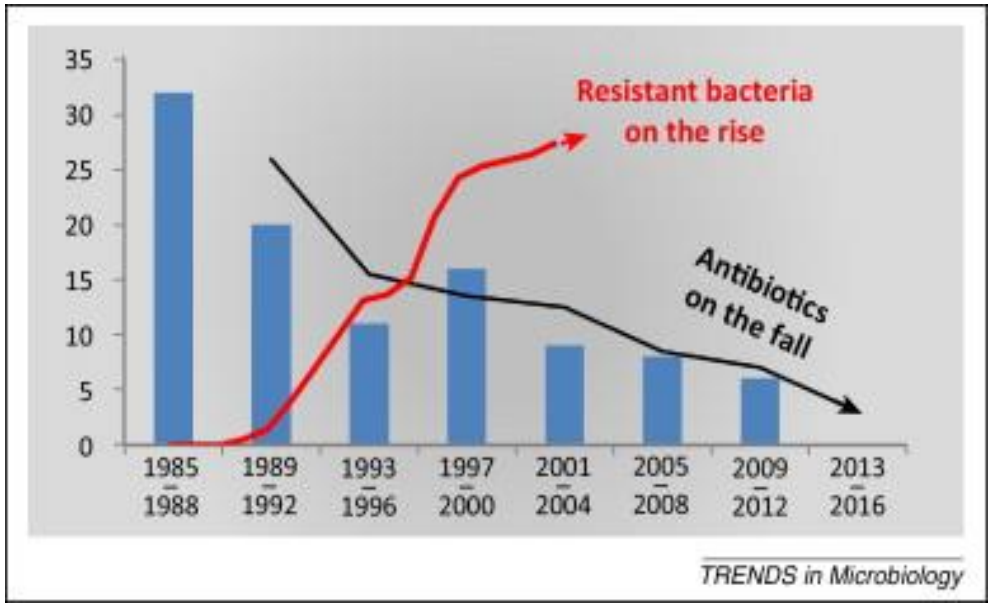


26%



European Antimicrobial Resistance Surveillance Network (EARS-Net), 2017

Antibiotic development and antimicrobial resistance



Solution ?

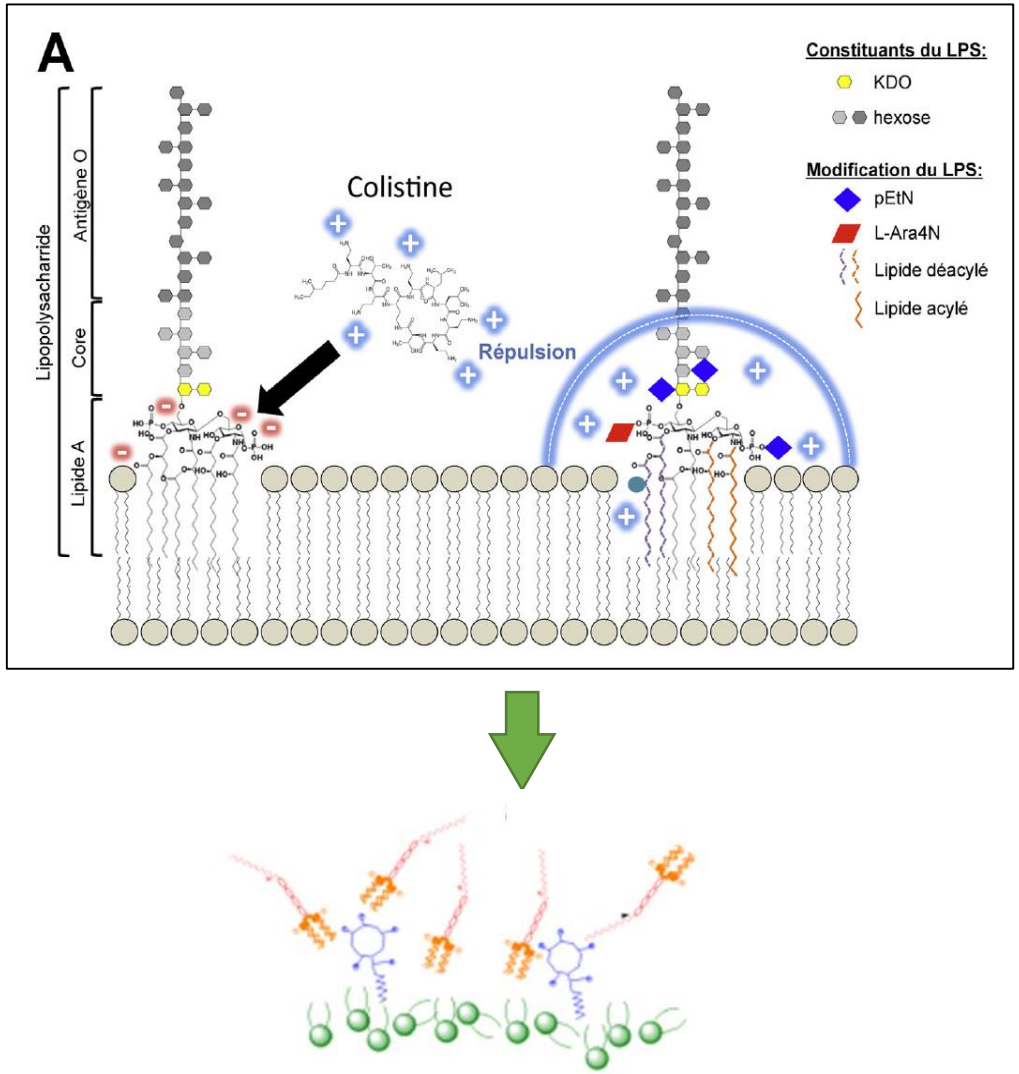
Combine 2 or 3 antibiotics

or

COLISTIN ⟵ Re-introduce ‘old’ antibiotics

Colistin (polymyxin E) & polymyxin B

- Polymyxins class; Cationic Antimicrobials Peptides (CAMPs)
- In 1970s, it was replaced by newer antibiotic because of its side effect (nephrotoxicity >20%)*
- Early 1990s, It is increasingly being used as a "Last resort drug" to overcome infections caused by multidrug-resistant GNB (MDR(-))
- In particular *P. aeruginosa, A. baumannii, K. pneumoniae & E. coli*
- Infections caused by GNB are the most difficult infections to treat because of their ability to develop into the intrinsic drug resistance



**Expert Rev Anti Infect Ther. 2012*

[A] Dortet L, et al. *Émergence de la résistance à la colistine chez les entérobactéries: une brèche dans le dernier rempart contre la pan-résistance*. Journal des Anti-infectieux (2016)

Polymyxin Resistance Reports

ELSEVIER

Journal of Cystic Fibrosis 7 (2008) 391–397

Journal of Cystic Fibrosis
www.elsevier.com/locate/jcf

Spread of colistin resistant non-mucoid *Pseudomonas aeruginosa* among chronically infected Danish cystic fibrosis patients ☆

Helle Krogh Johansen [a,b,*], Samuel M. Moskowitz [c], Oana Ciofu [b], Tacjana Pressler [a], Niels Høiby [a,b]

[a] Department of Clinical Microbiology, Dept. 9301 and Danish Cystic fibrosis Centre, Dept. 5003, Rigshospitalet, Copenhagen Ø, Denmark
[b] Institute of International Health, Immunology and Microbiology, Panum Institute, University of Copenhagen, Copenhagen Ø, Denmark
[c] Division of Pulmonary Medicine, Children's Hospital and Regional Medical Centre and University of Washington School of Medicine, Seattle, Washington 98195, USA

Received 30 September 2007; received in revised form 27 January 2008; accepted 4 February 2008
Available online 20 March 2008

www.nature.com/scientificreports

SCIENTIFIC REPORTS

OPEN

Evolved resistance to colistin and its loss due to genetic reversion in *Pseudomonas aeruginosa*

Received: 13 October 2015
Accepted: 20 April 2016

Ji-Young Lee, Young Kyoung Park, Eun Seon Chung, In Young Na & Kwan Soo Ko

JOURNAL OF CLINICAL MICROBIOLOGY, May 2009, p. 1611–1612
0095-1137/09/$08.00+0 doi:10.1128/JCM.02466-08

Decreased Susceptibility to Polymyxin B during Treatment for Carbapenem-Resistant *Klebsiella pneumoniae* Infection ▽

J Antimicrob Chemother 2012; **67**: 1607–1615
doi:10.1093/jac/dks084 Advance Access publication 22 March 2012

Journal of Antimicrobial Chemotherapy

Colistin resistance of *Acinetobacter baumannii*: clinical reports, mechanisms and antimicrobial strategies

Yun Cai, Dong Chai, Rui Wang*, Beibei Liang and Nan Bai

Department of Clinical Pharmacology, the PLA General Hospital, Beijing 100853, People's Republic of China

