NATURAL EVOLUTION OF BACTERIA IN THE ANTIBIOTIC ERA

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Short Course in Critical Infection SCCI 2023
SESSION 3 Are we keeping up with evolution
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THE UNIVERSITY OF SYDNEY

The Westmead Institute FOR MEDICAL RESEARCH

NSW GOVERNMENT

NATURAL EVOLUTION OF BACTERIA IN THE ANTIBIOTIC ERA

Evolution is a process that results in **changes in the genetic material** of a population over time. Evolution reflects the **adaptations of organisms to their changing environments** and can result in altered genes, novel traits, and new species (*https://www.nature.com/scitable/definition/evolution. 2014*).

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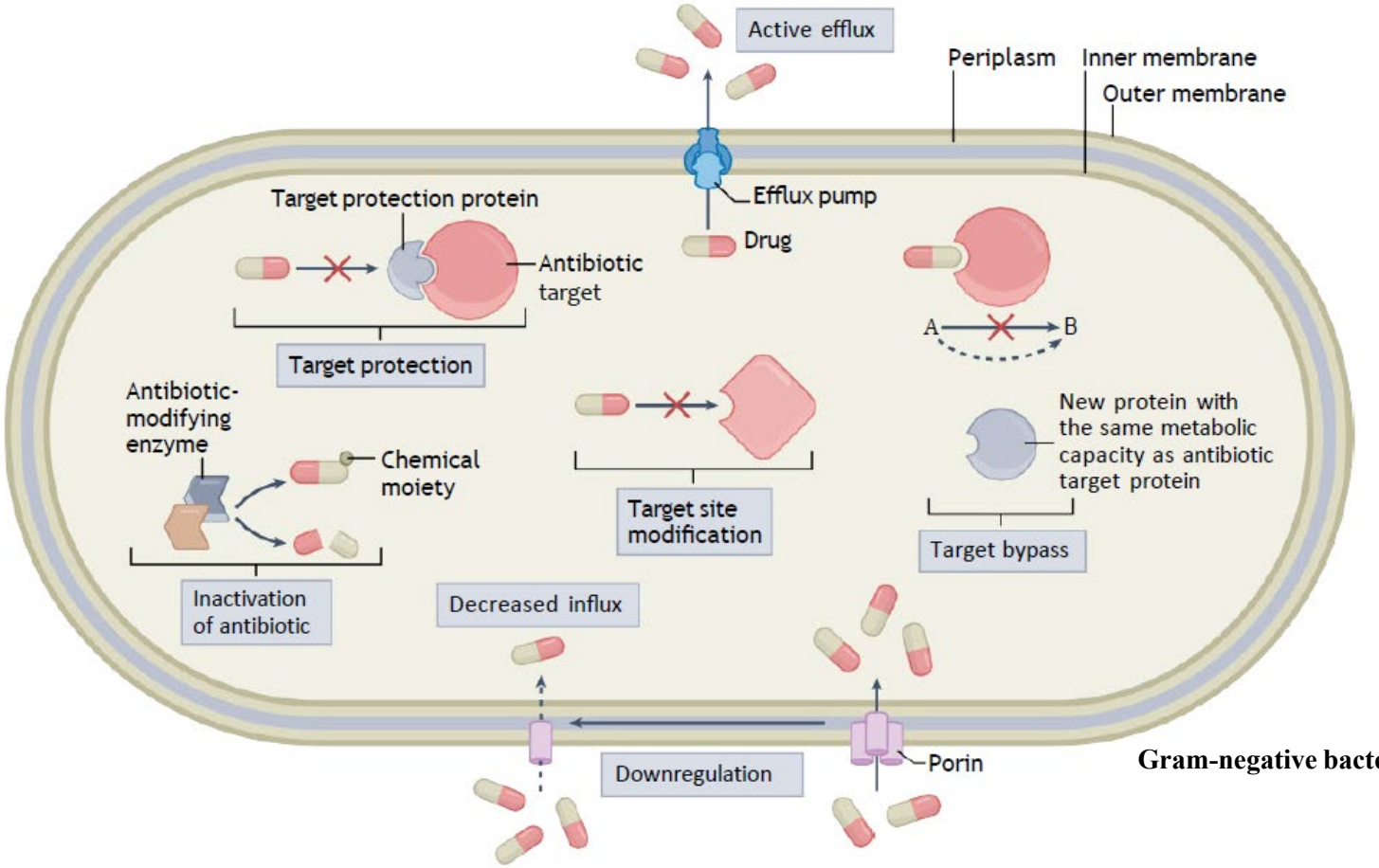
And "**antibiotics** are not only **selectors** but also **drivers** of bacterial genetic variation" (*Baquero et al. CMR, 2021*).

Mechanisms of antibiotic resistance

Intrinsic: cell uses genes it already possesses to survive antibiotic exposure.

Acquired: gain of new genetic material provides new capacities that mediate survival.



Darby et al. Nat Micro Rev. 2023.

Mechanisms of antibiotic resistance

Intrinsic

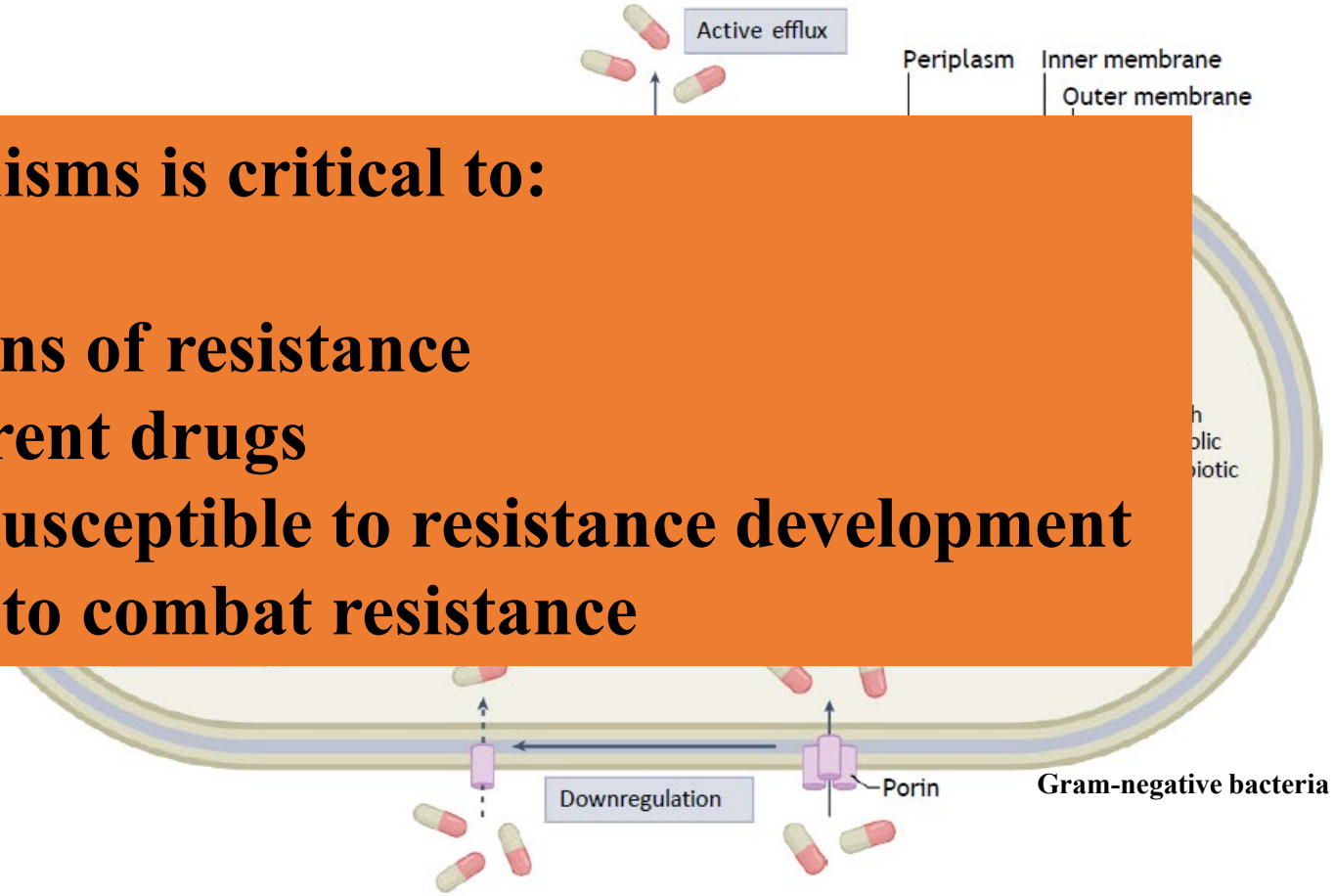
antibiotic

Acquire

new capa

Understanding these mechanisms is critical to:

- recognize global patterns of resistance
- improve the use of current drugs
- design new drugs less susceptible to resistance development
- design novel strategies to combat resistance



Darby et al. Nat Micro Rev. 2023.

Evolutionary pathways to AMR

AMR emergence, spread and maintenance is influenced by:

1. Mutation rate
2. Level of resistance conferred
3. Fitness cost
4. Strength of selective pressures
5. Epistatic interactions and compensatory evolution
6. Co-selection of other drug resistances
7. Epidemiological factors (e.g., host population biology/immunity, infection control measures)

Hughes and Andersson. Annu. Rev. Microbiol. 2017

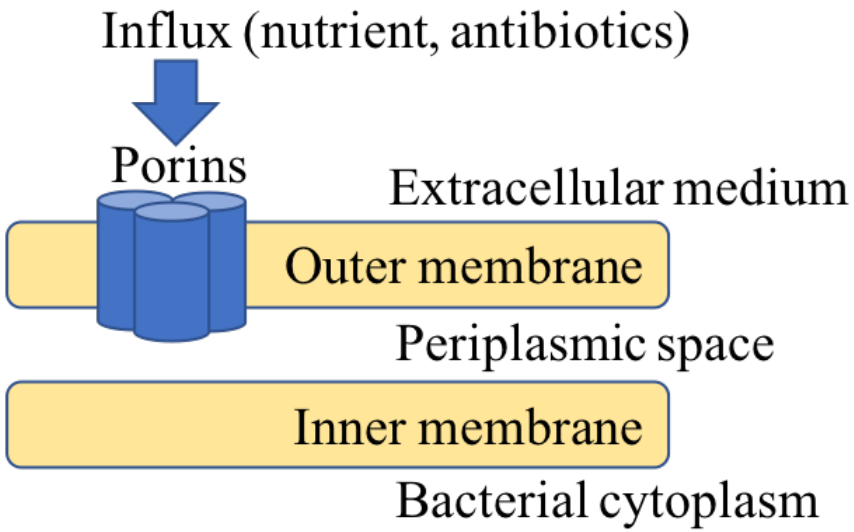
Example 1

Porin evolution: how bacteria are closing the door to antibiotics

1. Proteins located in the OM of G- forming channels

2. Passive transport of molecules: nutrients and antibiotics (e.g., β-lactams, quinolones)

3. In Enterobacterales, porins are the most abundant outer membrane proteins (OMPs)



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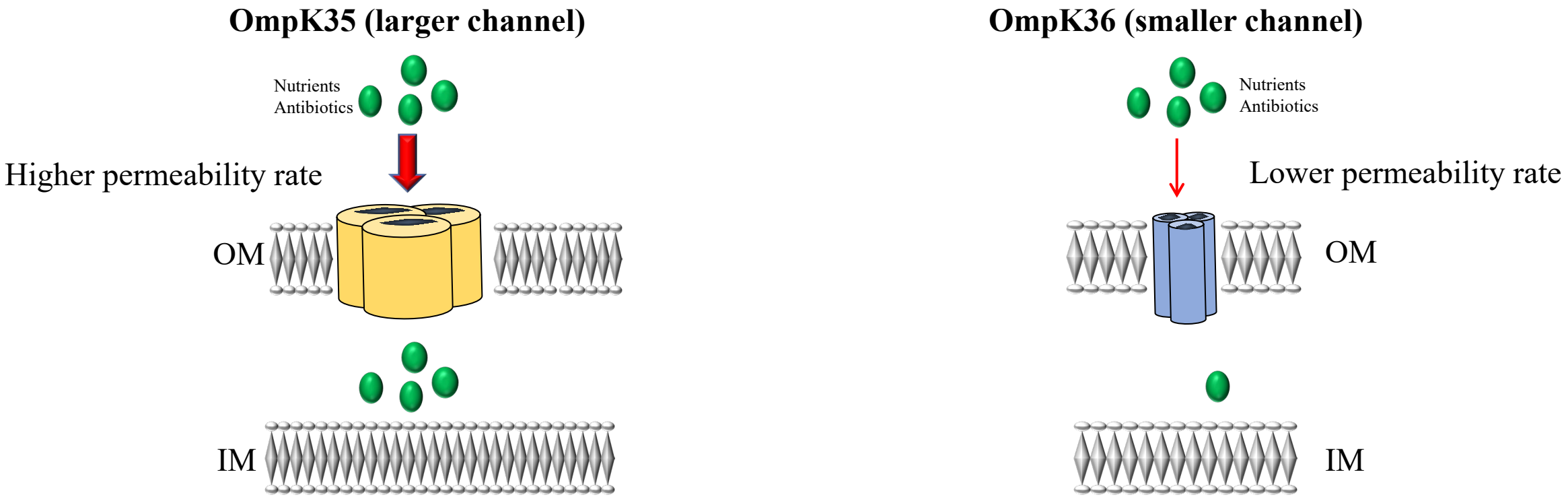
NHMRC BUILDING A HEALTHY AUSTRALIA

