Mutational activation of the CpxAR two-component system triggers antibiotic resistance in a clinical isolate of *Enterobacter aerogenes*

Muriel Masi* & Jean-Marie Pagès
Medical school of Marseille, Aix-Marseille University, France

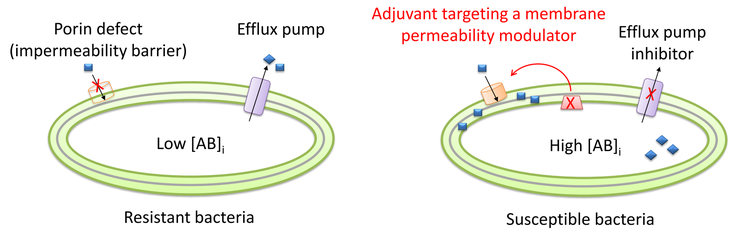
Introduction

Manipulating membrane permeability in Gram-negative bacteria to our therapeutic advantage involves increasing the influx or reducing the efflux of antibiotics. In this work, we sought to identify new drug targets to improve the influx thus the activity of poorly permeable antibiotics by using an approach of clinical relevance.

In a previous study, we reported the evolution of antibiotic resistance of four *Enterobacter aerogenes* strains, which were sequentially isolated during the clinical course of a patient. Comparative genomics of these isolates — herein referred as to P1 to P4, with P4 showing high level of resistance towards multiple classes of antibiotics — revealed a number of mutations, including one in the envelope stress-responsive two-component sensor *cpxA*, producing an Y144N substitution in the periplasmic domain of CpxA. This mutation was phenotypically and biochemically characterized in *Escherichia coli* K12. First, we showed that *cpxA*^{Y144N} is a gain-of-function (*cpxA**) mutation that constitutively activates the Cpx stress response pathway and confers a high level of resistance to various classes of antibiotics including β-lactams and aminoglycosides in. Then, we used a candidate approach to identify the Cpx regulon member(s) responsible for this phenotype and found that inactivation of the efflux gene *acrD* decreased resistance to aminoglycosides conferred by *cpxA**, while overexpression of porins restored susceptibility to β-lactams. The biochemical activities of the wild-type CpxA and CpxA^{Y144N} were characterized *in vivo* and showed that the mutant CpxA is altered in phosphotransfer reactions with CpxR. Finally, in order to understand the selection of this mutation and its competitive advantage regarding the patient's history, we set up an in vivo assay to monitor the activity of the β-lactamase AmpC in dependency of different signals and regulatory factors. Our results clearly show that the AmpR-mediated derepression of AmpC that follows cells exposure to β-lactams is dependent on the presence of both AmpG and CpxAR. Therefore, one can suggest that mutations located in the periplasmic domain of CpxA renders the Cpx response "locked on". Consistent with idea, the expression of CpxA^{Y144N} induced significant AmpC activity even in the absence of β-lactams.

Mutations in other two-component systems (TCS) — namely, PhoPQ and PmrAB — have been identified in another *E. aerogenes* clinical isolate. These results illustrate the impact of TCS on antibiotic-mediated stress adaptation and resistance and help to validate TCS as new targets for combination antibiotherapy.

Novel antibiotic targets using a genetic approach



- ***Objective:*** identify and connect the genes that control the OM permeability. Such gene products could be targeted by "adjuvant molecules" that would improve the activity of otherwise poorly permeable antibiotics.