BRAZ J INFECT DIS 2017;21(1):98–101

The Brazilian Journal of INFECTIOUS DISEASES
www.elsevier.com/locate/bjid

Brief communication

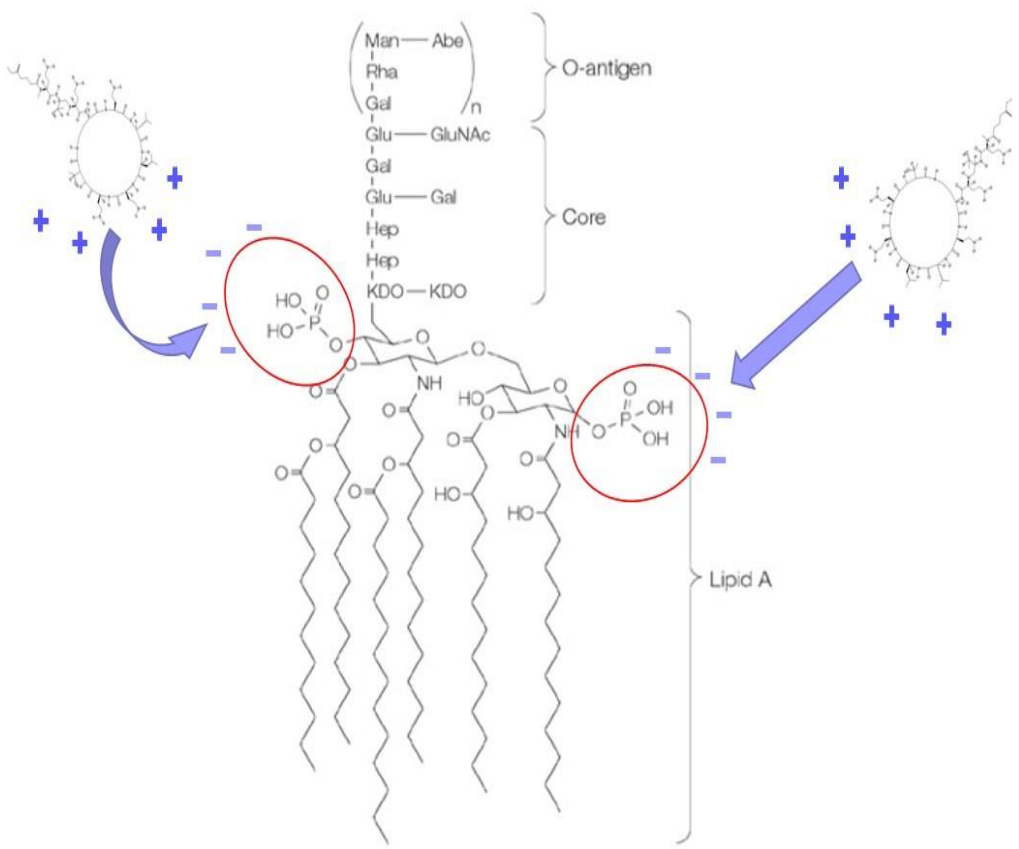
Emergence of colistin resistance in the largest university hospital complex of São Paulo, Brazil, over five years ☆

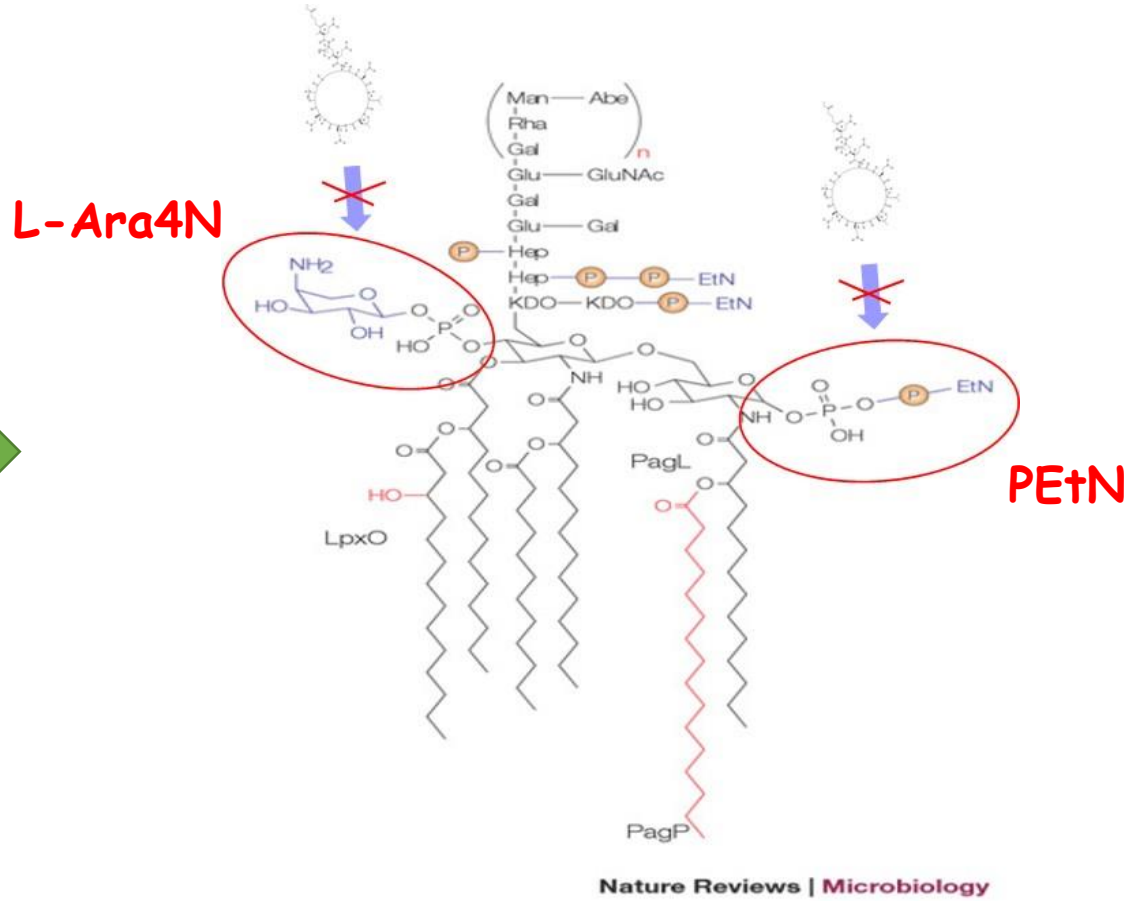
Flávia Rossi [a,b,], Raquel Girardello [a,b], Ana Paula Cury [a,b], Thais Sabato Romano Di Gioia [a,b], João Nóbrega de Almeida Jr [a,b], Alberto José da Silva Duarte [b]*

[a] Universidade de São Paulo, Hospital das Clínicas da Faculdade de Medicina, Divisão Laboratório Central, São Paulo, SP, Brazil
[b] Universidade de São Paulo, Faculdade de Medicina, Medicina Laboratorial – LIM-03, São Paulo, SP, Brazil

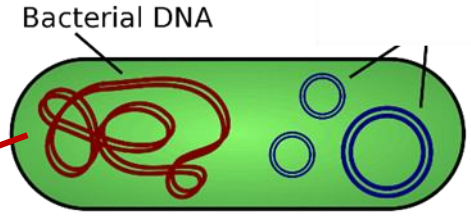
All Reports from clinical isolates

LPS Modification





Molecular Pathway of LPS Modification



Two-Component regulatory systems (TCS)

PhoQ
PmrB
mgrB
PhoP
P
PhoP
MicA
PmrA
PmrA
P
pmrD
Activation of PmrA/PmrB
*pagL
Deacylation of lipid A
MgrR
eptB
PEtN addition to KDO
etk
arnBCADTEF operon
L-Ara4N addition to the LPS
pmrCAB operon
PEtN addition to lipid A
cptA
PEtN addition to the LPS core
pmrR
lpxT
Phosphorylation of lipid A
lpxR
Deacylation of lipid A
pmrE
L-Ara4N addition to the LPS

Olaitan et al. 2014. **Frontiers in Microbiology** | Antimicrobials, Resistance and Chemotherapy

