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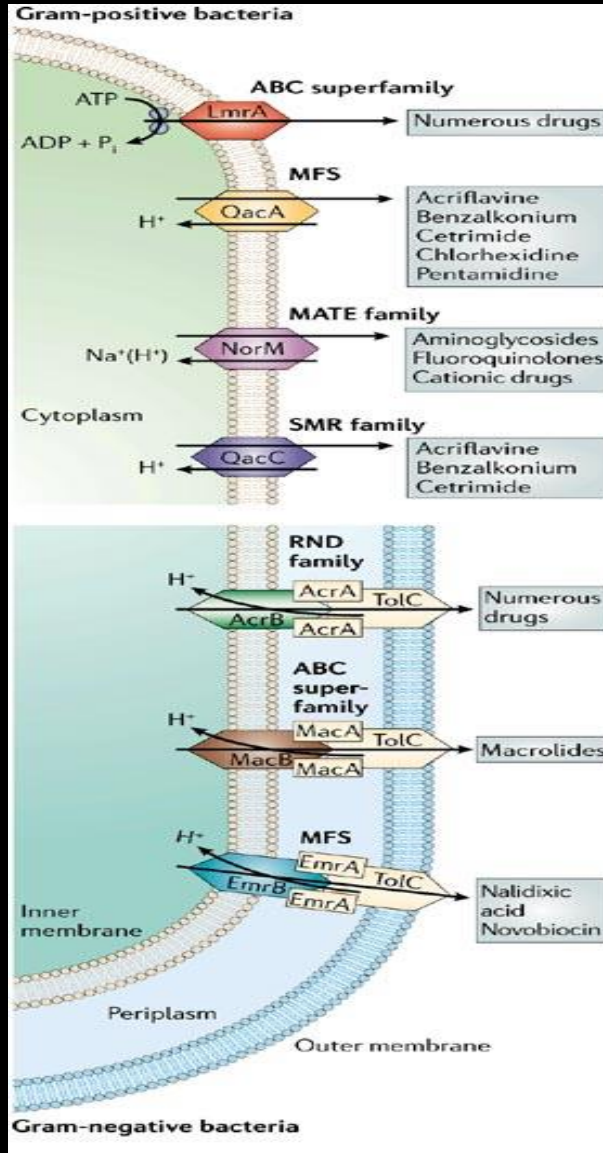
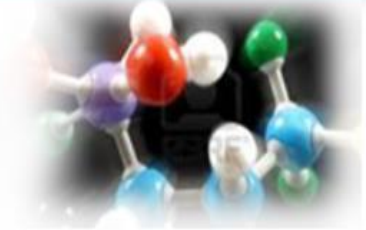


EFFLUX PUMPS OF BACTERIA ENERGETICS, GENETIC REGULATION; METHODS FOR PHYSIOLOGICAL ASSESSMENT

Leonard Amaral

Travel Medicine of the CMDT
Instituto de Higiene e Medicina Tropical
Universidade Nova de Lisboa

L Amaral, August 2014

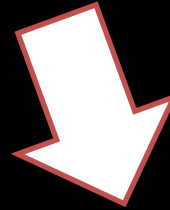




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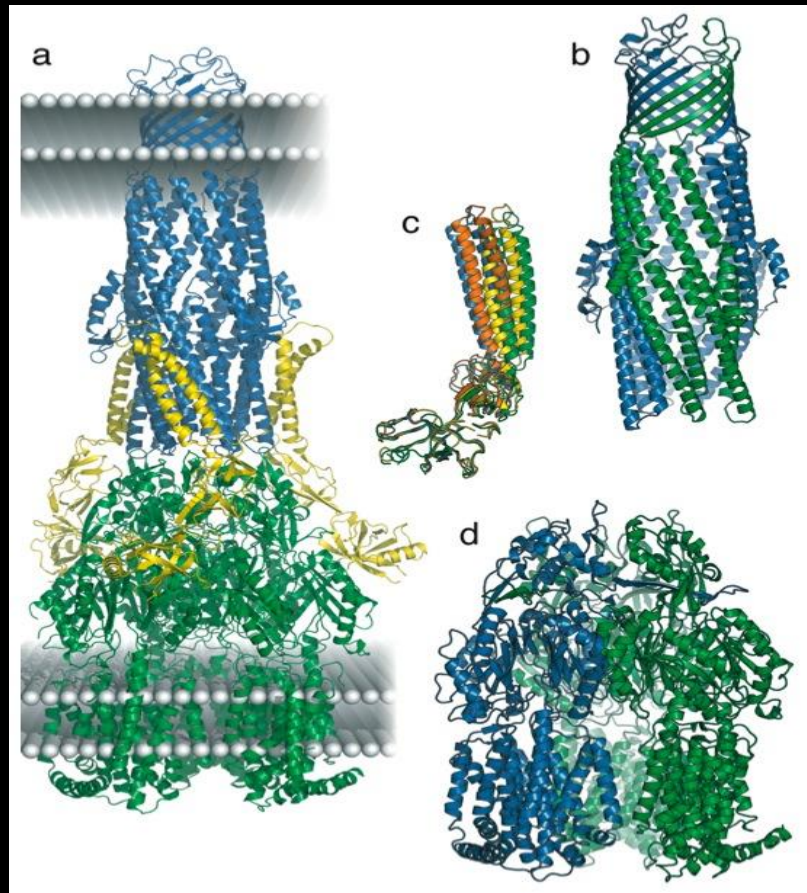
AcrAB-ToIC EFFLUX PUMP of *E. coli*