- ***Step-by-step rationale:***

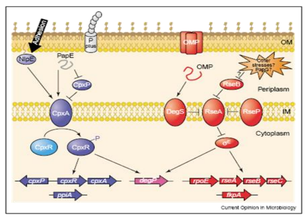
Clinical isolates → WGS → Gene identification → Construction of mutants → Phenotypic characterization → Target validation

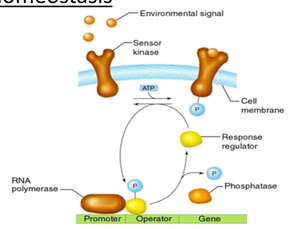
WGS revealed a mutational alteration of the HK CpxA in P4

Strain	P1	P2	P3	P4
Imipenem treatment	No (day 1)	Yes (day 7)	Yes (day 8)	Yes (day 12)
Susceptibilty to β-lactams	S	R	S	R
Porins (WB)	Yes	No	Yes	No
Efflux (WB)	Yes	Yes	Yes	Yes
Mutations				
Omp35 (OmpF homolog)	No	No	No	No
Omp36 (OmpC homolog)	No	Amber	No	Amber
Others	No	No	No	CpxA(Y144N)

Philippe *et al.*, PLoS One, 2016

CpxAR is a two-component regulatory system involved in envelop homeostasis

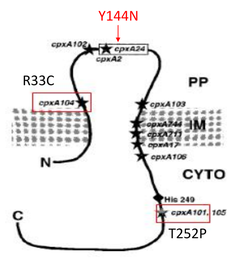




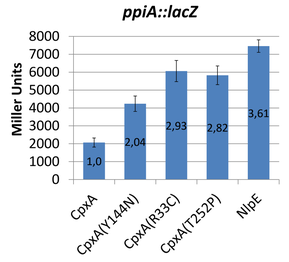
Results (I): Genetic and phenotypic characterization

cpxA(Y144N) is a *cpxA** allele

- During this decade, the Silhavy group provided the first links between the *cpx* locus and the secretion of aberrantly folded and/or targeted proteins. Mutations mapping to the *cpxA* locus were isolated in studies aimed at identifying protein trafficking factors that act on extracytoplasmic proteins. The *cpxA** mutations identified could suppress the toxicity associated with grossly misfolded and mislocalized mutant proteins and were shown to lead to activation of CpxA, ultimately causing up-regulated expression of the periplasmic protease DegP and degradation of the offending proteins.
- *cpxA* from *E. aerogenes* was cloned into the arabinose-inducible plasmid pBAD33 and mutant alleles were generated by site-directed mutagenesis. In order to investigate the effect of the Y144N mutation, we used 3 different MC4100 Δara *lacZ* fusions (*ppiA* and *cpxP*, which are primary downstream targets of the CpxAR regulon; *omrAB*, which is dependent on the EnvZ/OmpR regulon, but also a secondary downstream target of CpxAR via the MzrA connector). Consistent with previous results, *ppiA::lacZ* activity increased 2-3 fold in response to the overexpression of NlpE, CpxA(R33C) and CpxA(T252P). Similar activation was observed when the novel CpxA(Y144N) was overexpressed.

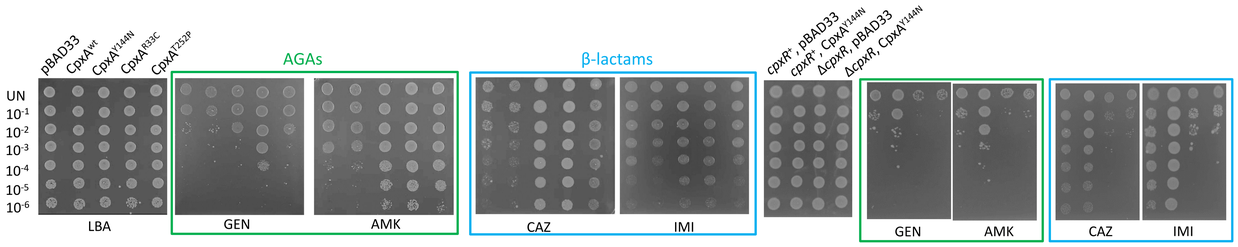


Adapted from Raivio & Silhavy, J. Bacteriol., 1997



Activation of the Cpx stress response confers resistance to some, but not all antibiotics