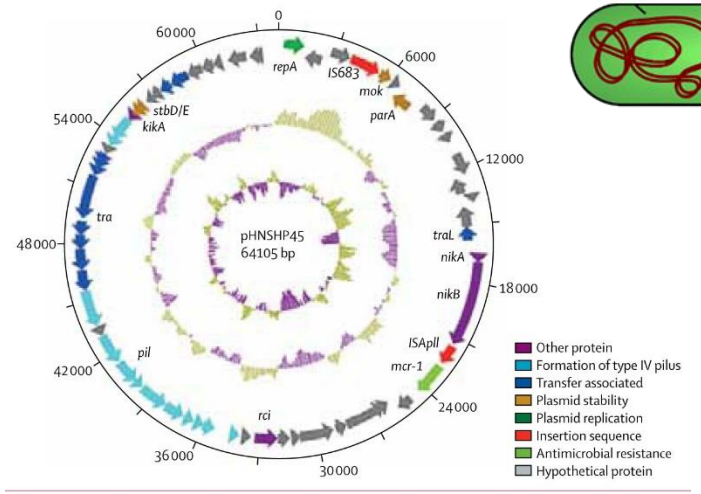
Plasmid-Mediated Resistance

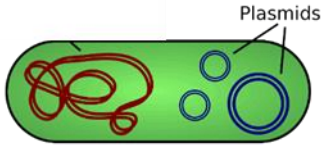
< Previous Article Volume 16, No. 2, p161–168, February 2016 Next Article >

Articles

Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study

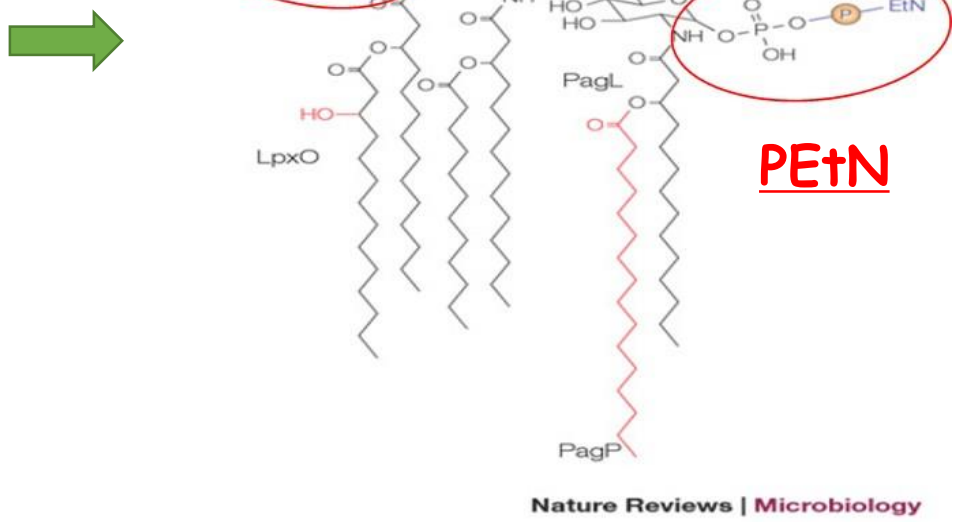
Yi-Yun Liu, BS†, Yang Wang, PhD†, Prof Timothy R Walsh, DSc, Ling-Xian Yi, BS, Rong Zhang, PhD, James Spencer, PhD, Yohei Doi, MD, Guobao Tian, PhD, Baolei Dong, BS, Xianhui Huang, PhD, Lin-Feng Yu, BS, Danxia Gu, PhD, Hongwei Ren, BS, Xiaojie Chen, MS, Luchao Lv, MS, Dandan He, MS, Hongwei Zhou, PhD, Prof Zisen Liang, MS, Prof Jian-Hua Liu, PhD, Prof Jianzhong Shen, PhD





	Year	Positive isolates (%)/number of isolates
Escherichia coli		
Pigs at slaughter	All	166 (20·6%)/804
Pigs at slaughter	2012	31 (14·4%)/216
Pigs at slaughter	2013	68 (25·4%)/268
Pigs at slaughter	2014	67 (20·9%)/320
Retail meat	All	78 (14·9%)/523
Chicken	2011	10 (4·9%)/206
Pork	2011	3 (6·3%)/48
Chicken	2013	4 (25·0%)/16
Pork	2013	11 (22·9%)/48
Chicken	2014	21 (28·0%)/75
Pork	2014	29 (22·3%)/130
Inpatient	2014	13 (1·4%)/902
Klebsiella pneumoniae		
Inpatient	2014	3 (0·7%)/420

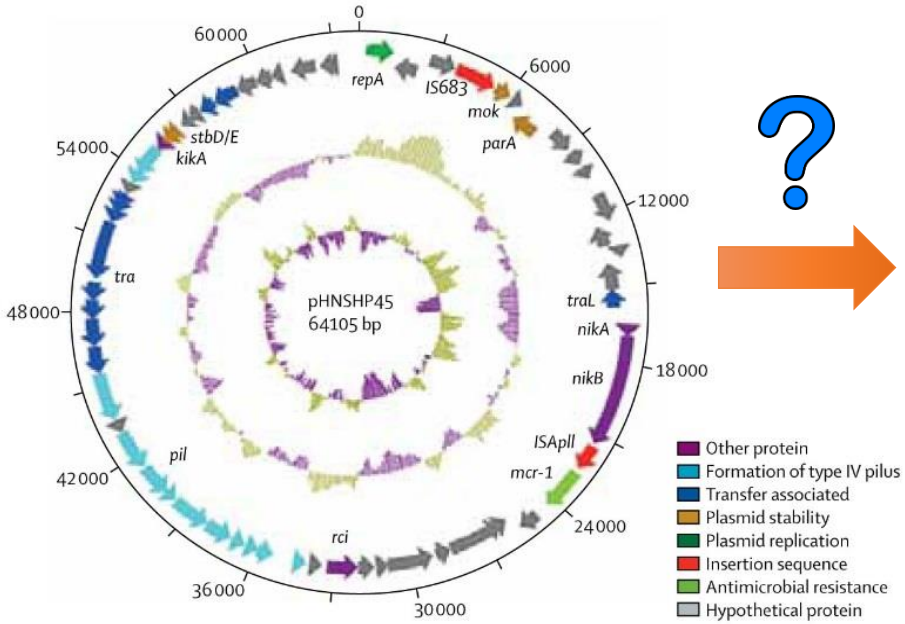
Table 2: Prevalence of colistin resistance gene *mcr-1* by origin

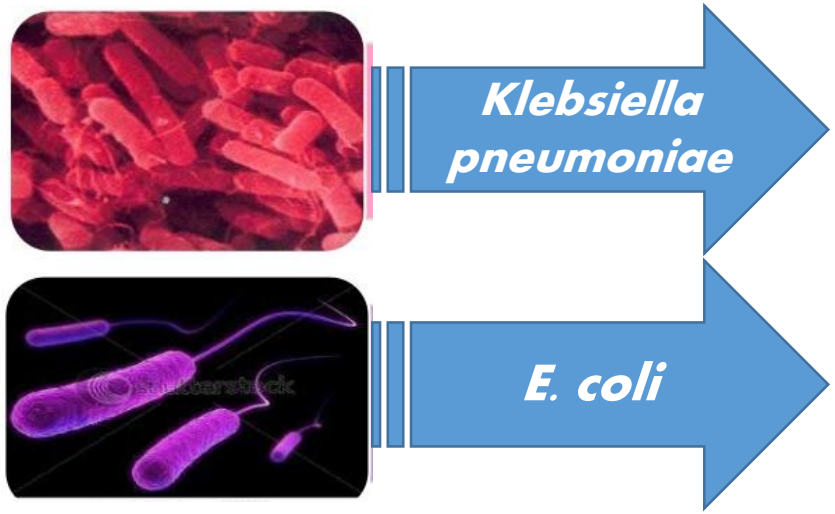


MCR : Mobilizable Colistin Resistance

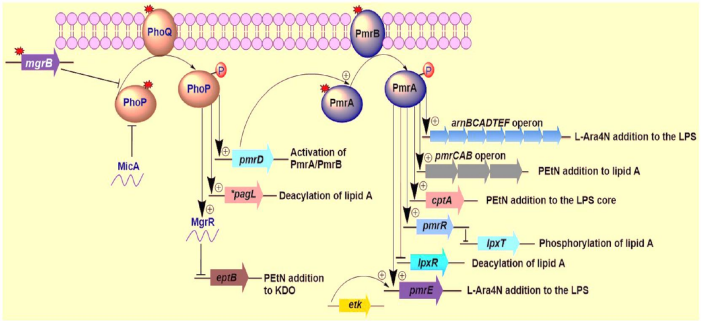
→ **Phosphoethanolamine transferase (addition of PEtN to lipid A)**