PROTON MOTIVE FORCE
DEPENDENT



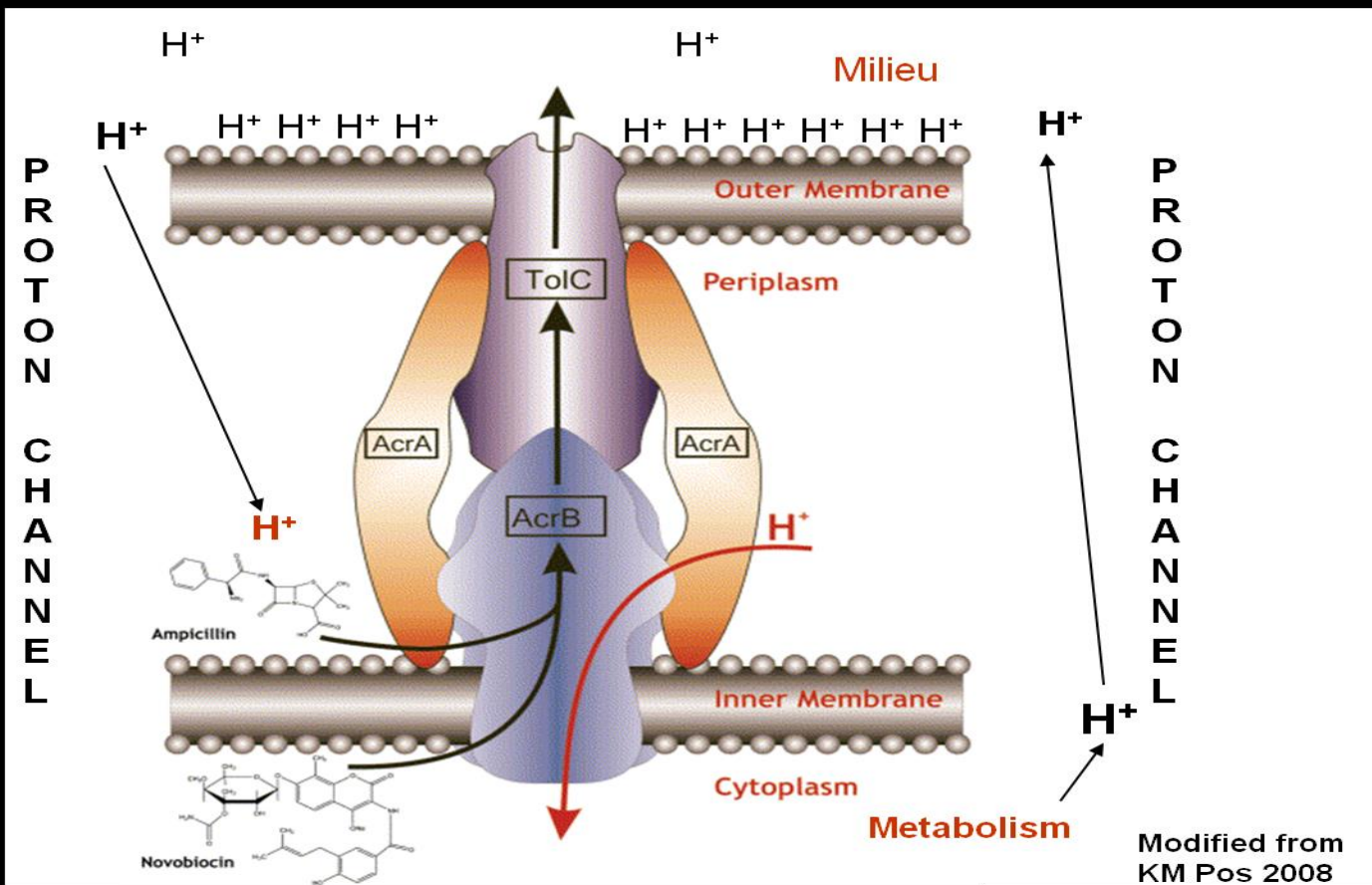
The AcrAB-TolC Efflux Pump of Gram-Negative Bacteria