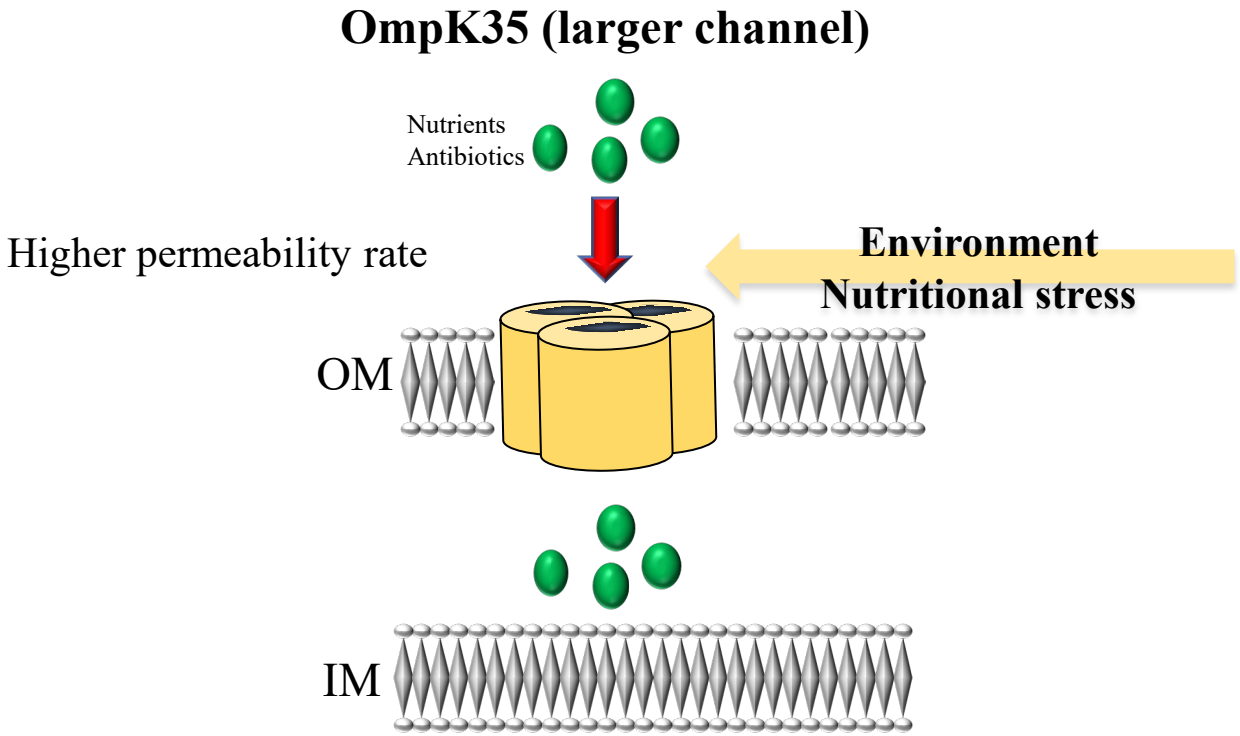
Fajardo-Lubian et al. PloS Pathogens. 2019.

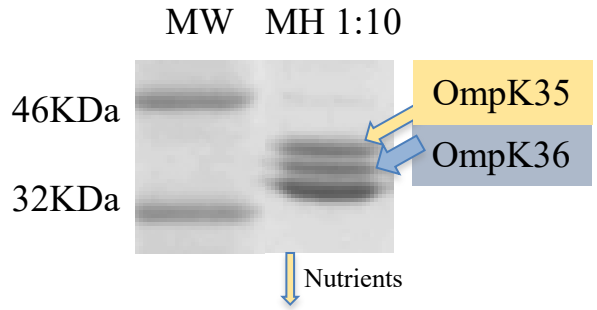
Major OMPs (porins) in *Klebsiella pneumoniae*



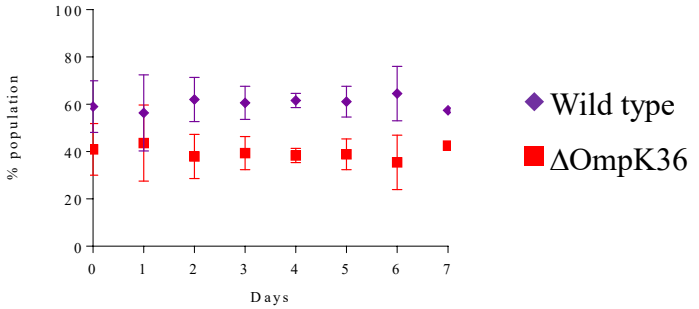
IM: Inner membrane
OM: Outer membrane
MW: Molecular Weight
MH: Mueller Hinton media: high nutrient conditions
MH 1:10: Mueller Hinton media diluted: nutrient-limited conditions

OmpK35 is the main non-specific channel in nutrient-poor conditions (environment)





Competition in conditions mimicking environment-low nutrient



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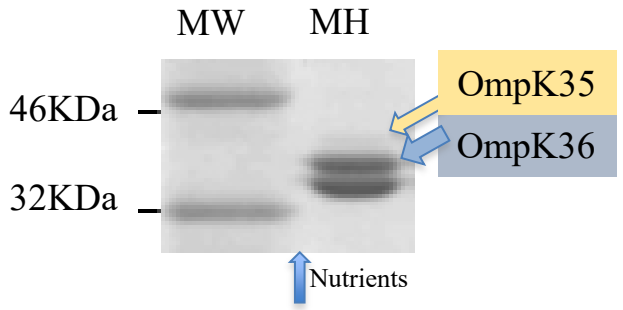
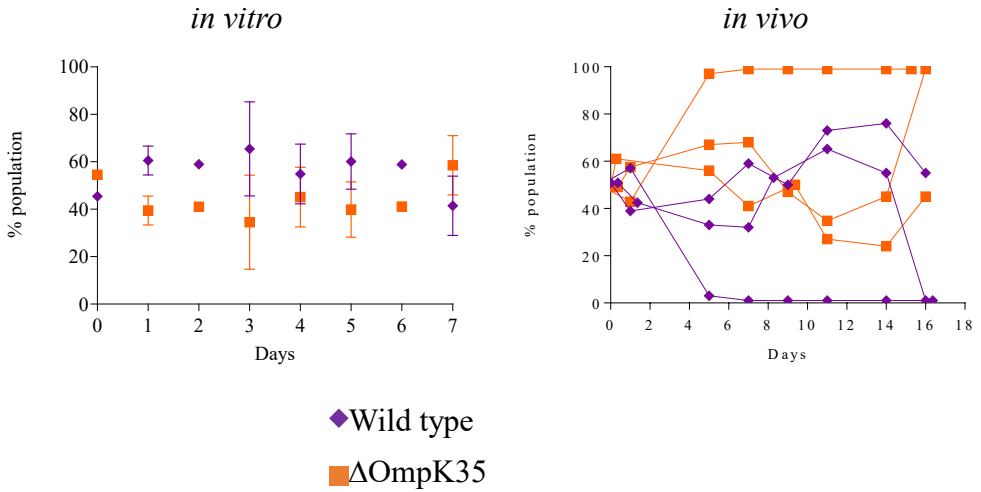
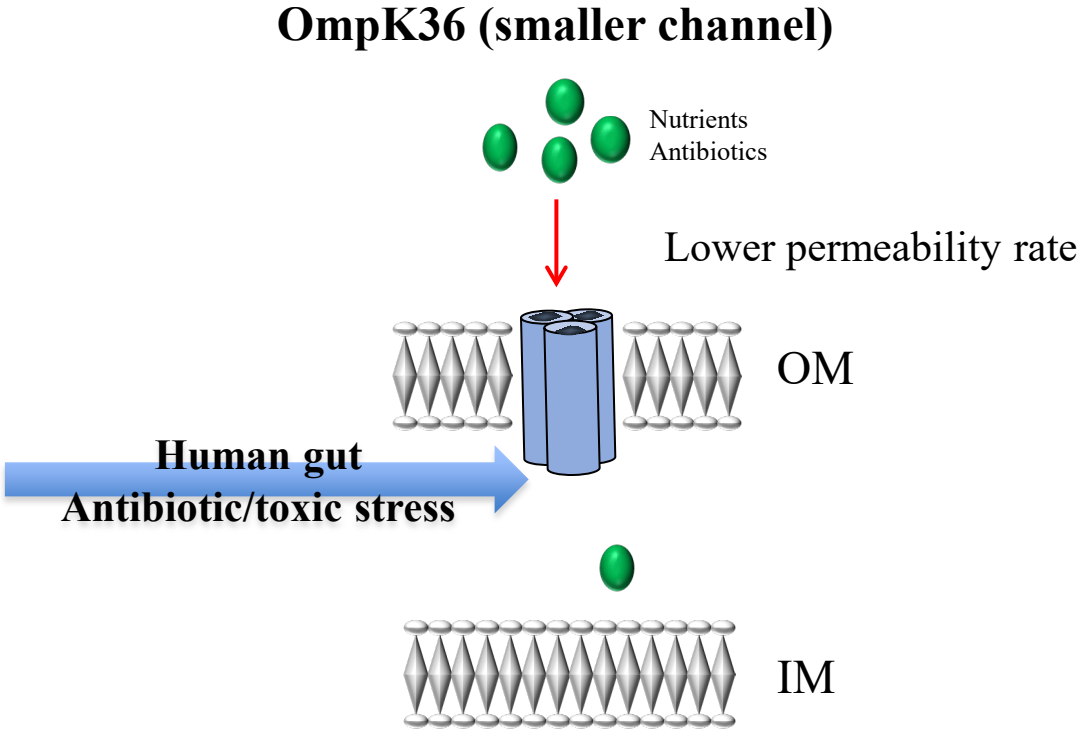
Fajardo-Lubian et al., PloS Pathogens, 2019

Porin loss and host adaptation: OmpK35 not essential for survival *in vivo*







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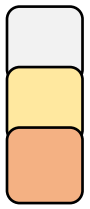
Fajardo-Lubian et al., PloS Pathogens, 2019

OmpK36 is a major determinant to acquired AMR in *K. pneumoniae*

	Antibiotics (MIC, mg/L)					
Strains	**ETP**	**MEM**	**CXM**	**FOX**	**CEF**	**CFZ**
Wild type	0.015	0.03	4-8	8	8	2
ΔOmpK35	0.03	0.06	8	16	8-16	4
ΔOmpK36	0.0625	0.06	16	16-32	32	8-16
ΔOmpK35/36	1	0.12-0.25	32	64	64	64

No resistance gene



WT MIC value
2 to 3-time fold
≥ 4-time fold

MIC. Minimal Inhibitory Concentration. ETP, Ertapenem (S ≤ 0.5, R > 1). MEM, Meropenem (S ≤ 2, R > 8). CXM, Cefuroxime (S ≤ 8, R > 8). FOX, Cefoxitin (S ≤ 8, R ≥ 32). CEF, Cephalothin (S ≤ 8, R ≥ 32). CFZ, Cefazolin (S ≤ 2, R ≥ 8).