MCR-1





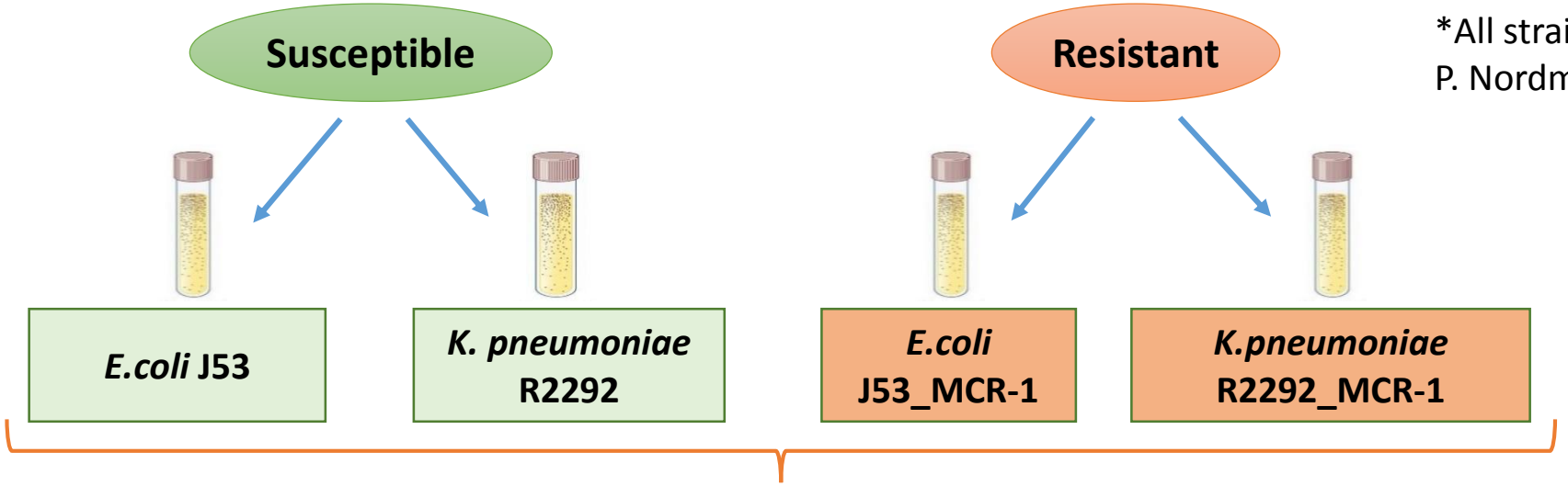
Two-Component regulatory systems (TCS)



Objective

Role of MCR-1 in the development of additional adaptive resistance to polymyxins by an original approach of sequential time-kill study

*All strains were kindly provided by P. Nordmann



Population Analysis Profils (PAPs)

MICs Determination (After TKC)

1st Time-Kill Curve

2nd Time-Kill Curve

3rd Time-Kill Curve

Biomolecular analysis

DNA Sequencing
- To determine the order of nucleotides in specific DNA
- Genome mutations

RT – qPCR Analysis
- RNA Extraction → cDNA
- Quantitatively measure gene expression based on RNA transcript levels

MICs Result (mg/L)

Bacteria / Antibiotic	*E. coli* J53		*K. pneumoniae* R2292	
	WT	+ MCR-1	WT	+ MCR-1
Colistin (CST)	0,25	2-4	0,25	2
Polymixin B (PMB)	0,25	2	0,25	2

WT : Wild-type (non-carrying-MCR-1)
+ MCR-1 : inserted by plasmid MCR-1
Susceptible : MICs < 2 µg/mL
Resistant : MICs ≥ 2 µg/mL

TIME-KILL CURVE ANALYSIS

- Colistin (CST) & Polymyxin B (PMB) shown rapid and concentration-dependent bacterial killing during Time-Kill Curve (TKC)

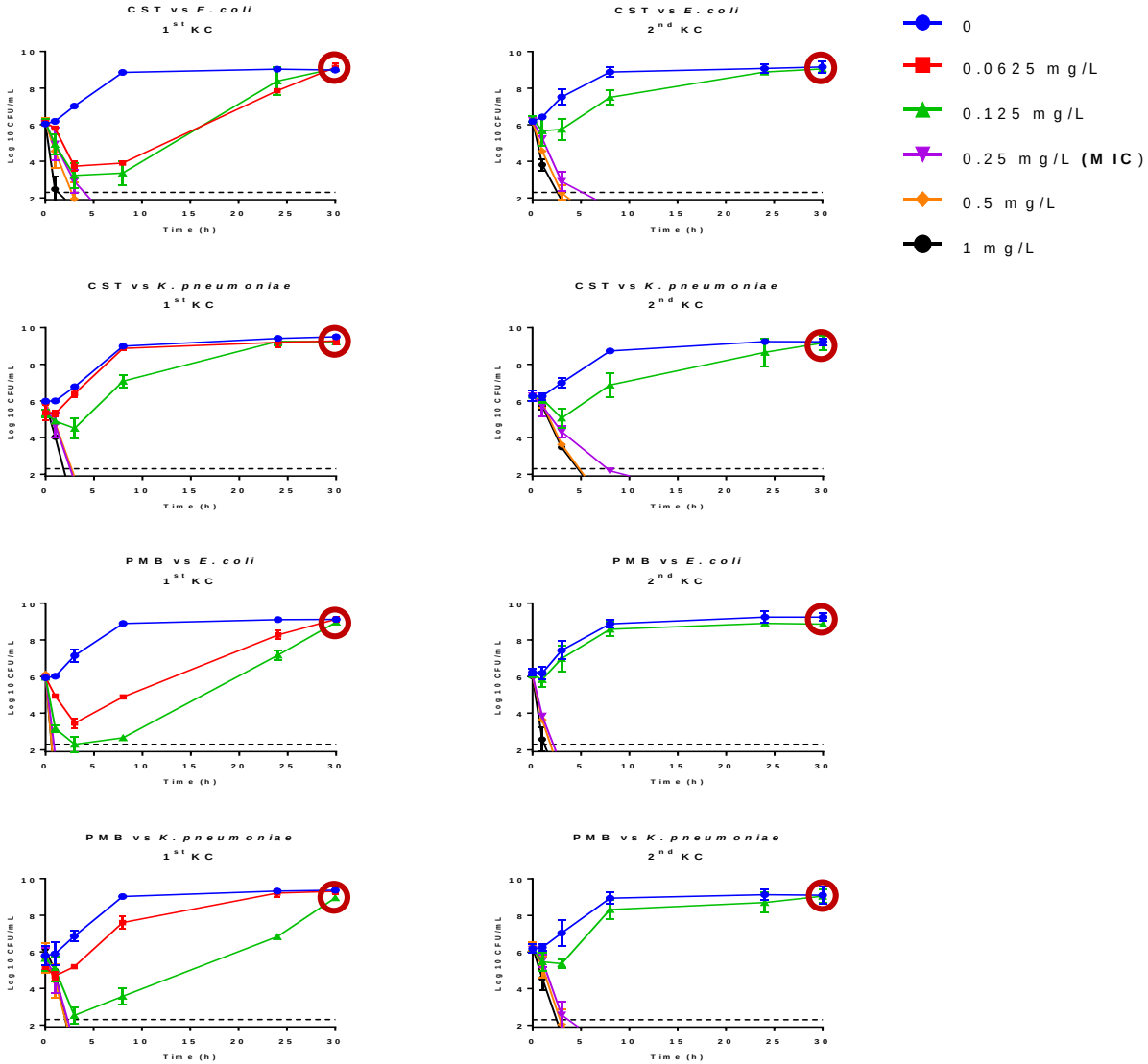
- The highest concentration of antibiotic where bacterias can regrowth over 10^6 CFU/mL after 30 hours considered as **MAXIMUM REGROWTH CONCENTRATION**

- For all WT Strains (not-carrying-MCR-1), the regrowth was stable and observed at 0,5x MIC (0.125 mg/L) in both of 1st and 2nd TKC