- It has recently been suggested that bactericidal antibiotics, including aminoglycoside antibiotics (AGAs) and toxic small molecules, such as hydroxyurea (HU), kill bacteria by generating reactive oxygen species (ROS) via a process requiring activation of the Cpx stress response (Kohanski *et al.*, Cell, 2008).
- A dominant *cpxA** mutation that constitutively activates the Cpx stress response confers a high level of resistance to both HU and AGAs in a CpxR-dependent manner (Mahoney *et al.*, J. Bacteriol., 2013). This suggests an opposite protective role for Cpx.
- Consistently to the latter hypothesis, we show that the overexpression of 3 *cpxA** mutations (R33C, T252P and Y144N) confer resistance to AGAs and β-lactams. This effect was not observed upon overexpression of the wild-type CpxA, indicating that increased antibiotic resistance resulted from the activation of the Cpx stress response.
- In addition, the removal of the cognate regulator CpxR abolished the increased resistance conferred by activated CpxA kinases, thus demonstrating that resistance was due to an « on-pathway » phosphorylation of CpxR and concomitant changes in the Cpx regulon.



Multiple Cpx regulon members may be responsible for antibiotic resistance

Candidate approach:

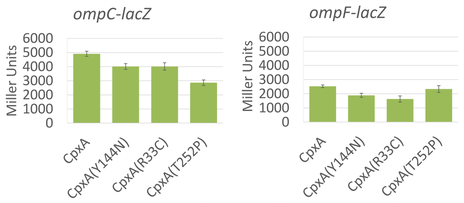
- Construct null mutants of members upregulated by the Cpx response
- Overexpress members downregulated by the Cpx response

Upregulated genes	Downregulated genes
cpxP	*efeU*
rdoA-dsbA	*mdtABCD*
psd	*ompF*
ydeH	*tsr*
ppiD	*aroK*
ycfS	*aer*
yccA	*csgDEFG*
spy	*glpABC*
ybaJ	
ung	
yebE	
yqjA	
ppiA	
ydeH	
htpX	
ftnB	
degP	
ompC	
acrD	
secA	

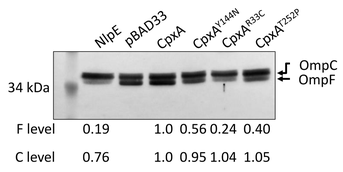
Price & Raivio, J. Bacteriol., 2009

Role of OmpF in Cpx-mediated antibiotic resistance

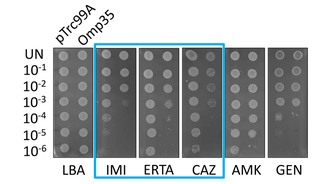
- The Cpx envelope stress response system regulates the expression of outer membrane porins OmpF and OmpC in *E. coli* (Batchelor *et al.*, J. Bacteriol., 2005).
- Here, Western Blot analysis showed that overexpression of all 3 CpxA* affect porins' expression (OmpF↓).
- Overexpression of Omp35 (OmpF ortholog in *E. aerogenes)* from an IPTG-inducible promoter in cells producing CpxA(Y144N) restored susceptibility to β-lactams but not to AGAs, demonstrating that reduction of OmpF levels due activated CpxA* is responsible for the increased to resistance to this class of antibiotics. This is consistent with the fact that OmpF is the preferred route for the translocation of β-lactams across the outer membrane.



Chromosomal *ompC*- and *ompF-lacZ* fusions are a gift from T. Ferenci (Univ. of Sydney).