Results replicated in three *K. pneumoniae* strains

Fajardo-Lubian et al., PloS Pathogens, 2019

OmpK36 is a major determinant of high-level AMR in *K. pneumoniae*

	Antibiotics (MIC, mg/L)														
Strains	**ETP**	**MEM**	**CXM**	**FOX**	**CEF**	**CFZ**	**ETP**	**MEM**	**IPM**	**ETP**	**MEM**	**IPM**	**ETP**	**MEM**	**IPM**
Wild type	0.015	0.03	4-8	8	8	2	0.25	0.125	1	8	8	4	16	8	8
ΔOmpK35	0.03	0.06	8	16	8-16	4	0.5	0.125	1	8	8	4	32	32	16
ΔOmpK36	0.0625	0.06	16	16-32	32	8-16	1	0.25	1	8	8	4	32	32	32
ΔOmpK35/36	1	0.12-0.25	32	64	64	64	8	2	1	64	32	64	128	128	128
	No resistance gene						**CTX-M-15 Non-carbapenamase**			**IMP-4 Carbapenamase**			**KPC Carbapenamase**		

WT MIC value
2 to 3-time fold
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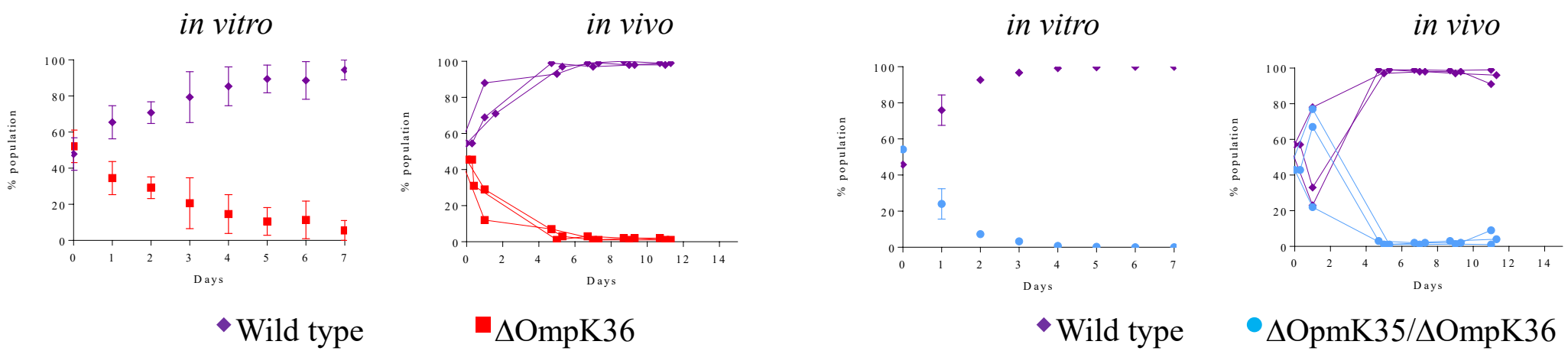
Results replicated in three *K. pneumoniae* strains

Fajardo-Lubian et al., PloS Pathogens, 2019

OmpK36 is a major determinant of high-level AMR in Enterobacterales……

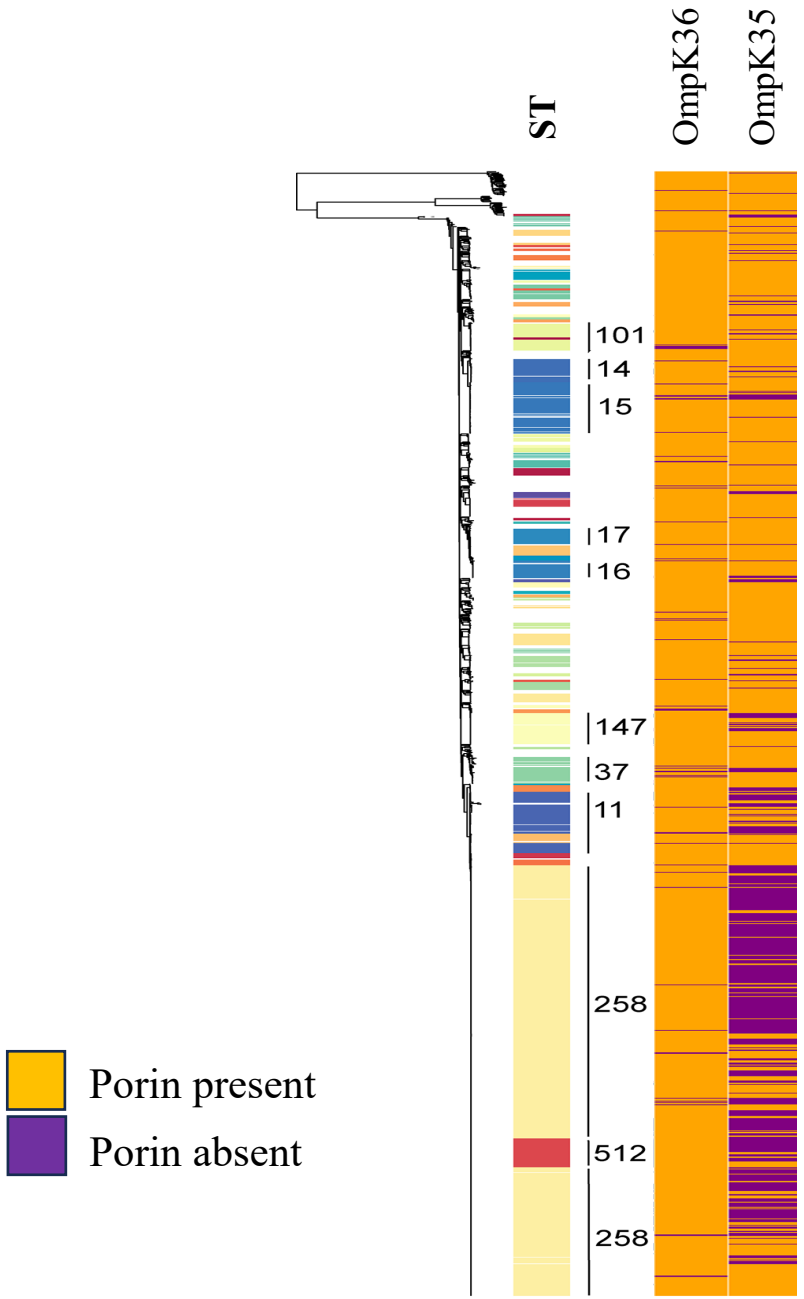
Strains	Antibiotics (MIC, mg/L)								
	ETP	MEM	IPM	ETP	MEM	IPM	ETP	MEM	IPM
Wild type	0.25	0.125	1	8	8	4	16	8	8
ΔOmpK35	0.5	0.125	1	8	8	4	32	32	16
ΔOmpK36	1	0.25	1	8	8	4	32	32	32

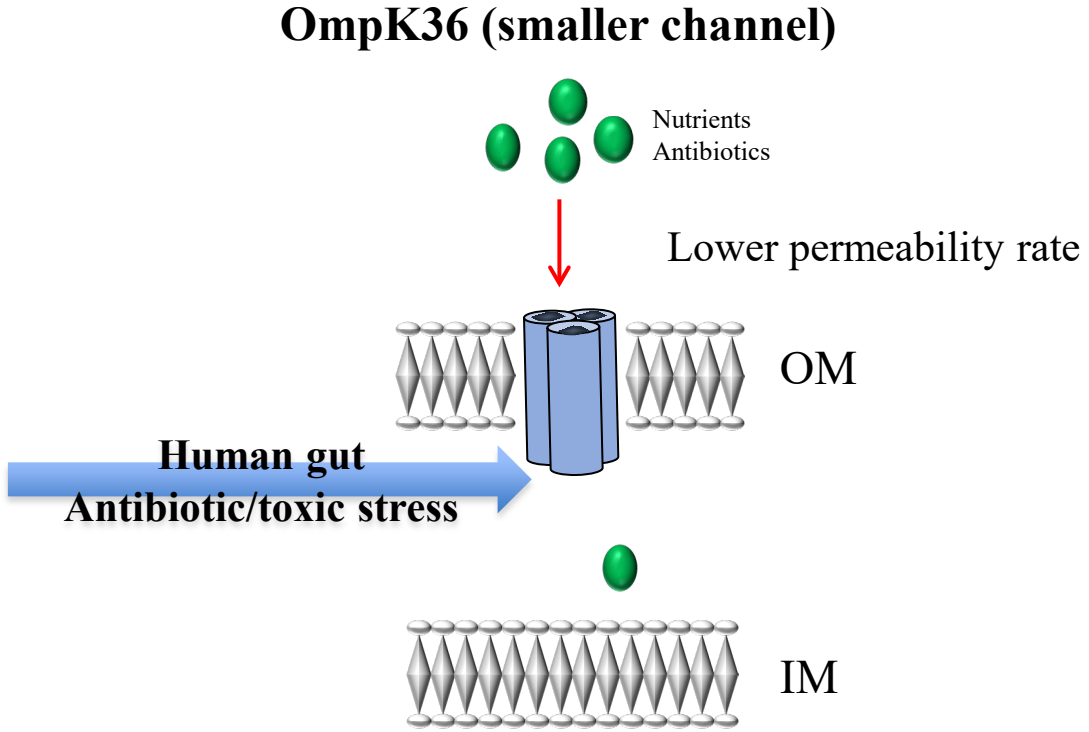
……But the loss of OmpK36 and the loss of the two major porins also reduces the influx of nutrients: negative impact on bacterial fitness



Fajardo-Lubian et al., PloS Pathogens, 2019

Porin loss and host adaptation: OmpK35 not essential for survival *in vivo*, but OmpK36 is

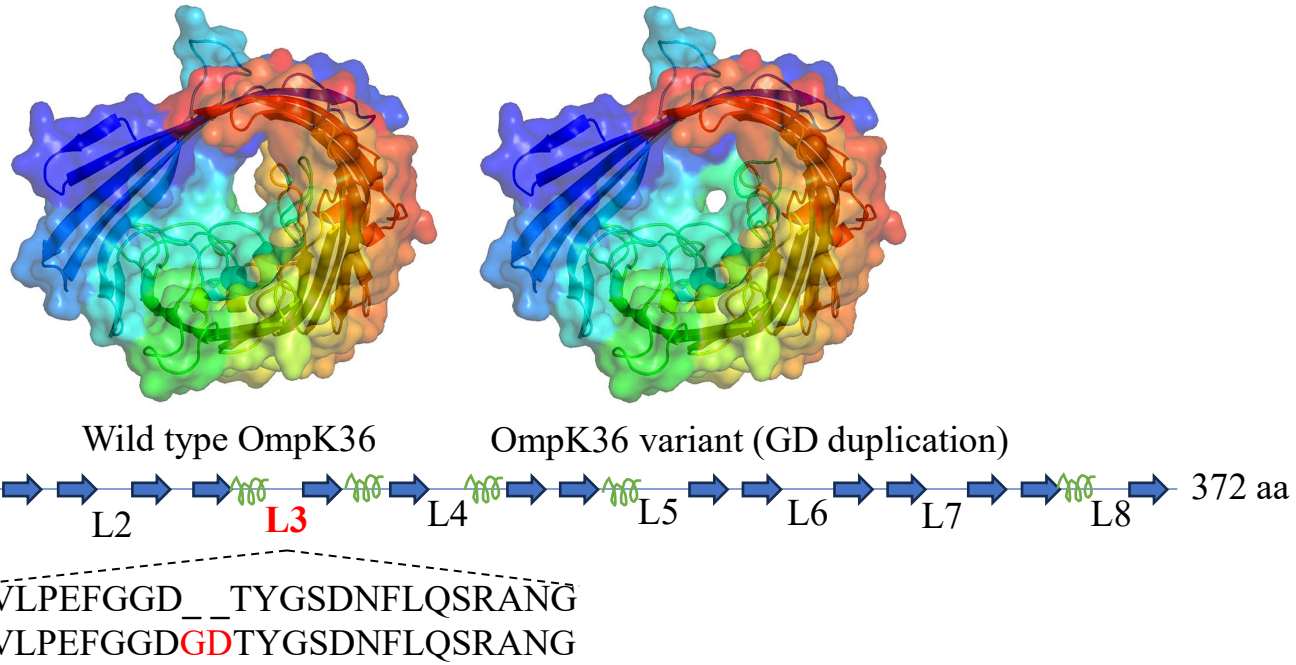




IM: Inner membrane
OM: Outer membrane

Fajardo-Lubian et al., PloS Pathogens, 2019

Functional porins with less permeability
Channel restriction of OmpK36: mutations in the constriction region