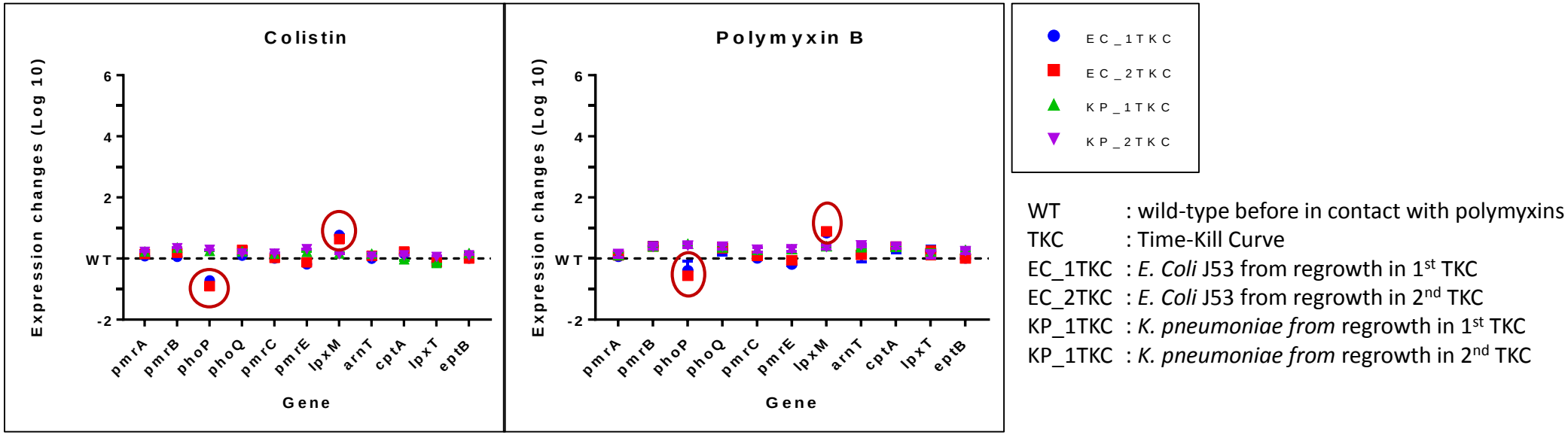
No Adaptation was found

RT-qPCR Analysis

Sequential Time-Kill Curve
Polymyxins vs Wild-type



Genes expression level after sequential TKC for WT strains



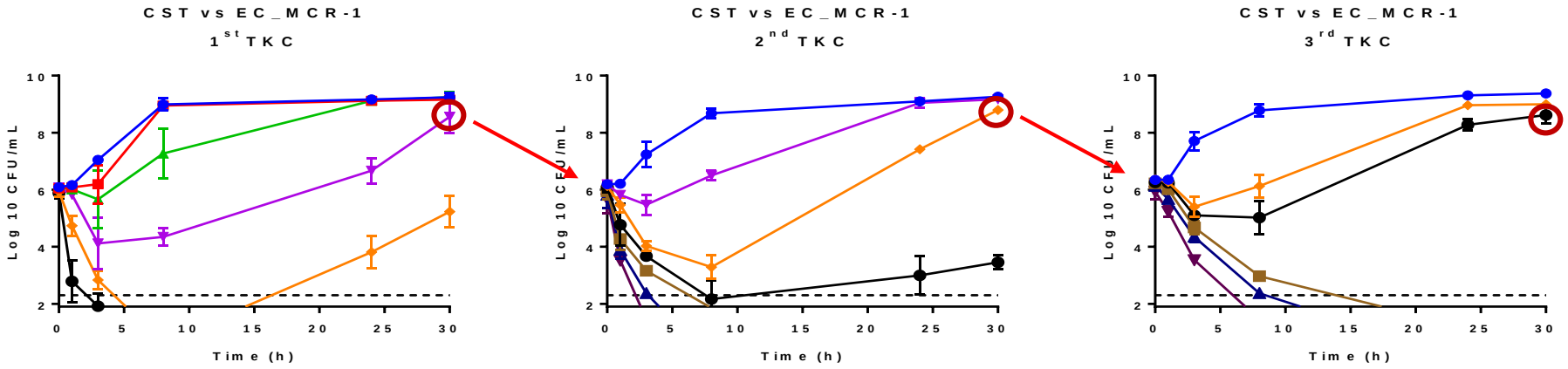
WT : wild-type before in contact with polymyxins
TKC : Time-Kill Curve
EC_1TKC : *E. Coli* J53 from regrowth in 1st TKC
EC_2TKC : *E. Coli* J53 from regrowth in 2nd TKC
KP_1TKC : *K. pneumoniae from* regrowth in 1st TKC
KP_1TKC : *K. pneumoniae from* regrowth in 2nd TKC

Fig. Relative expression of genes for all WT strains after sequential Time-Kill Curves (n=3)

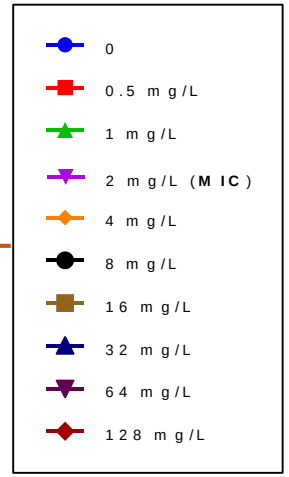
- No different gene expression was shown between 1st and 2nd TKC for both species
- Down-expression of *phoP* and over-expression of *lpxM* for *E. coli* in CST & PMB
- Presumably were triggered by polymyxins pressure

Sequential Time-Kill Curve
Colistin vs MCR-1 transconjugants

COLISTIN vs *E. coli*_MCR-1

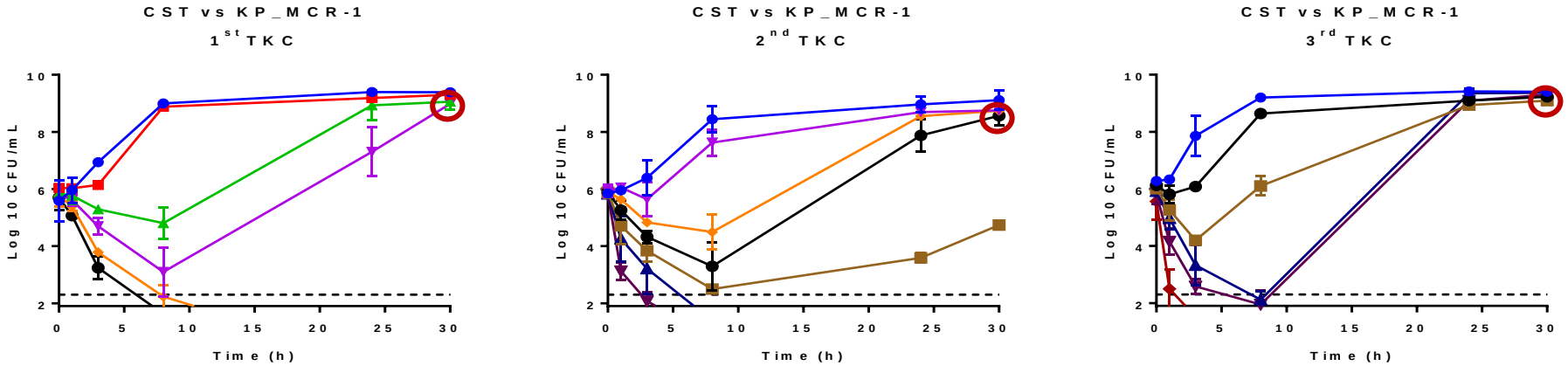


- Maximum regrowth concentrations are:
  2x MIC (4 mg/L) for 2nd TKC
  and
  4x MIC (8 mg/L) for 3rd TKC



***K. Pneumoniae* with MCR-1** develop to high-level colistin resistance **HIGHER** than ***E.coli*_MCR_1**

COLISTIN vs *K. pneumoniae*_MCR-1

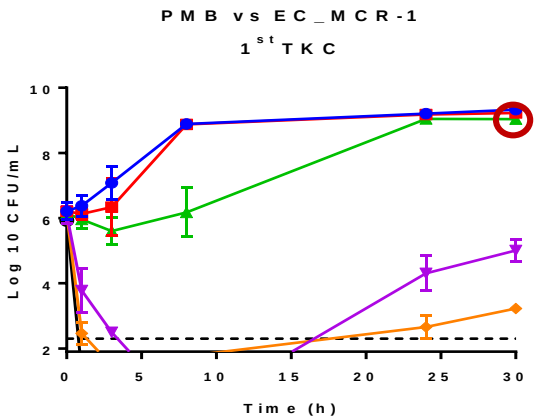


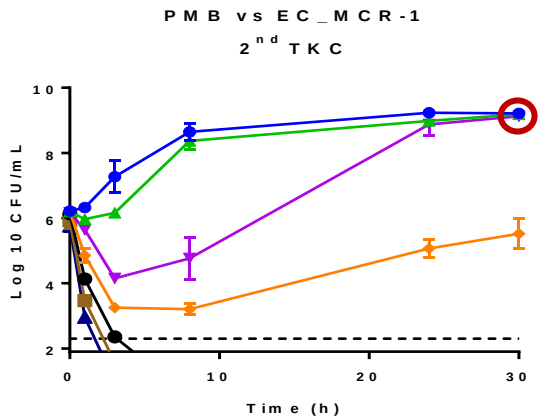
- Maximum regrowth concentration increased gradually up to
  4x MIC (8 mg/L) for 2nd TKC
  and
  32x MIC (64 mg/L) for 3rd TKC

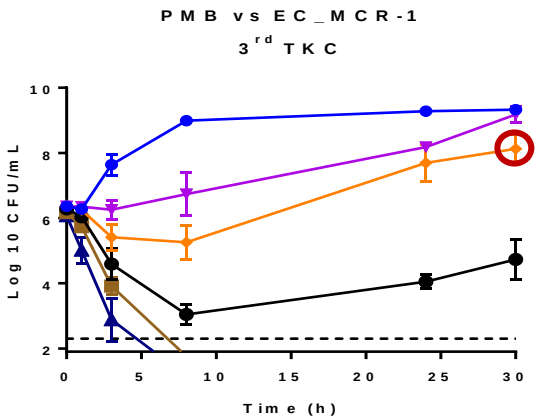
Sequential Time-Kill Curve
Polymyxin B vs MCR-1 transconjugants

Regrowth ?