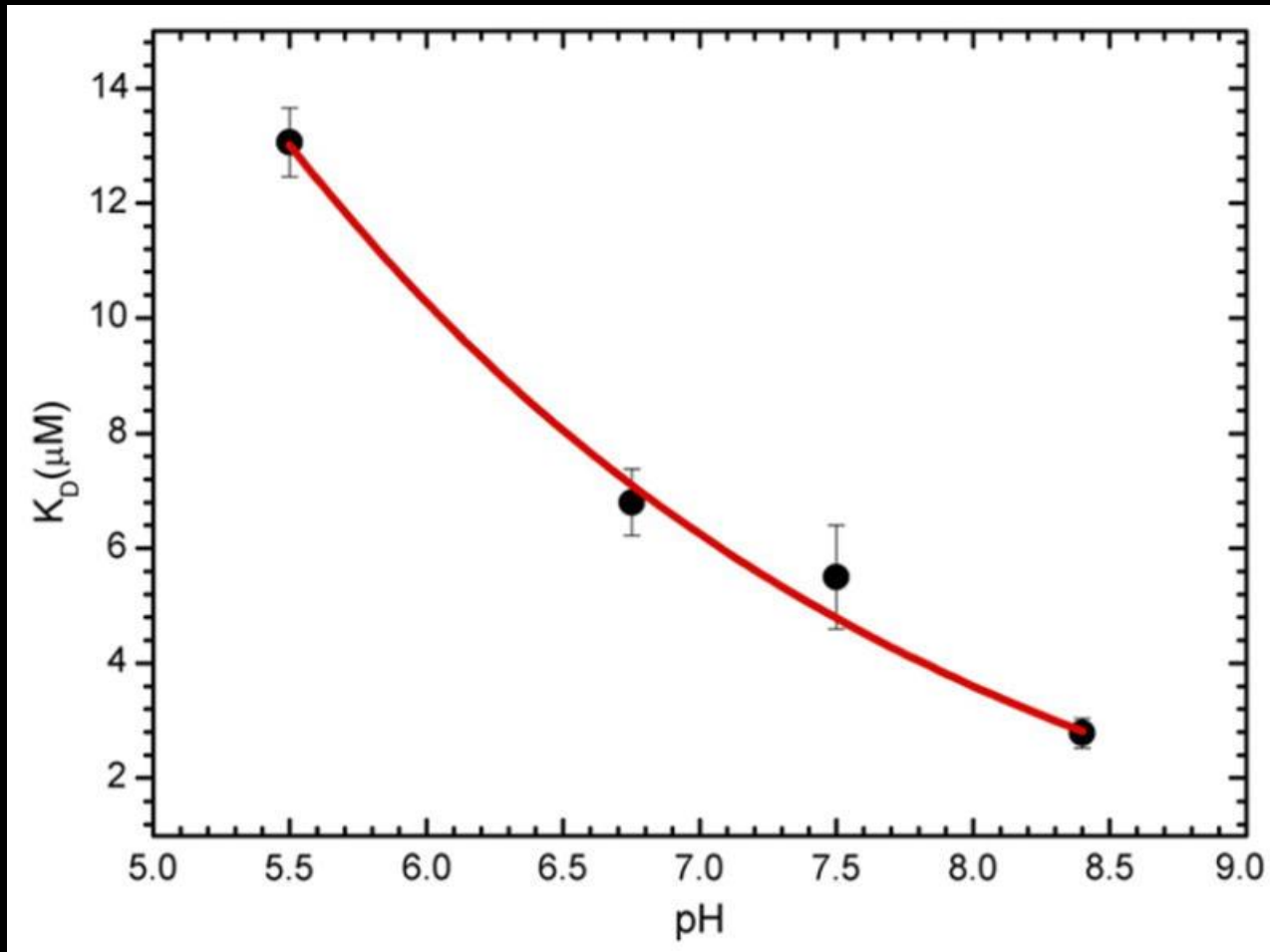
- a) AcrAB-TolC
- b) TolC
- c) Fusion Protein (AcrA)
- d) AcrB



Model of the AcrAB Efflux Pump of Gram-negative bacteria.