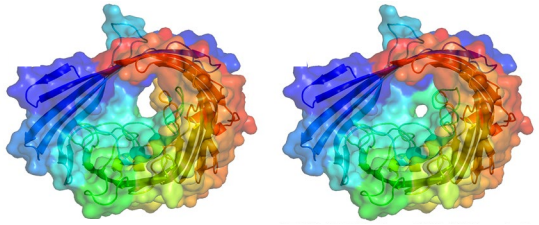


α-barrel
β-barrel
L: loop

G: glycine
D: aspartic acid

Fajardo-Lubian et al., PloS Pathogens, 2019

Functional porins with less permeability
Similar level of resistance



Wild type OmpK36 OmpK36 variant (GD duplication)

DVLPEFGGD_ _TYGSDNFLQSRANG
DVLPEFGGDGDTYGSDNFLQSRANG

Strains	Antibiotics (MIC, mg/L)								
	ETP	MEM	IPM	ETP	MEM	IPM	ETP	MEM	IPM
ATCC13883	0.25	0.125	1	8	8	4	16	8	8
ΔOmpK35	0.5	0.125	1	8	8	4	32	32	16
ΔOmpK36	1	0.25	1	8	8	4	32	32	32
OmpK36GD	1	0.25	1	8	8	4	32	16	16
ΔOmpK35/36	8	2	1	64	32	64	128	128	128
ΔOmpK35/36GD	4	1	1	32	32	16	128	128	64
	CTX-M-15 Non-carbapenamase			**IMP-4 Carbapenamase**			**KPC Carbapenamase**		

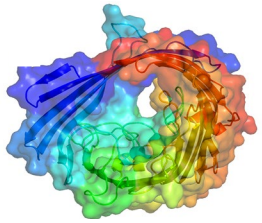
α-barrel
β-barrel
L: loop

ETP. Ertapenem. MEM. Meropenem.
WT: wild type ATCC 13883 *K. pneumoniae*
MIC. Minimal Inhibitory Concentration
Broth microdilution method in Mueller Hinton Broth (cation adjusted)

WT MIC value
2 to 3-time fold
≥ 4-time fold

Fajardo-Lubian et al., PloS Pathogens, 2019

Functional porins with less permeability
Fitter and no pathogenicity attenuation



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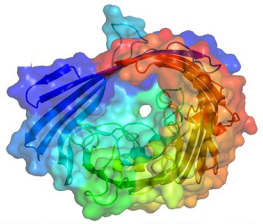
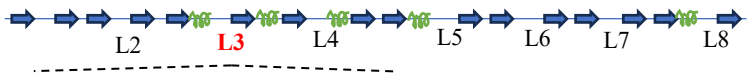
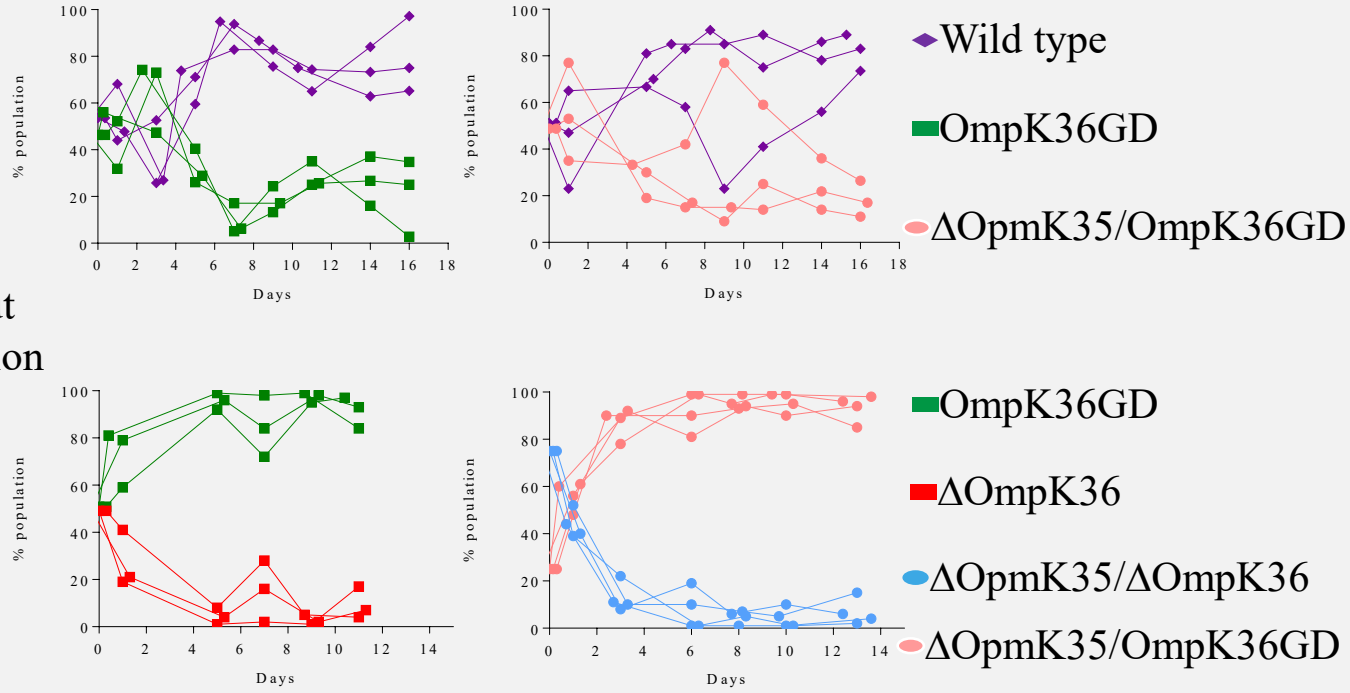


OmpK36 variant (GD duplication)