Polymyxin B vs *E. coli*_MCR-1

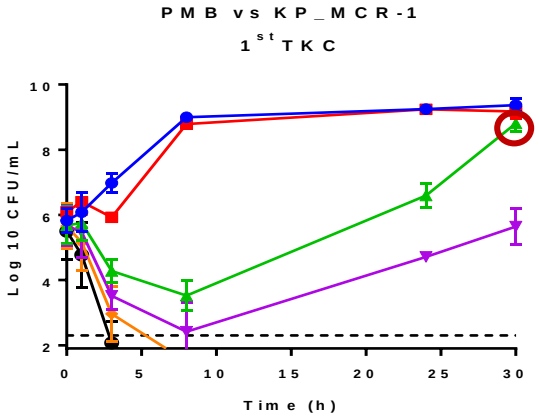


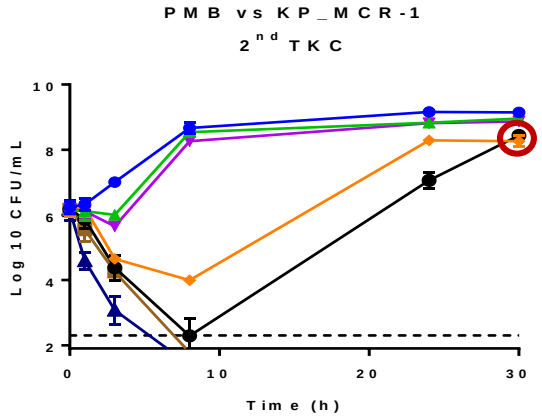


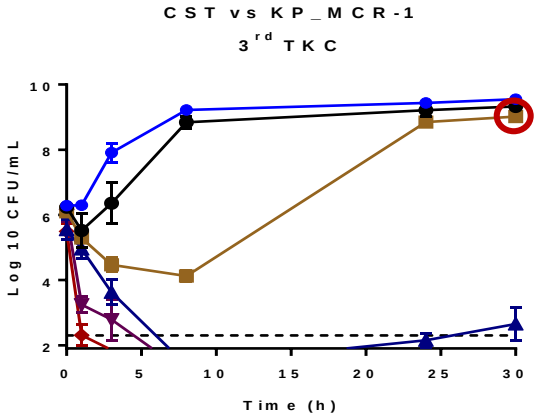


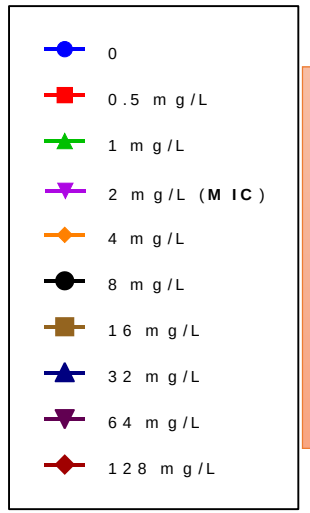
NO ANTIBIOTIC DEGRADATION was found AFTER 30 HOURS

Polymyxin B vs *K. pneumoniae*_MCR-1







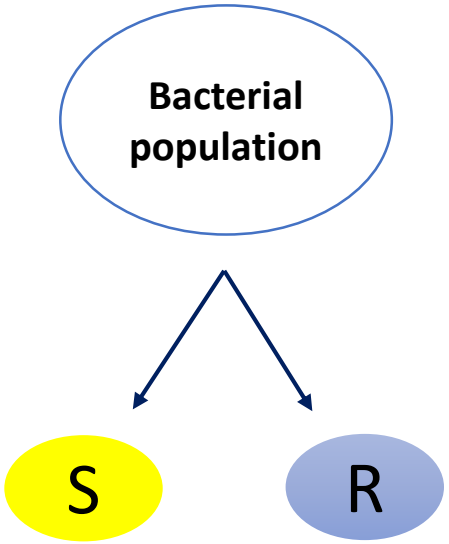


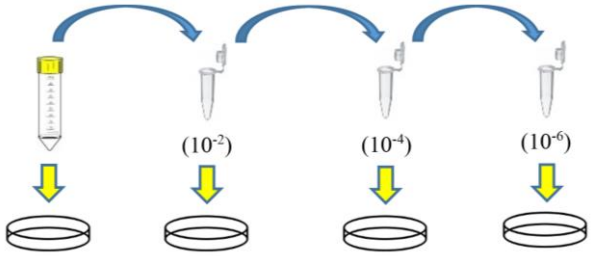
- Maximum regrowth concentrations are :
  1x MIC (2 mg/L) for 2nd TKC
  and
  2x MIC (4 mg/L) for 3rd TKC

Both **MCR-1 transconjugants strains** adapted rather slowly in PMB than in CST

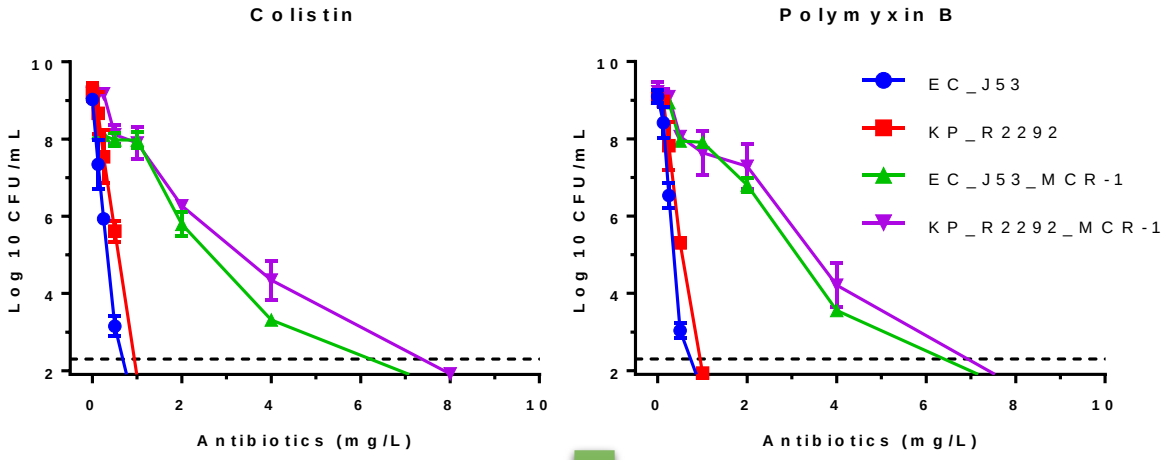
- Maximum regrowth concentrations are :
  4x MIC (8 mg/L) for 2nd TKC
  and
  8x MIC (16 mg/L) for 3rd TKC

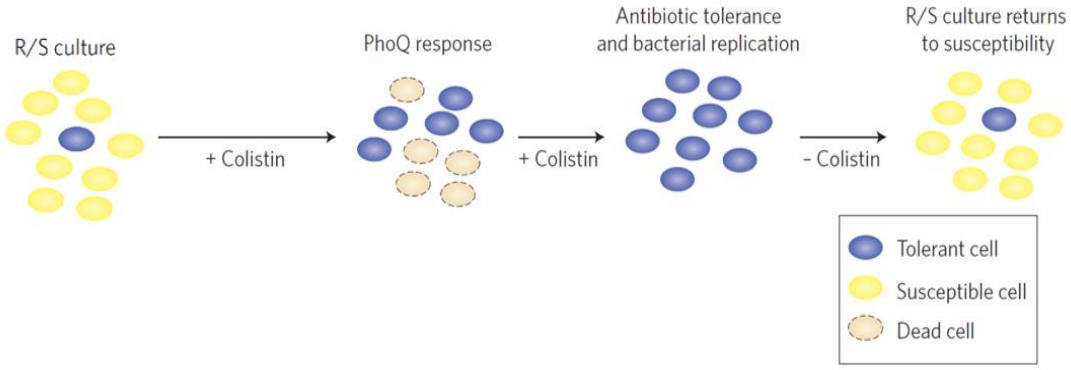
Population analysis profiles (PAPs)





Spiral plating in MH agar with various concentrations of ATB
(0, 0.125, 0.25, 0.5, 1, 2, 4, 8, 10 mg/L)





Both carrying-MCR-1 strains had NO ABLE TO GROW in the presence of antibiotics at concentrations higher than 2 mg/L (cutoff at 10^6 CFU/mL)