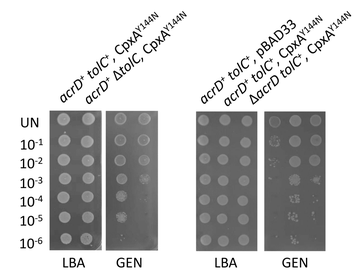
WB analysis of whole cell lysates (arabinose 0.2% final; 2h @ 37°C);
Eq 0.2 ODU/lane + loading control with α-OmpA antibodies
Mini SDS-PAGE 4M urea
α-loop 3 polyclonal antibodies (lab collection)



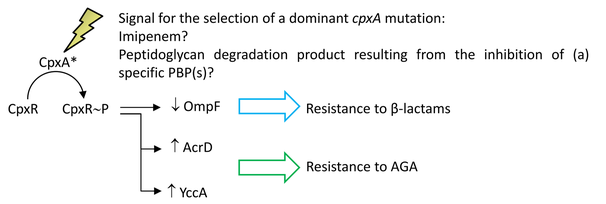
MC4100 Δ*ara* pBAD33-CpxA^{Y144N}

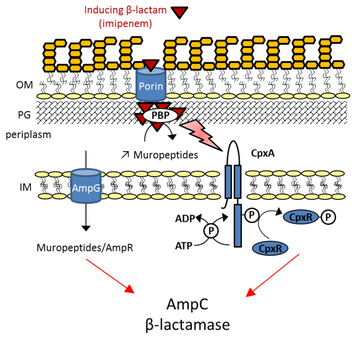
Role of efflux in Cpx-mediated antibiotic resistance

- AcrD is a TolC-dependent efflux pump specific for AGA.
- Removing acrD or tolC restored susceptibility to AGAs even in the presence of *cpxA** alleles, suggesting that AcrD contributes to resistance to AGAs in *cpxA** backgrounds.

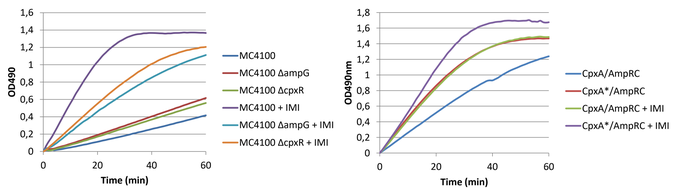


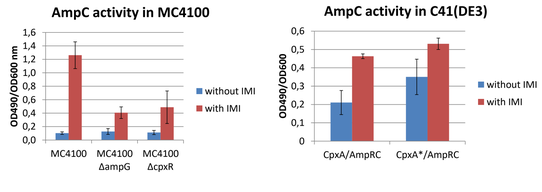
Emergence of antibiotic resistance in isolate P4





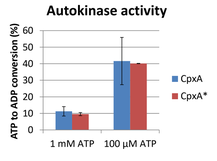
- In the majority of *Enterobacteriaceae* (although not in *E. coli*), the chromosome-encoded AmpC β-lactamase is repressed by AmpR in standard culture conditions, but induced in response to the addition of sub-inhibitory concentrations of β-lactams (namely, imipenem and cefoxitin).
- The interaction of these β-lactams with specific PBPs in the periplasm yields to their functional inhibition and to abnormal PG recycling with a local increase of muropeptides.
- For decades, derepression of AmpC has been reported to be dependent on the IM permease AmpG, which retrotranslocates PG degradation products back in the cytoplasm. Interestingly, recent studies also describe the contribution of TCS to AmpC expression in several unrelated bacterial species.
- In order to test the above model, we set up an inducible AmpC-mediated nitrocefin assays using (i) *E. coli* MC4100 and derivative mutants carrying pACYC184-ampRC; (ii) C41(DE3) pET24a+-cpxA/cpxA* + pACYC184.



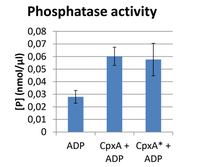


Results (II): Biochemical characterization

- Sensor kinases of TCS perform 3 reactions: autophosphorylation on a specific His; phosphate transfer on a specific Asp in their cognate response regulator; dephosphorylation of the regulator to shut down the activation pathway.
- *cpxA** alleles that have been characterized are defective in their phosphatase activity.



ADP-Glo Assay (Promega)



Pi colorimetric assay (Sigma)