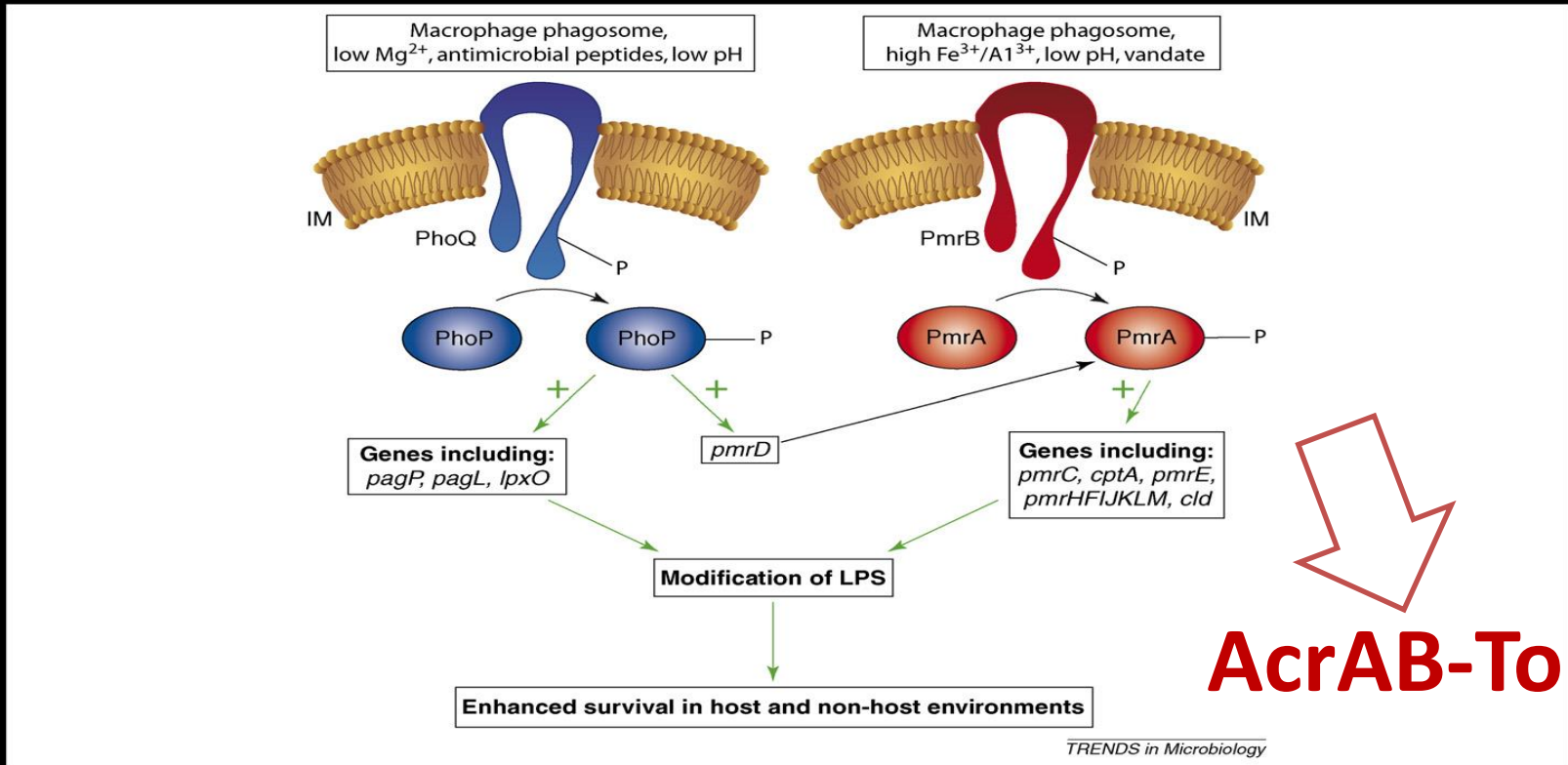
Dissociation of AcrAB substrate and pH



H. Nikaido 2007



PhoPQ & PmrAB



AcrAB-ToIC



Effect of pH on accumulation of EB by *Enterobacter aerogenes*

pH5



0 mg/L EB



0,5 mg/L EB



1,5 mg/L EB



2,5 mg/L EB

pH8



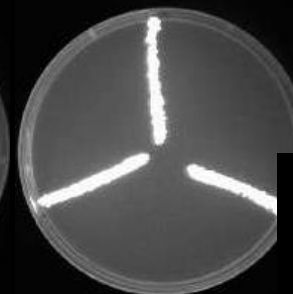
0 mg/L EB



0,5 mg/L EB



1,5 mg/L EB



2,5 mg/L EB

EA ATCC 13048

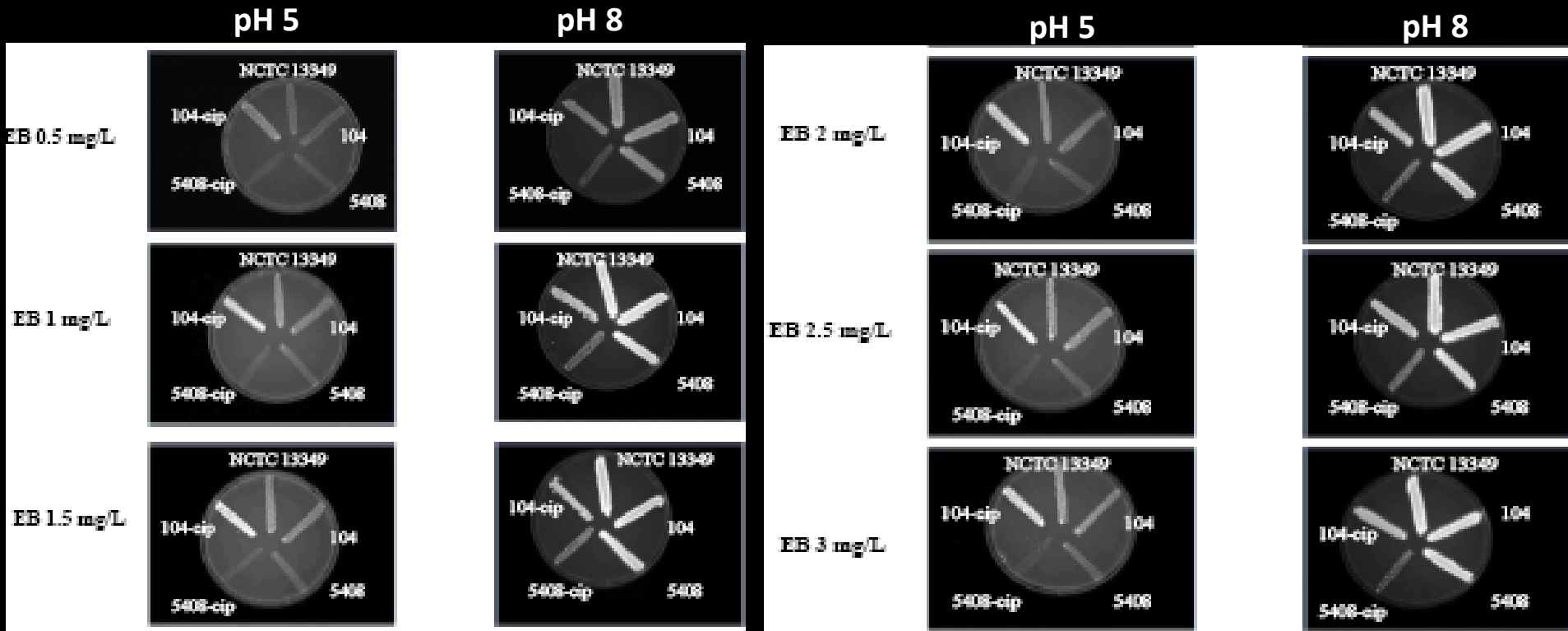


EA27

CM64



Accumulation of EB at pH5 and pH8 by Salmonella





Effect of pH on the MIC of antibiotics on *Salmonella* Enteritidis serovar Typhimurium

CIPROFLOXACIN

STRAIN	pH 5	pH 7	pH 8
104	15.6	0.19	0.156
104 cipro	>2000	62.5	6.25
5408	15.6	0.19	0.156
5408 cipro	>2000	62.5	6.25

NORFLOXACIN

104	62.5	1.9	0.625
104 cipro	500	250	62.5
5408	62.5	3.9	1.25
5408 cipro	1000	500	250



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Literature

- **Armen Y. Mulkidjanian**

Biochimica et Biophysica Acta 1757 (2006) 415–427.