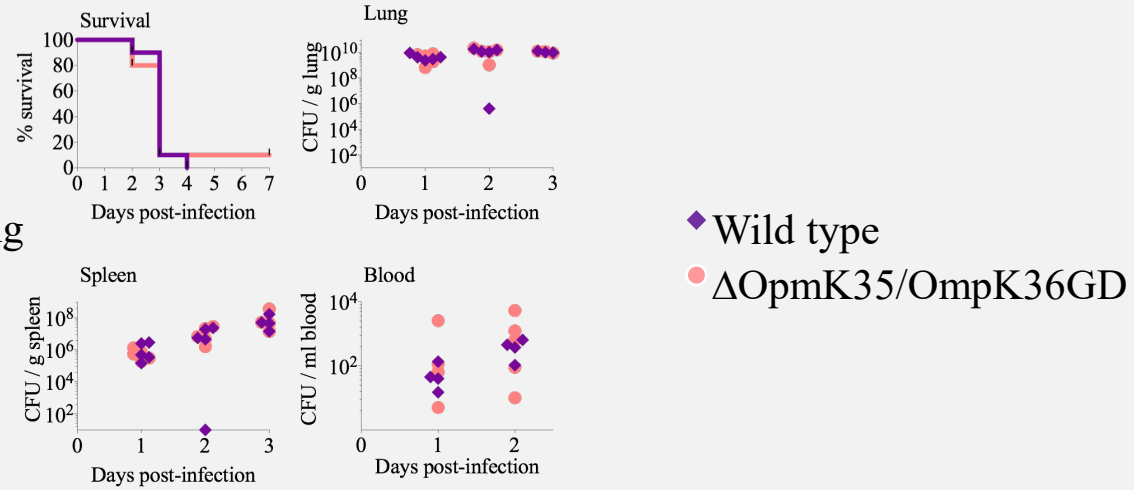


DVLPEFGGD_ _TYGSDNFLQSRANG
DVLPEFGGDGDTYGSDNFLQSRANG

Mouse gut colonization



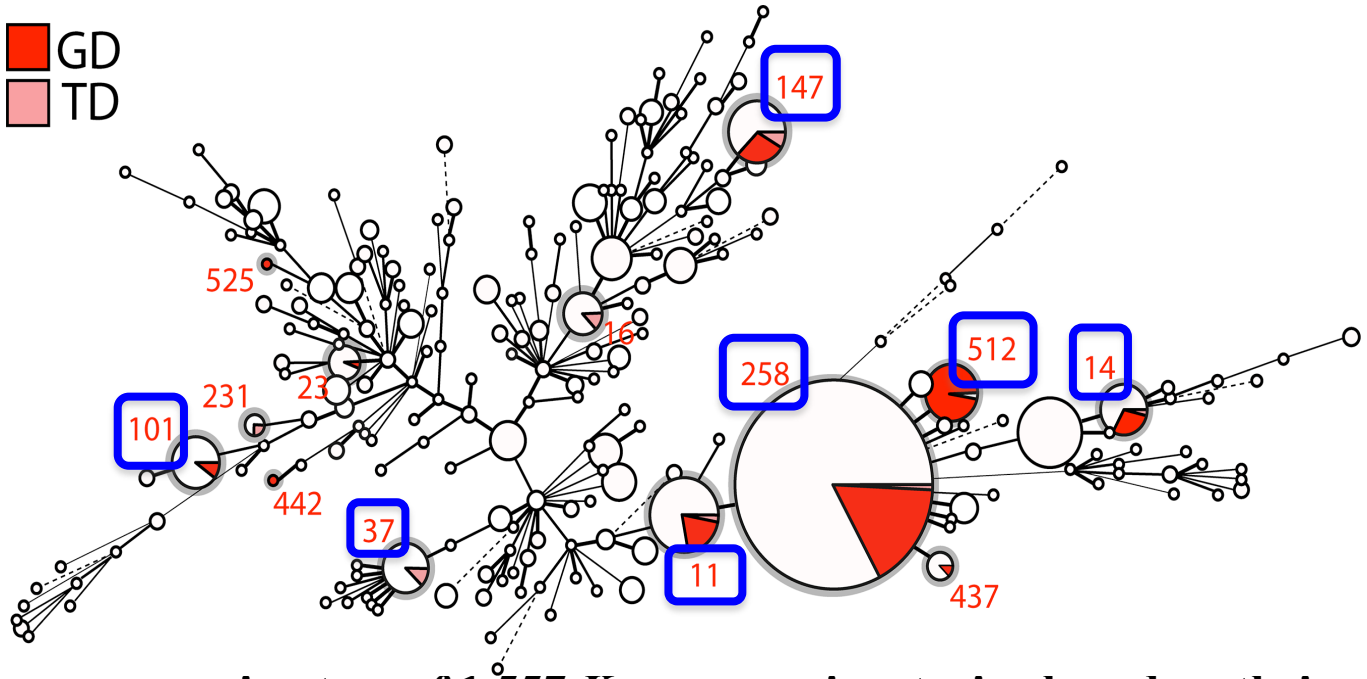
Mouse lung infection



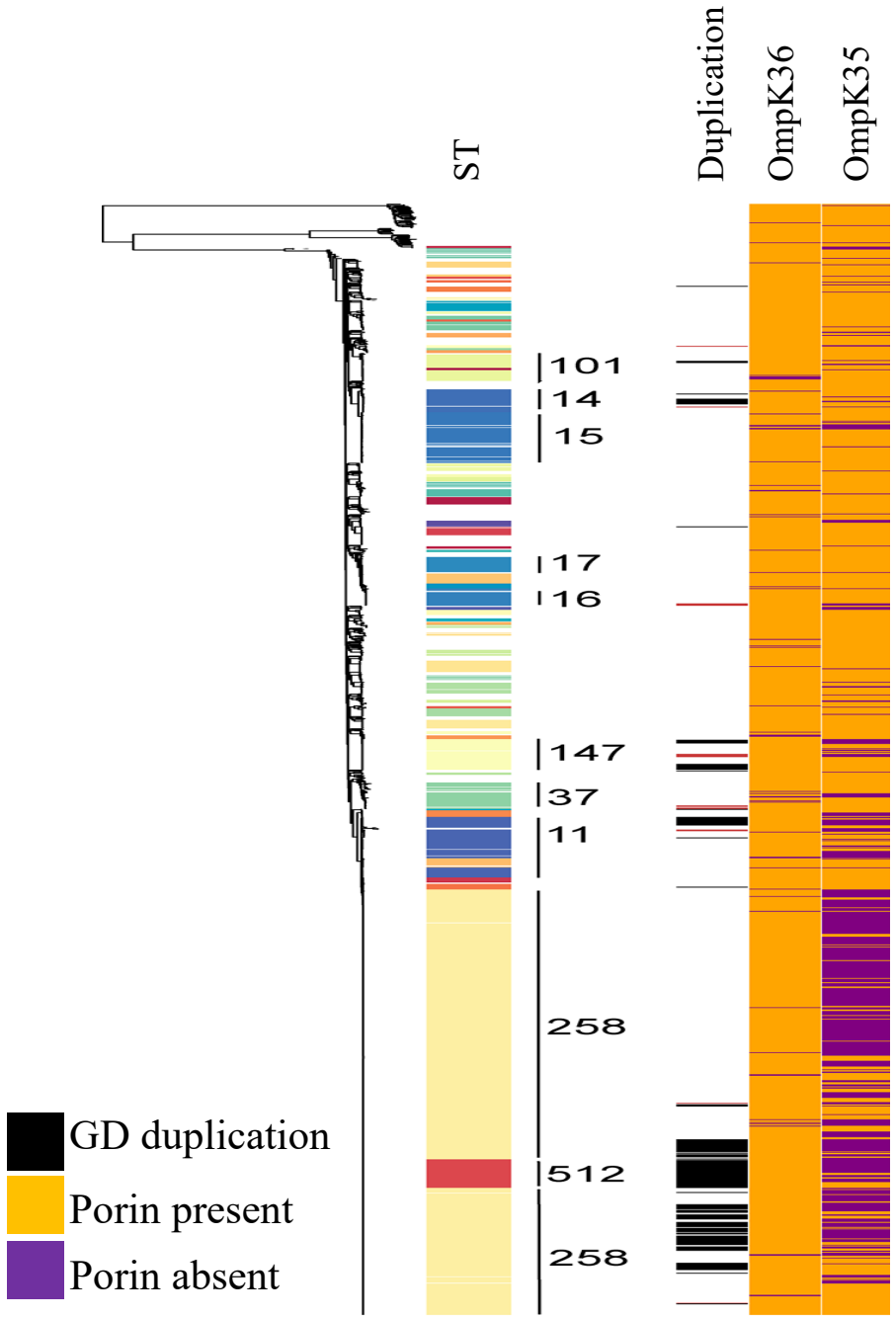
Fajardo-Lubian et al., PloS Pathogens, 2019

GenBank sequences suggest convergent evolution in OmpK36_Loop 3 variants in unrelated strains

The two common variants (+GD and +TD) are distributed across the whole spectrum of diversity of *K. pneumoniae*, including in most major epidemic clones



Minimum spanning tree of 1,557 *K. pneumoniae* strains based on their MLST profile



G: glycine
D: aspartic acid
T: threonine

Fajardo-Lubian et al., PloS Pathogens, 2019

Are there other mutations?

Mutations of channel constriction zone of OmpK36 (and orthologue porins)

Charged residues	*K. pneumoniae* (OmpK36)	*E. coli* (OmpC)	*K. aerogenes* (Omp36)	ECC (OmpE36)	*Citrobacter spp.* (OmpC)
DLYGK	E	E, T, Y, N	I, E	E	E
TYMRV	-	-	-	-	-
AWTRL	-	-	-	-	-
WTDVLP	-	-	-	-	-
PEFGGDTYG Loop 3, L3	**GGDTYD** **GGDDTYG** ***GGDGDTYG** **GGDTDTYG** **GGDSDTYG** **GGDSDTYGTYG** **GGDSDTYDTYG** **GGDSDTYGSDTYG**	**GGDTYD** **GGDTYS**	**DGDTYG** **GGDSDTYG** **GGDTYS**		**DGDTYG** **GGDTYD** **GGDTYT**
QSRANG		C, L			

Red. Conserved charged residues involved in pore constriction (based on Bornet et al. 2004).
Green. Conserved charged residues involved in pore constriction, hot spot for duplications.
Blue, mutations in L3.
ECC. Enterobacter Cloacae Complex.
-: no substitution or mutation found.
*: most prevalent duplication found.

Unpublished

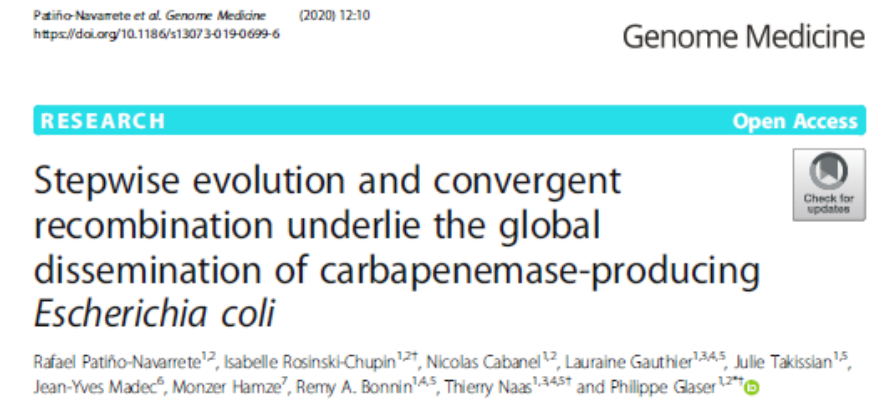
Are other antibiotics affected?

Ciprofloxacin MIC

Strain	MIC (mg/L)
WT	0.0625
ΔK35	0.125-0.25
ΔK36	0.125
ΔK35ΔK36	0.25-0.5
K36GD	0.125
ΔK35K36GD	0.25

Unpublished

Is this happening in other *Enterobacterales*?



Predictor of subsequent AMR acquisition

Conclusions (Part I)

1. Host-adaptation in *K. pneumoniae* (loss of OmpK35) seems common in unrelated strains: many *K. pneumoniae* have dispensed with OmpK35 porin, required for an environmental life cycle.
2. The highly specific variation in the inner channel of OmpK36 provides carbapenem resistance at no cost to colonising ability, competitiveness or pathogenicity and can be expected to be an increasingly common feature of host-adapted ‘high-risk’ clones.

Conclusions (Part I)

1. Host-adaptation in *K. pneumoniae* (loss of OmpK35) seems common in unrelated strains: many *K. pneumoniae* have dispensed with OmpK35 porin, required for an environmental life cycle.
2. The highly specific variation in the inner channel of OmpK36 provides carbapenem resistance at no cost to colonising ability, competitiveness or pathogenicity and can be expected to be an increasingly common feature of host-adapted 'high-risk' clones.

What does this mean for therapy?

1. If we detect these mutations: early recognition of 'silent' colonization by "high-risk" clones (host-adapted/pathogenic strains)
2. Compensatory mutations: other porin(s) expressed? Can use/design other antibiotics that enter these channels?
3. Select antibiotics to avoid this route of entry?
4. Advanced therapies: porins are bacteriophage receptors.

Example 2

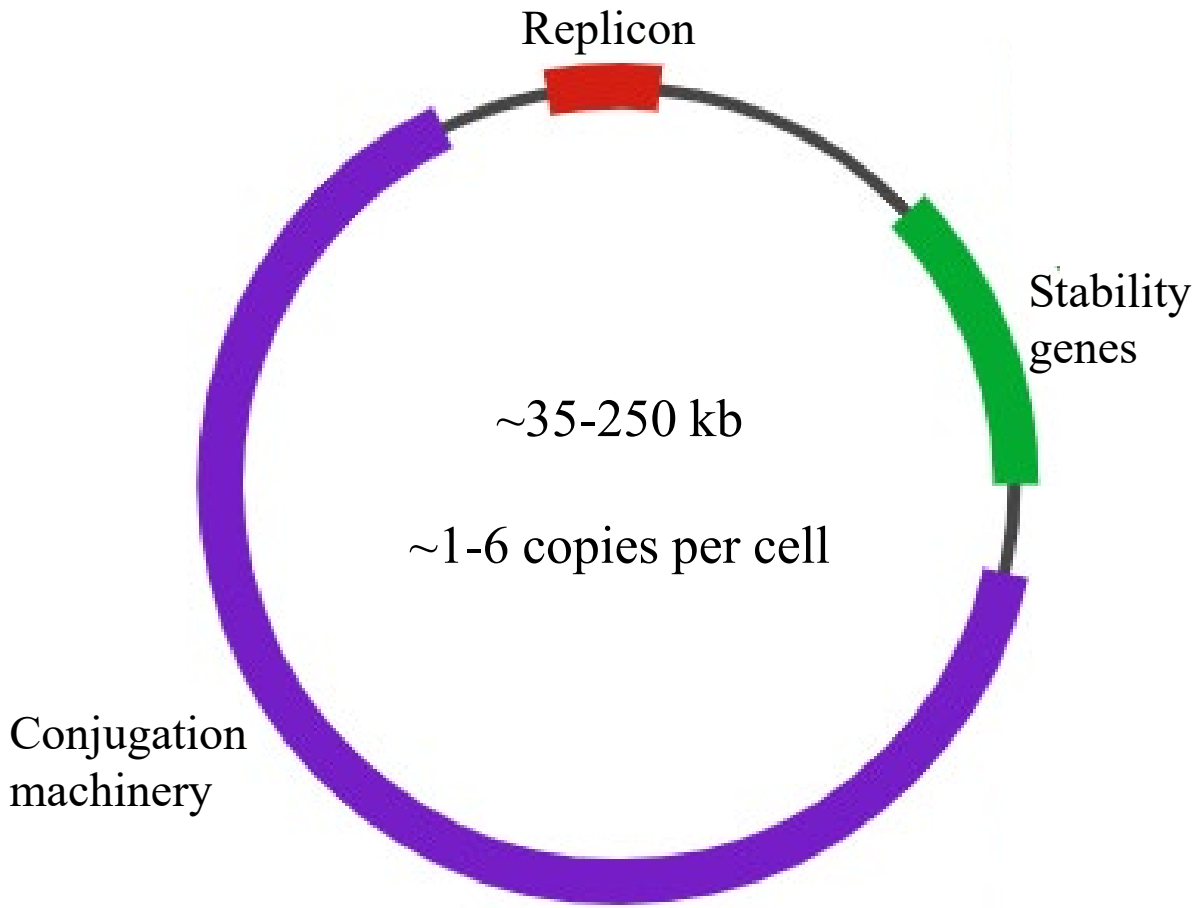
Chromosomal integration of AMR genes

AMR genes in Gram-negative organisms commonly disseminate on plasmids, but critical AMR genes are increasingly reported on bacterial chromosomes.

Aim. To define the extent of chromosomal *bla*$_{\text{CTX-M}}$ carriage in *E. coli* in Australia.

Conjugative plasmids spread AMR

AMR genes mostly spread on large, low-copy plasmids.