REGROWTH in SEQUENTIAL TKC WERE NOT CAUSED by RESISTANT SUBPOPULATION

MICs (mg/L) after Sequential Time-Kill Curve

Strain	Colistin	Polymyxin B
EC	0,25	0,125
EC_1st TKC	0,25	0,25
EC_2nd TKC	0,25	0,25
EC_MCR-1	2	2
EC_MCR-1_1st TKC	8	4
EC_MCR-1_2nd TKC	16	8
EC_MCR-1_3rd TKC	32	16
KP	0,25	0,25
KP_1st KC	0,25	0,25
KP_2nd KC	0,25	0,25
KP_MCR-1	2	2
KP_MCR-1_1st TKC	16	8
KP_MCR-1_2nd TKC	64	16
KP_MCR-1_3rd TKC	512	128

LOW-Level Resistance

HIGH-Level Resistance

LOW-Level Resistance

HIGH-Level Resistance

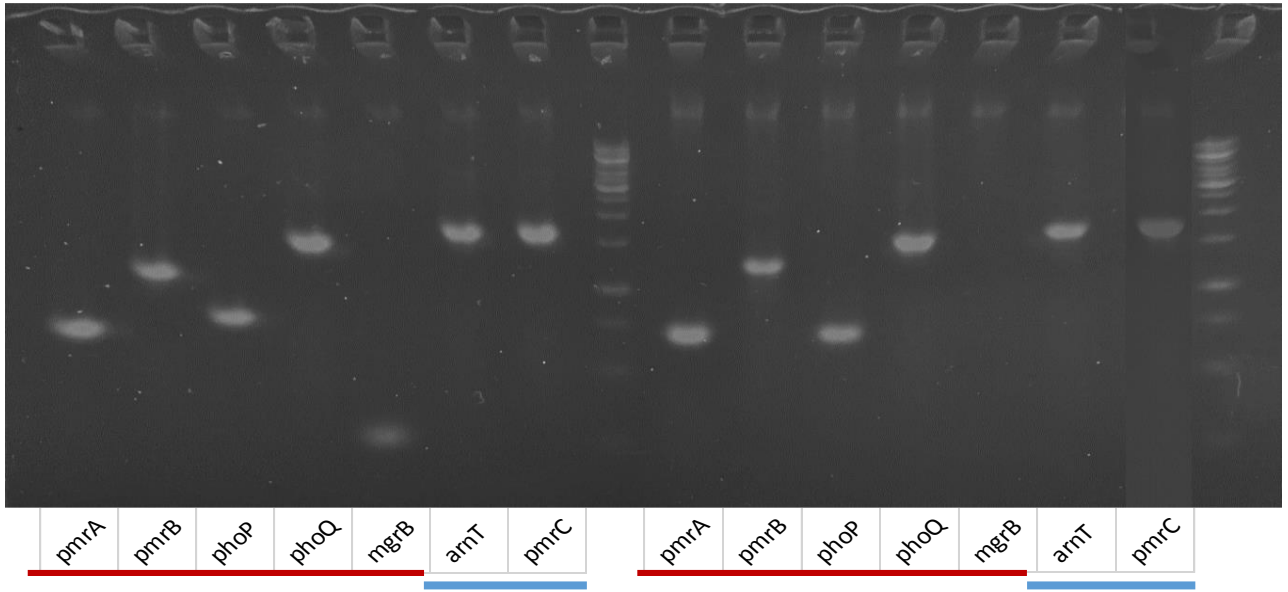
EC : *E. Coli* J53
KP : *K. Pneumoniae* R2292
EC_MCR-1 : *E. Coli* carrying-MCR-1
KP_MCR-1 : *K. Pneumoniae* carrying-MCR-1

The presence of MCR-1 may able to develop High-level polymyxin resistance (HLPR)

***K. Pneumoniae*_MCR-1 highly adapted in CST & PMB high concentration**

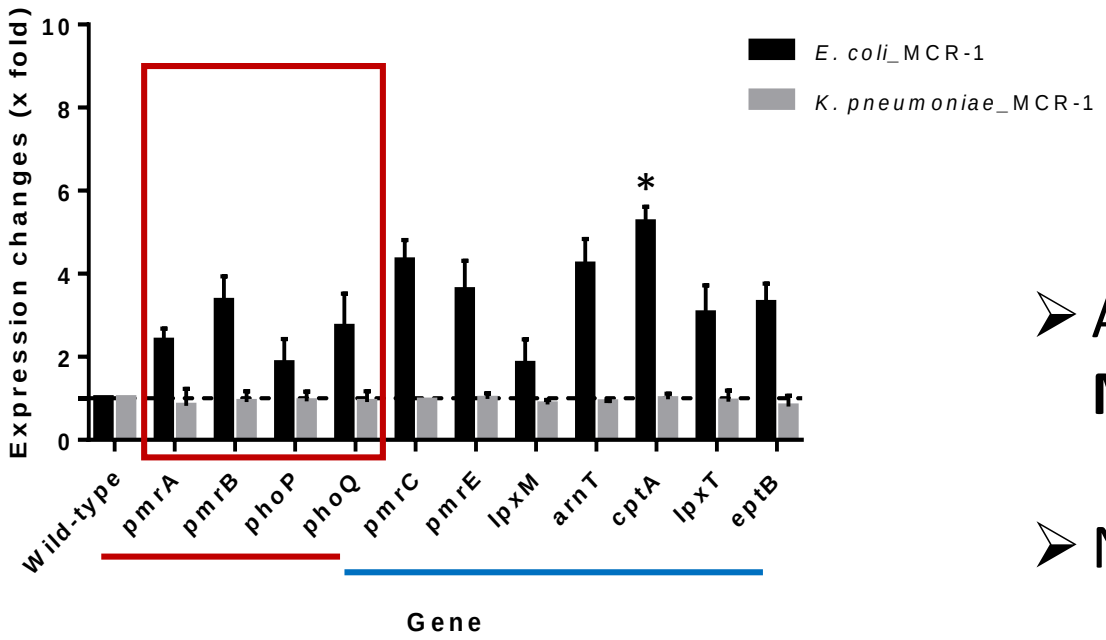
BIOMOLECULAR STUDIES

DNA Sequencing



- 7 genes were determined
- Analysis was performed for all strains before and after sequential TKC
- **NO mutations were found**

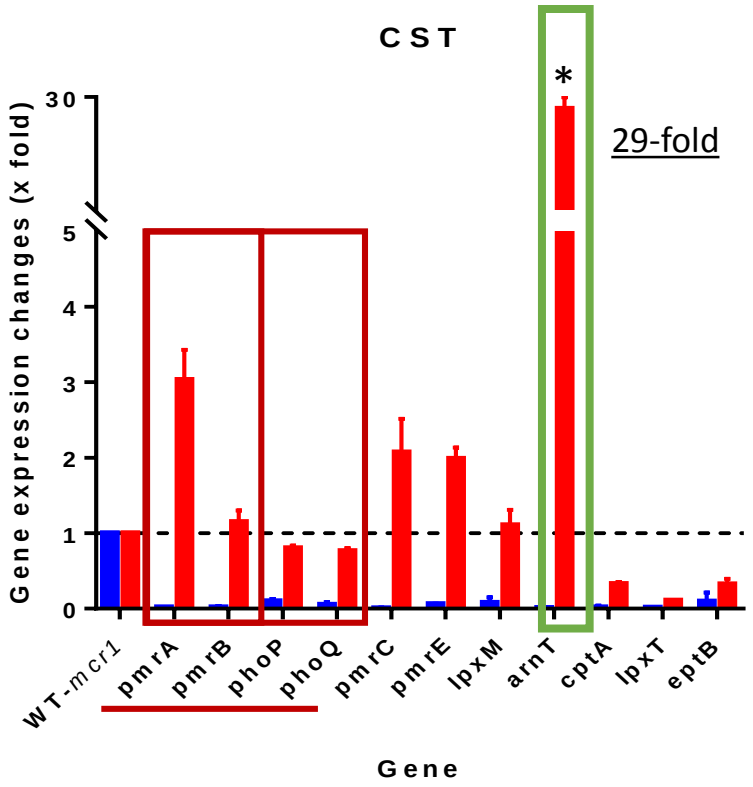
Gene expression profiles by RT-qPCR
Before Sequential TKC
(*no contact with antibiotic*)