Biochemistry (Moscow), Vol. 70, No. 2, 2005, pp. 251-256.

- **Klaas M Pos**

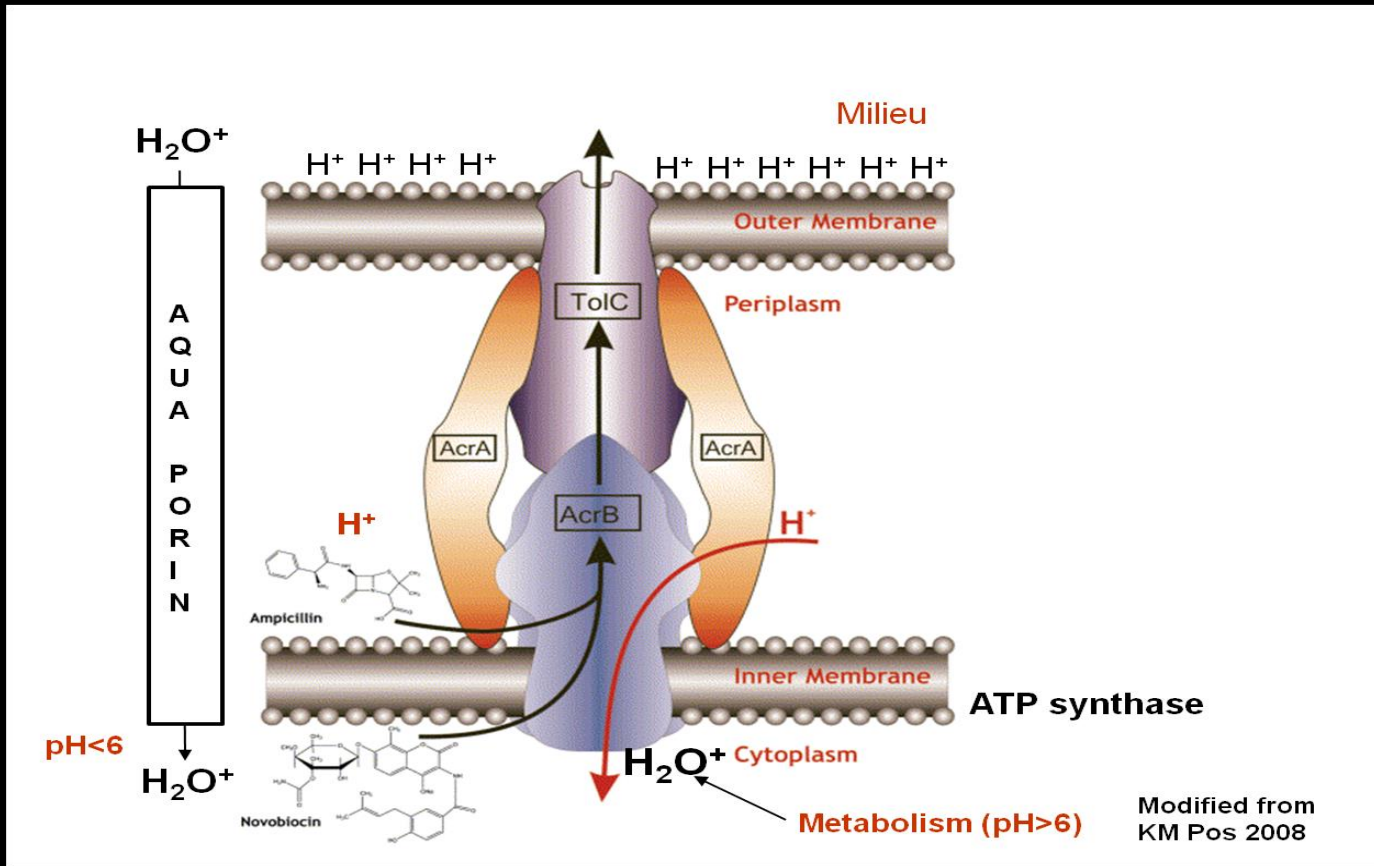
Drug transport mechanism of the AcrB efflux pump Biochimica et Biophysica Acta 1794 (2009) 782–793-

- **Seeger MA et al.**

Crucial Role of Asp408 in the Proton Translocation Pathway of Multidrug Transporter AcrB: Evidence from Site-Directed Mutagenesis and Carbodiimide Labeling. Biochemistry 2009, 48, 5801–5812.