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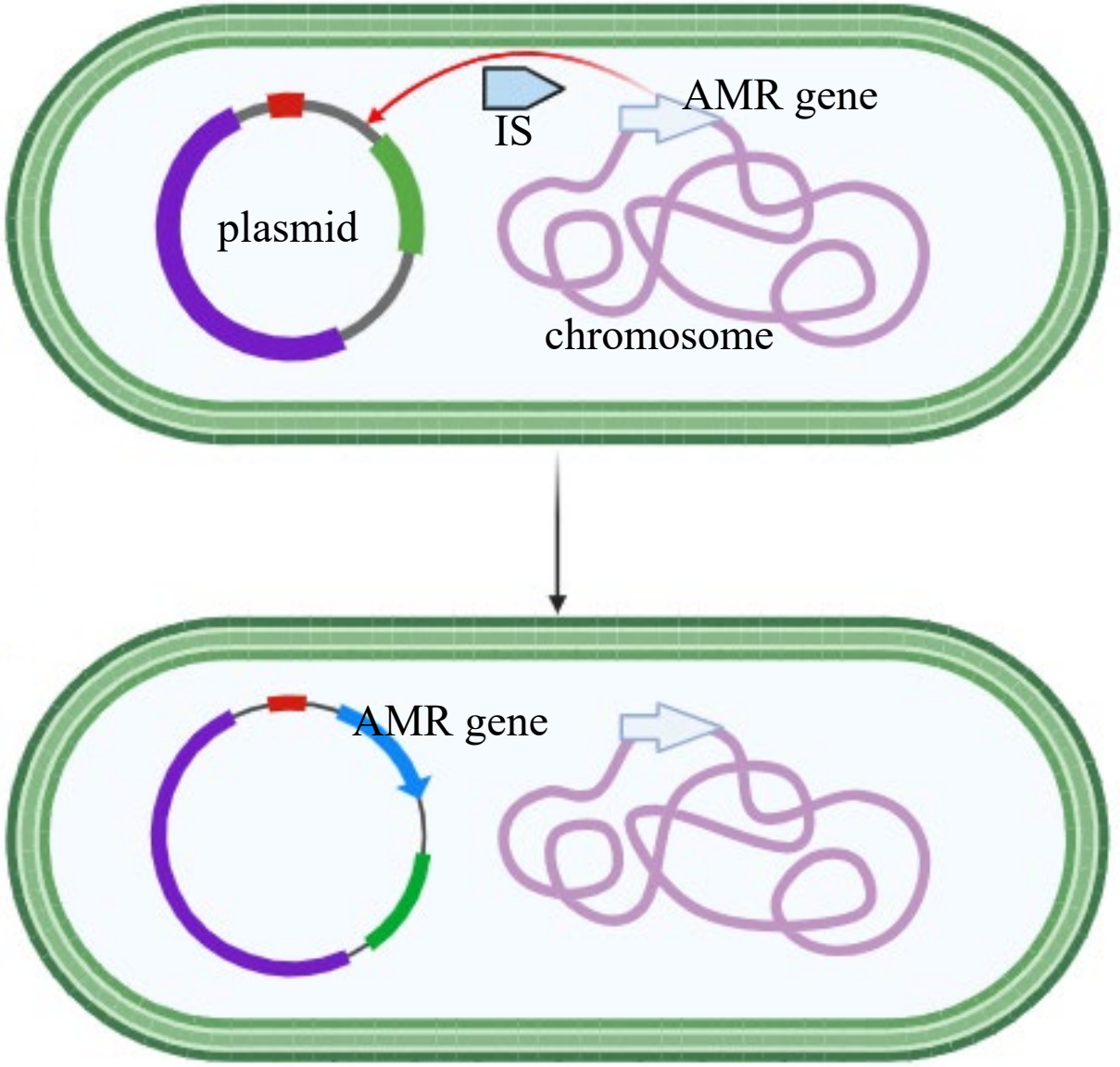
Conjugative plasmids spread AMR

Plasmid-borne AMR genes are derived by capture of chromosomal segments (e.g., $bla_{CTX\text{-}M}$ genes from *Kluyvera* spp.).
Insertion into plasmids, mediated by different mobile elements (e.g., insertion sequences, IS).



Resistance on plasmid

- Higher expression
- Increased copy number
- Evolution to extend resistance phenotype

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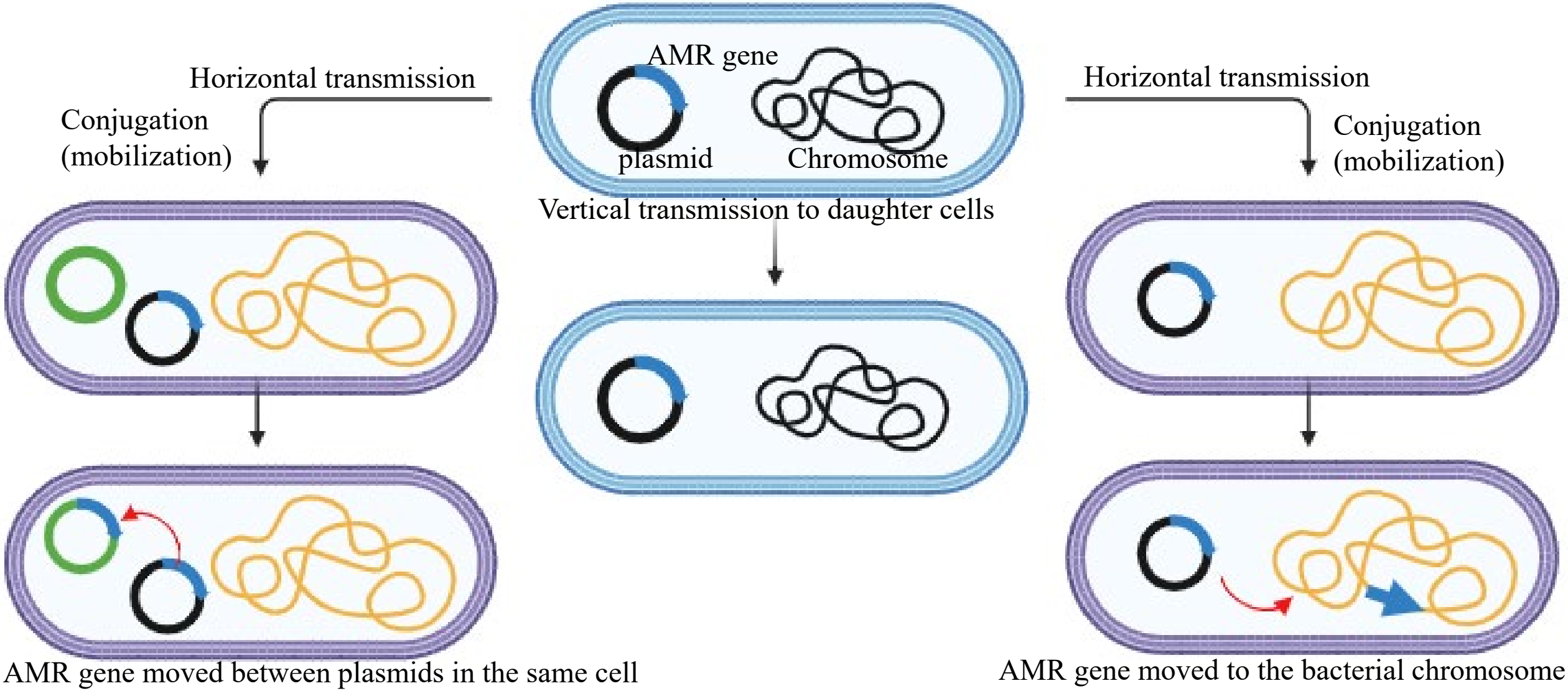
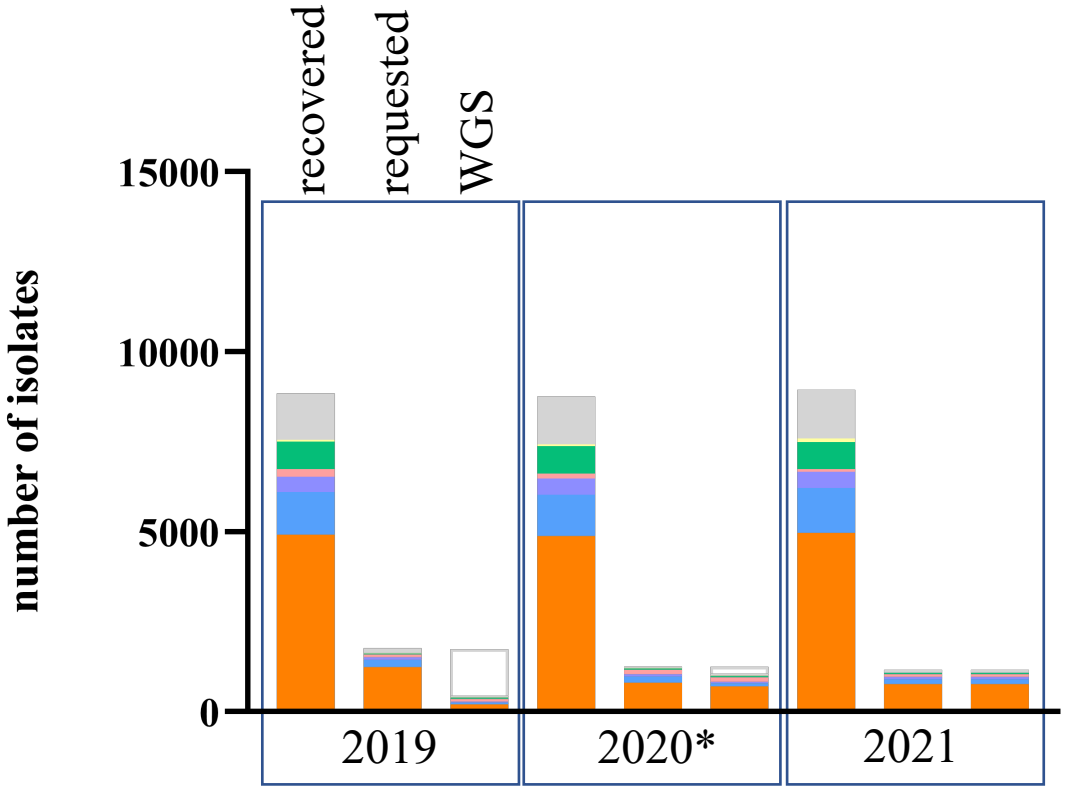
Conjugative plasmids spread AMR

"Mobile" AMR genes can travel between bacterial cells/species on different types of plasmids

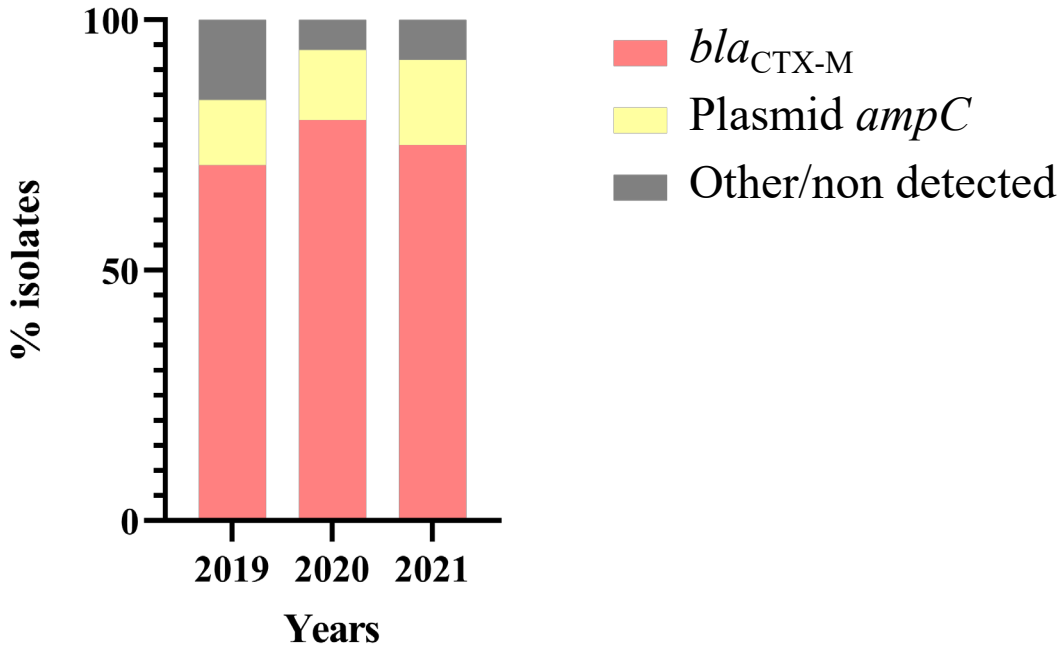


National survey of antibiotic resistance in Gram-negative pathogens (GnSOP, 2019-2021)