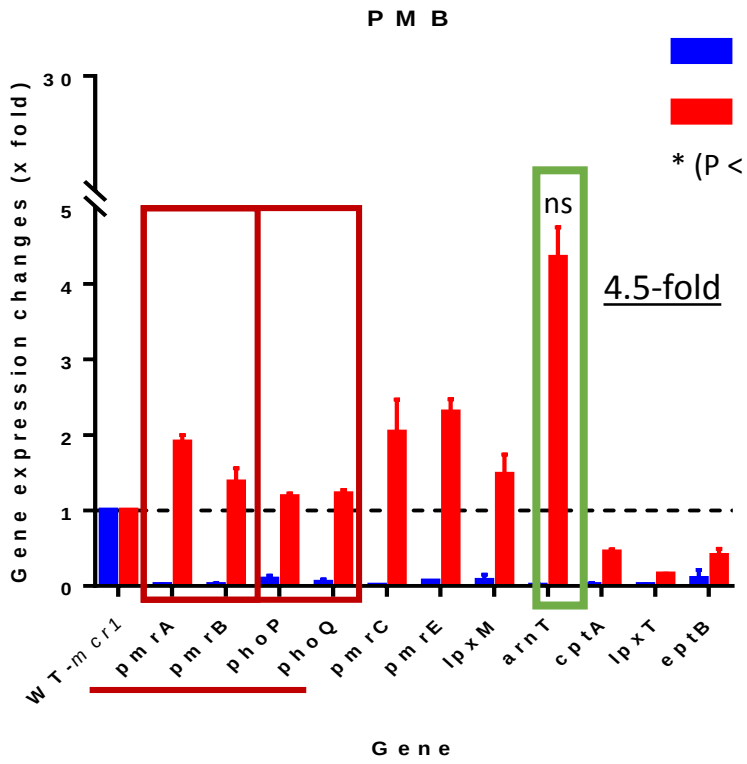


- All genes had over-expressed in ***E.coli*** since **MCR-1** plasmid was firstly inserted
- NO overexpression in ***K.pneumoniae*_MCR-1**

Fig. Relative expression of genes for *E.coli* J53 and *K.pneumoniae* R2292 carrying-MCR-1 before Sequential Time-Kill Curves was performed (n=3)
*($P < 0.05$)

Gene expression profiles AFTER 3rd Time-Kill Study (*Antibiotic challenge*)

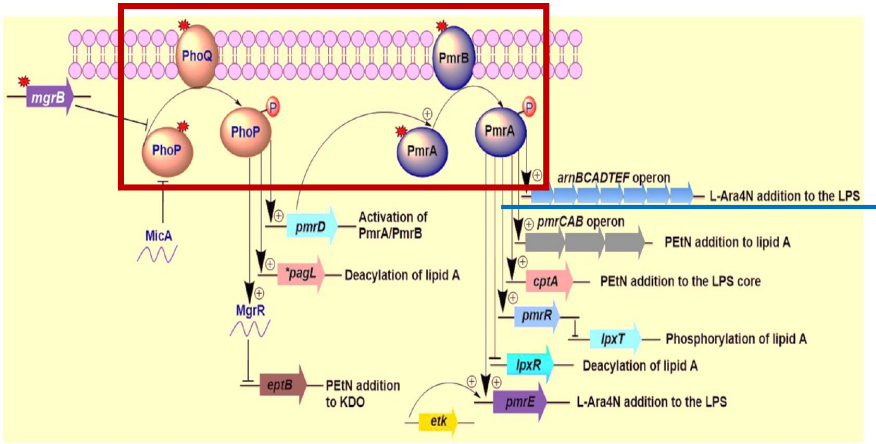




EC_MCR-1_3TKC

KP_MCR-1_3TKC

* (P < 0.05)

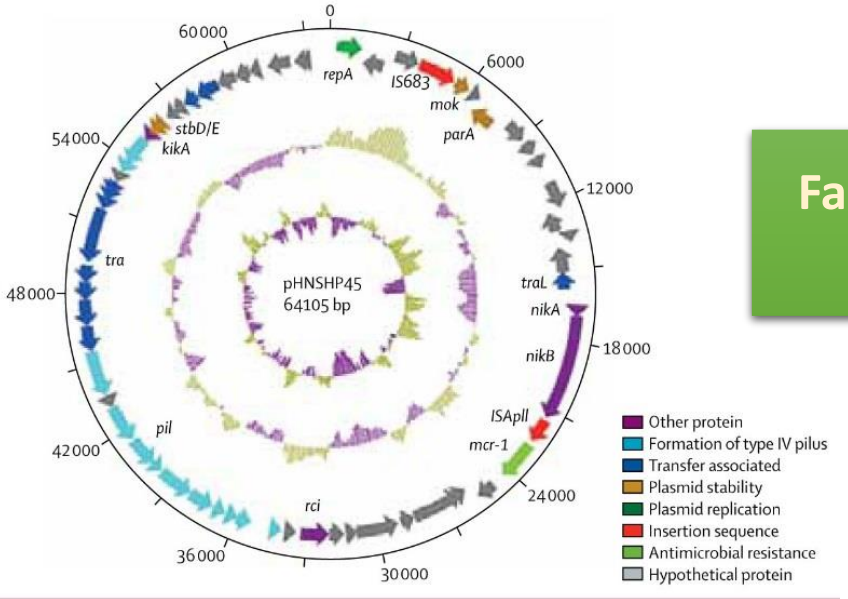


arnT → 4-amino-4-deoxy-L-Arabinose (L-Ara4N) addition to LPS

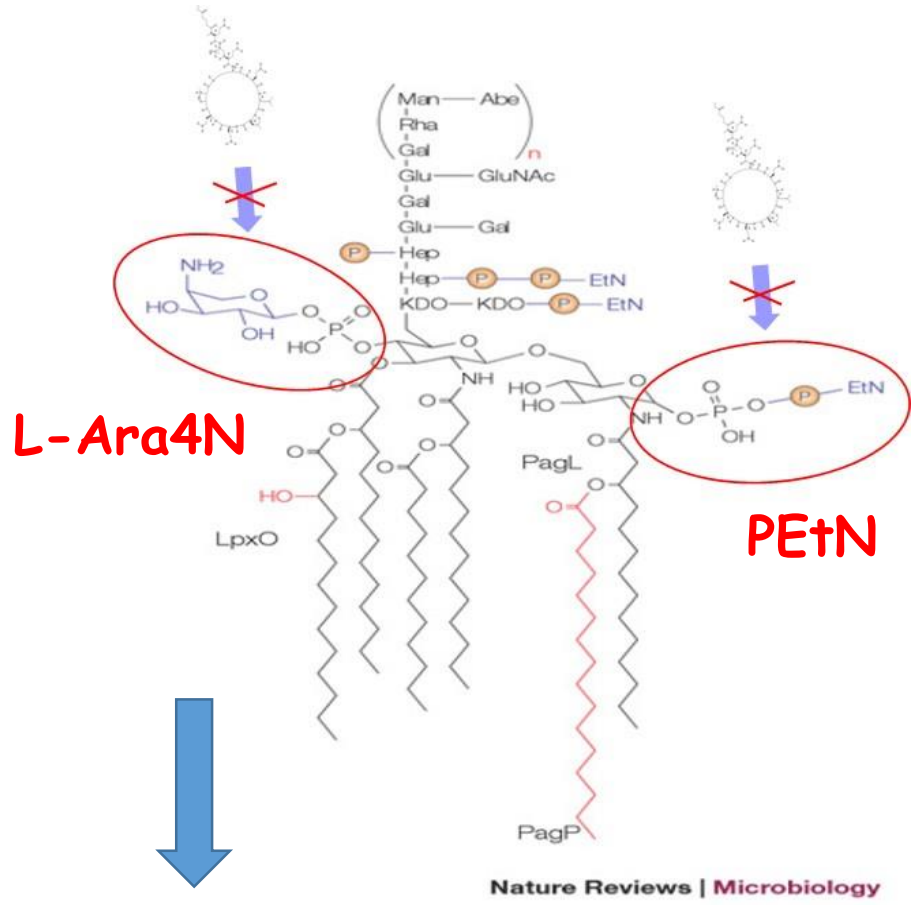
- For ***E. coli*_MCR-1**, all genes were **down-expressed** in CST & PMB high concentration
- *arnT* extremely **over-expressed** up to 29-fold for ***K. pneumoniae*_MCR-1** in CST, but only 4,5-fold in PMB

KP_MCR-1 highly adapted in CST than in PMB

Plasmid-Mediated Resistance



Facilitated L-Arabinose addition to LPS ?



MCR : Mobilizable Colistin Resistance

→ **Phosphoethanolamine transferase (addition of PEtN to lipid A)**

***K. pneumoniae*_MCR-1 well adapted better than EC_MCR-1 in both polymyxins antibiotics**

CONCLUSION

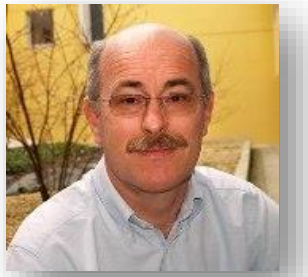
- The presence of MCR-1 facilitated the step-by-step resistance
- Polymyxin B less induce the resistance than in colistin

PERSPECTIVE

- Reversibility study (up to 2-6 months)
- Whole genome sequencing
- Structural changes of lipid A

Acknowledgements

Special Thanks



Pr William COUET

Dr Julien BUYCK

Déclaration de conflit d'intérêt

Pour cette présentation, je déclare n'avoir aucun conflit d'intérêt.

MERCI !