E. coli, the most predominant pathogen

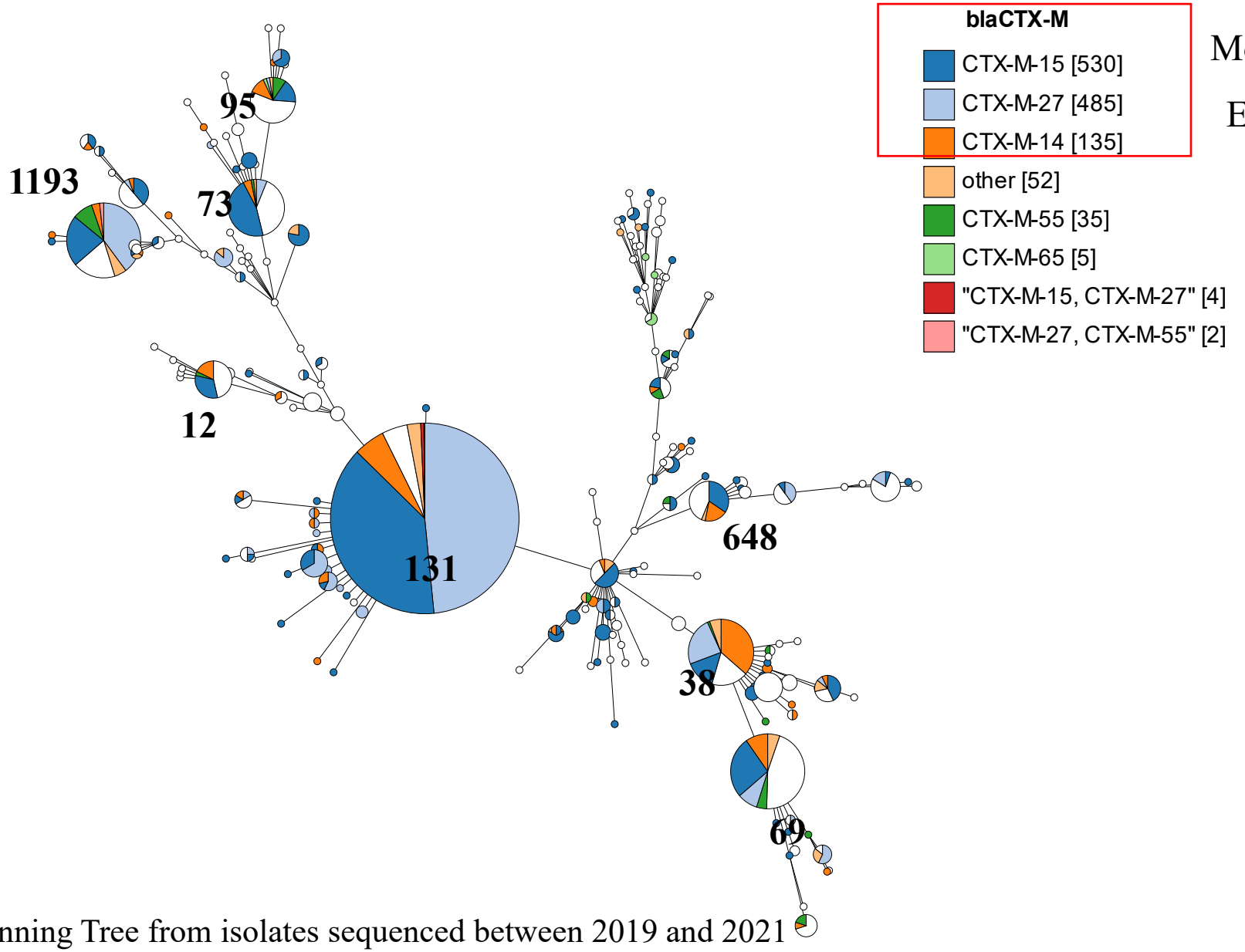


bla_{CTX-M}, the most prevalent ESBL in *E. coli* sepsis episodes



*GnSOP 2020, extra funding covering Illumina short read sequencing for chromosomal integration of resistance genes and PacBio long read sequencing

ST131 is the most prevalent drug resistant *E. coli* population recovered from sepsis episodes in Australia (2019-2021)

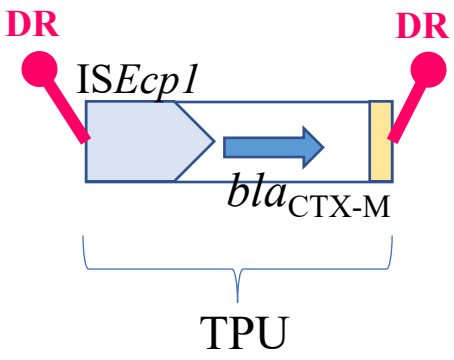


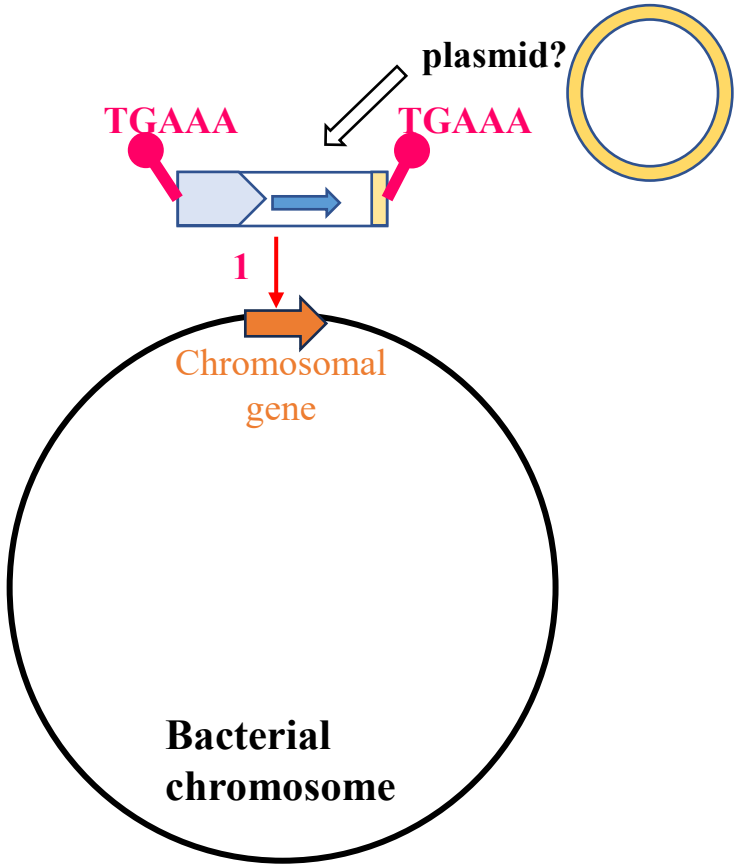
Most common ESBL genes

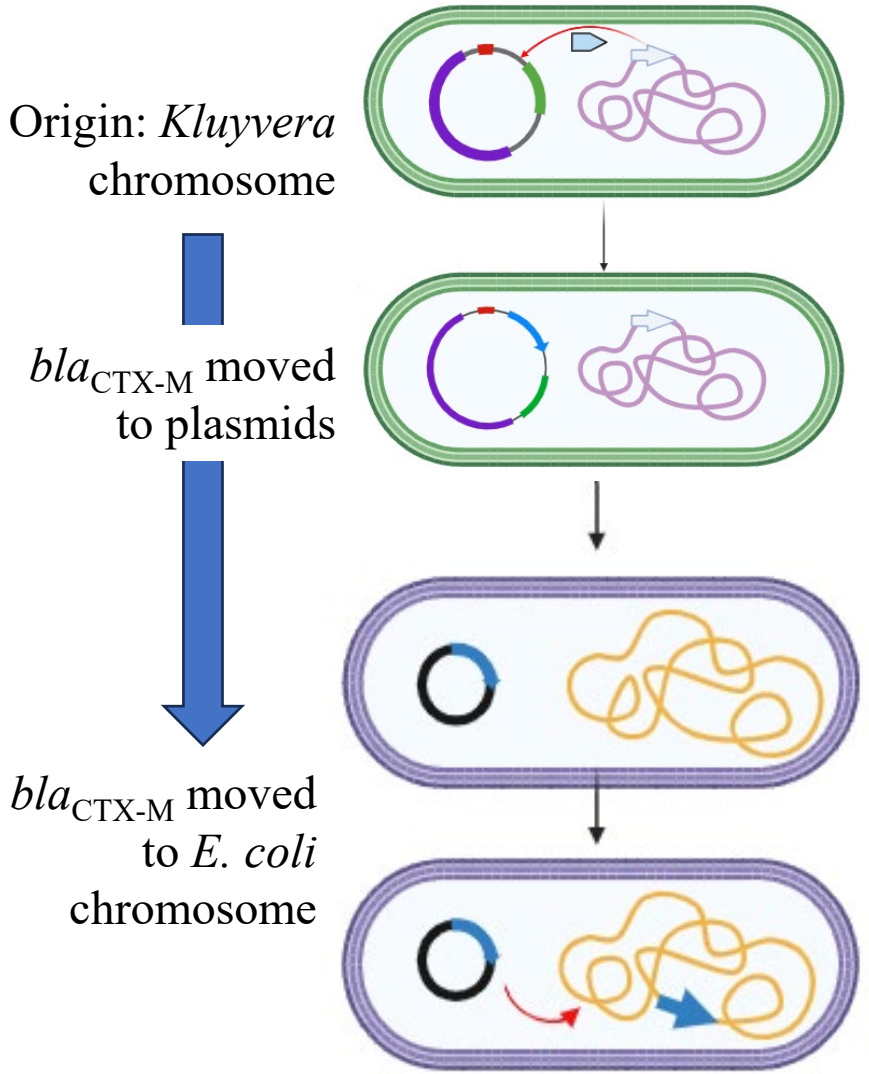
E. coli MLST-Minimum Spanning Tree from isolates sequenced between 2019 and 2021

How *bla*$_{CTX-M}$ genes move: IS*Ecp1* and TPUs

- IS*1380* family, 1,656 bp, ~14 bp IR
- Can pick up regions (containing an AMR gene) adjacent to IR_R
- Not clear how this works or how end of region picked up is selected
- Different regions can be transferred in different events as "transposition units" – TPU
- Creates 5-7 bp direct repeats (DR) flanking TPU.

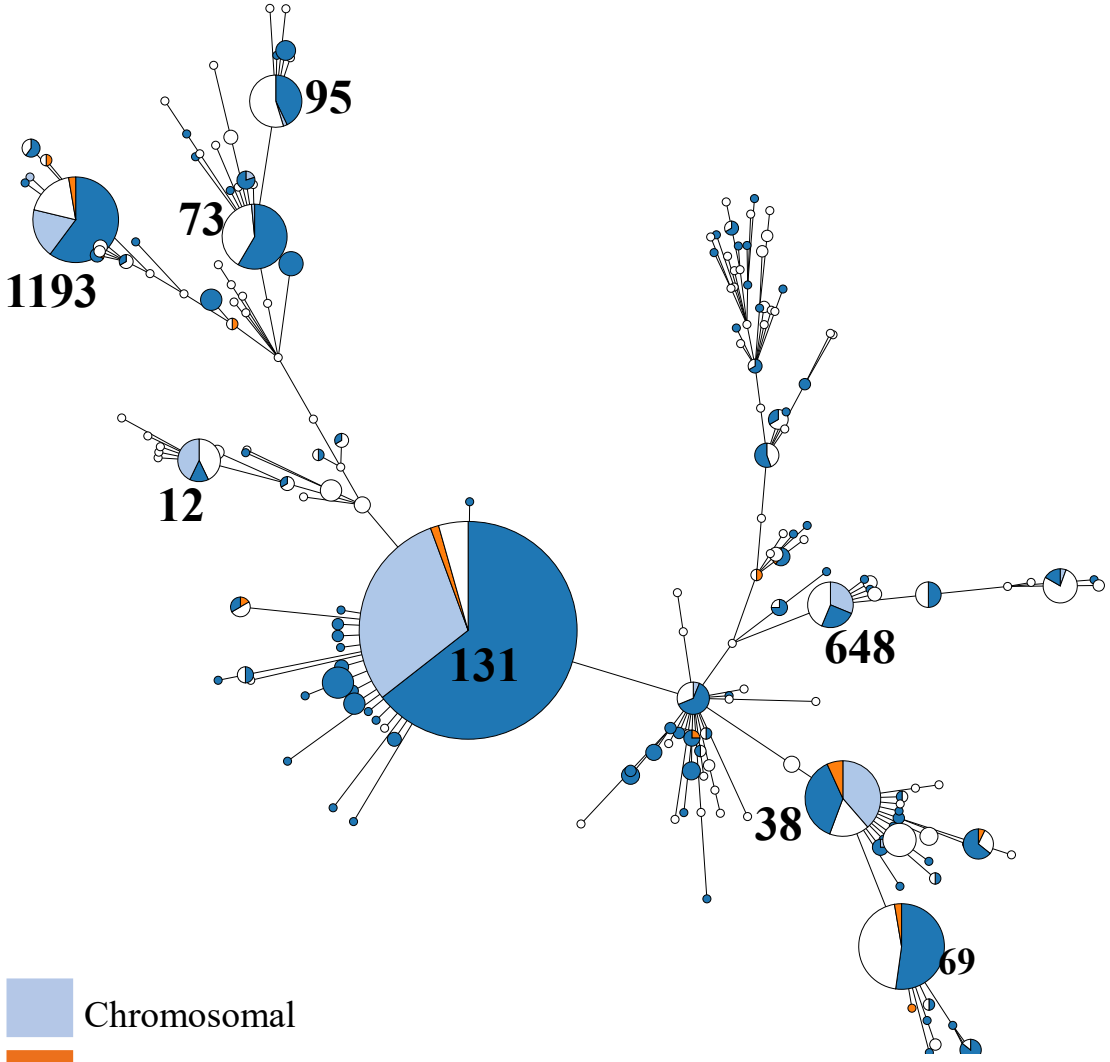






TPU: Transposition Unit. IR: inverted repeat. DR: direct repeat.

Proportion of chromosomal *bla*$_{CTX-M}$ in *E. coli* population recovered from sepsis episodes in Australia



At least a third of total *E. coli* sepsis isolates have *bla*$_{CTX-M}$ integrated in the bacterial chromosome

Escherichia coli	ESBL gene	Chromosomal (% of analysed)
ST12	*bla*$_{CTX-M-14}$/*bla*$_{CTX-M-15}$	**100%**
ST38	*bla*$_{CTX-M-14}$	***96%**
ST131-FimH30	*bla*$_{CTX-M-14}$	***87%**
ST131-FimH30-Rx	*bla*$_{CTX-M-15}$	***24%**
ST131-FimH41	*bla*$_{CTX-M-15}$/*bla*$_{CTX-M-27}$	***80%**
ST131-other FimH	*bla*$_{CTX-M-14}$/*bla*$_{CTX-M-15}$/*bla*$_{CTX-M-27}$	***69%**
ST648	*bla*$_{CTX-M-15}$	**100%**
ST1193	*bla*$_{CTX-M-15}$	***78%**

*Analysis in progress. The location of *bla*$_{CTX-M}$ cannot always be determined. Short and long read sequencing

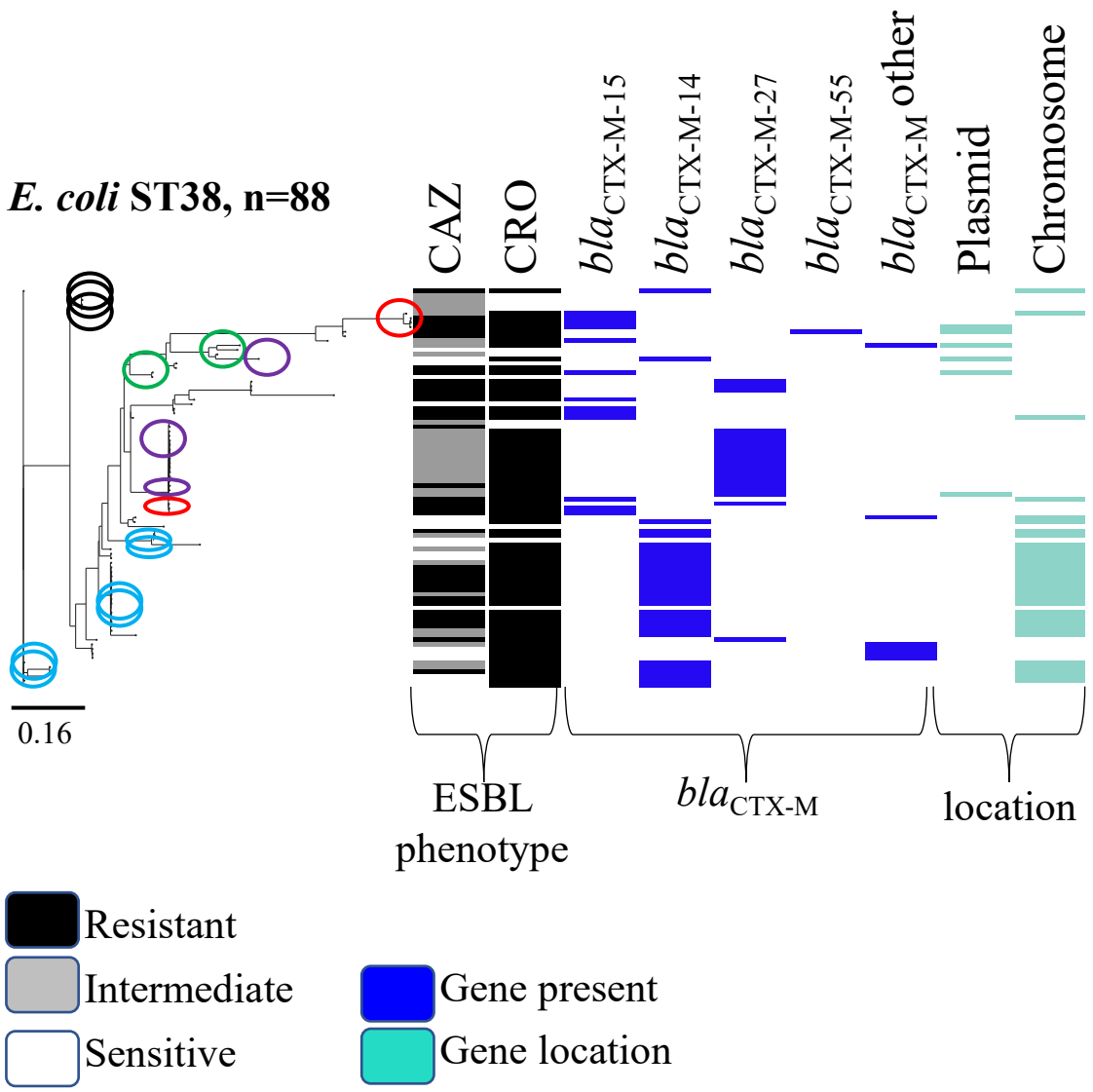
Chromosomal location of ESBL genes in most common *E. coli* sequence types (STs) in Australia (2019-2021)

Chromosomal location of ESBL genes.		
BACTERIAL GENE INTERRUPTED	***E. coli***	**ESBL gene***
bglA (glucosidase), *dtd* (tRNA deacylase), fimbrial type 1 protein	ST12	$bla_{\text{CTX-M-15, -14}}$
rbsR (transcriptional regulator), *yicI* (hydrolase), *glgX* (hydrolase), *mgtC/sapB* (magnesium transporter), *spoT* (synthase/hydrolase), *btuB* (outer membrane transporter), *dacD* (carboxypeptidase).	ST38	$bla_{\text{CTX-M-14}}$
SHPF domain protein and hypothetical proteins	ST131-FimH30	$bla_{\text{CTX-M-14}}$
Nucleoside transporter, *LacI* (transcriptional regulator)	ST131-FimH41	$bla_{\text{CTX-M-15}}$
gspD (Type II secretion system protein), prophage regions	ST131-FimH41	$bla_{\text{CTX-M-27}}$
mdtN (efflux pump), *recQ* (recombinase), *nanM* (carbon metabolism)	ST648	$bla_{\text{CTX-M-15}}$

*Some isolates carry multiple copies of $bla_{\text{CTX-M}}$. Summary of ongoing analysis of isolates from WGS data from 2019, 2020 and 2021.

Effect of *bla*$_{CTX-M}$ location on bacterial growth

Not obvious correlation between *bla*$_{CTX-M}$ location, AMR gene copy number and bacterial growth rates



CAZ: ceftazidime, CRO: ceftriaxone

Isolate_AGAR_ID	dt (min)	*bla*$_{CTX-M\ gene}$	Location (copies)
19GNB-0037	24.00±1.52	No resistance	
19GNB-0295	22.55±0.42	No resistance	
20GNB-0549	26.16±0.69	No resistance	
19GNB-1664	25.34±0.80	*bla*$_{CTX-M-15}$	plasmid (1)
20GNB-0362	22.50±0.91	*bla*$_{CTX-M-143}$	plasmid (1)
19GNB-1023	22.27±0.54	*bla*$_{CTX-M-15}$	chromosome (1)
19GNB-0329	27.96±1.51	*bla*$_{CTX-M-15}$	chromosome (1)
20GNB-0341	26.77±0.72	*bla*$_{CTX-M-27}$	plasmid (1)
20GNB-1037	24.53±0.67	*bla*$_{CTX-M-27}$	plasmid (1)
21GNB-0301	26.80±0.74	*bla*$_{CTX-M-14a}$	plasmid (1)
19GNB-0077	25.94±7.51	*bla*$_{CTX-M-14a}$	chromosome (1)
20GNB-0205	24.32±0.26	*bla*$_{CTX-M-14b}$	chromosome (1)
20GNB-1162	23.76±1.02	*bla*$_{CTX-M-14b}$	chromosome (2)
20GNB-0487	24.25±1.32	*bla*$_{CTX-M-14a}$	chromosome (3)
19GNB-0742	25.96±0.60	*bla*$_{CTX-M-14a}$	chromosome (6)
19GNB-0736	22.88±0.60	*bla*$_{CTX-M-14a}$	chromosome (7)

Growth conditions: LB broth, no antibiotics, 37°C, 96-well plate. dt: doubling time (calculated during log phase, between OD_{600} 0.02-0.09)

Other examples in the literature

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Chromosomal integration of *bla*$_{CTX-M}$ genes in diverse *Escherichia coli* isolates recovered from river water in Japan

Ryota Gomi [a,*], Masaki Yamamoto [b], Michio Tanaka [b], Yasufumi Matsumura [b]

RESEARCH ARTICLE

Amplification of the Chromosomal *bla*$_{CTX-M-14}$ Gene in *Escherichia coli* Expanding the Spectrum of Resistance under Antimicrobial Pressure

Eun-Jeong Yoon,[a,b,c] You Jeong Choi,[a,b] Dokyun Kim,[a,b] Dongju Won,[a] Jong Rak Choi,[a] Seok Hoon Jeong[a,b]

RESEARCH ARTICLE

Extended-Spectrum β-Lactamase Genes Traverse the *Escherichia coli* Populations of Intensive Care Unit Patients, Staff, and Environment

Robert A. Moran,[a] Liu Baomo,[b]* Emma L. Doughty,[a] Yingyi Guo,[b] Xiaoliang Ba,[c] Willem van Schaik,[a] Chao Zhuo,[b] Alan McNally[a]

Journal of Global Antimicrobial Resistance 17 (2019) 53–57

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Short Communication

Characterisation of chromosomally-located *bla*$_{CTX-M}$ and its surrounding sequence in CTX-M-type extended-spectrum β-lactamase-producing *Escherichia coli* isolates

Kouta Hamamoto[a,b], Itaru Hirai[a,c,*]

RESEARCH ARTICLE
Clinical Science and Epidemiology

Beneficial Chromosomal Integration of the Genes for CTX-M Extended-Spectrum β-Lactamase in *Klebsiella pneumoniae* for Stable Propagation

Eun-Jeong Yoon,[a,b] Bareum Gwon,[a,b] Changseung Liu,[a,b] Dokyun Kim,[a,b] Dongju Won,[a] Sung Gyun Park,[a] Jong Rak Choi,[a] Seok Hoon Jeong[a,b]

Conclusions (Part II)

1. Some global epidemic STs might be permanently incorporating AMR genes into their core genome and might not compromise their growth: **stable, long-term reservoir of AMR**?.
2. Might current antibiotic use policies (*less antibiotic use to reduce AMR*) be less effective if bacteria continue incorporating AMR genes in their chromosomes?.
3. Advanced therapies:
    a. Plasmid therapy, if AMR gene is located in plasmid.
    b. Bacteriophage therapy, if AMR gene is located in chromosome.

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Dr. Elena Martinez

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