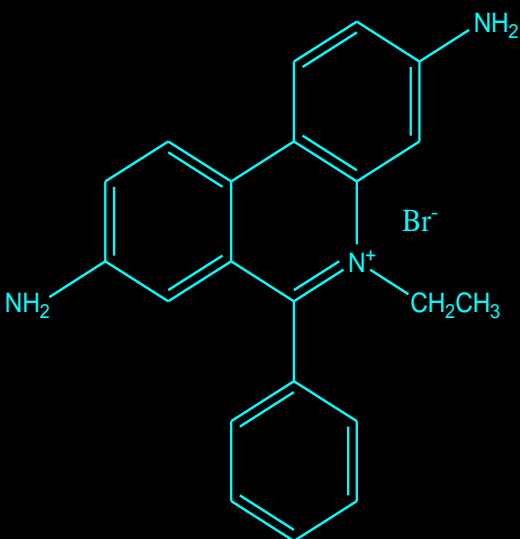
UP-DATED Model of the AcrAB Efflux Pump of Gram-negative bacteria



Modified from KM Pos 2008



Automated EB method for bacteria

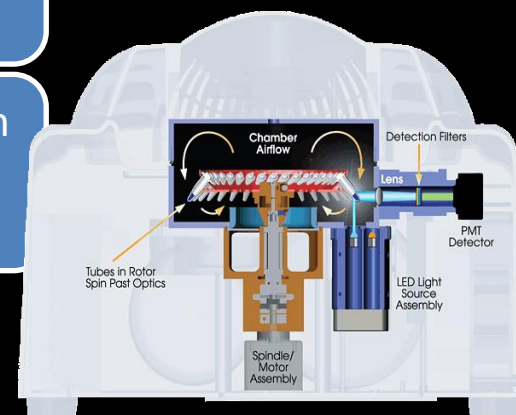
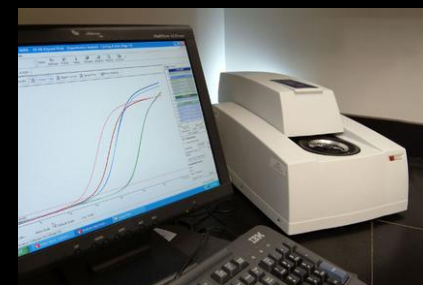


Ethidium Bromide
(EB)

transport of fluorescent substrates (EB) through the cell envelope of living bacterial cells

common substrate of bacterial efflux pumps

emits weak fluorescence in aqueous solution (outside cells) and becomes strongly fluorescent when concentrated in periplasm

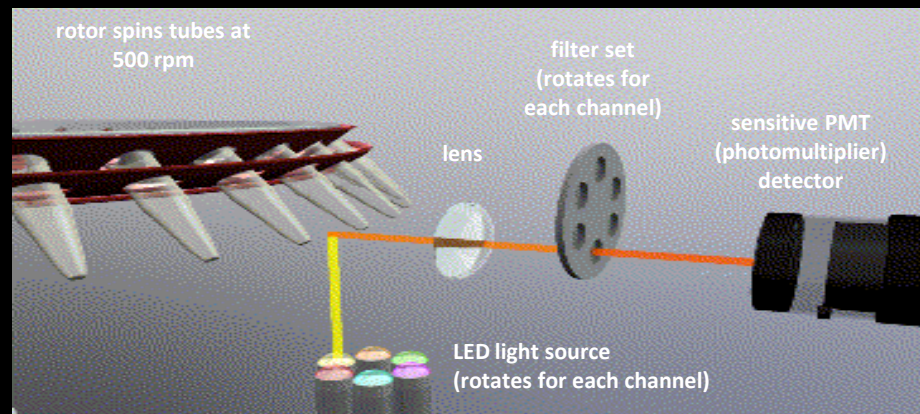
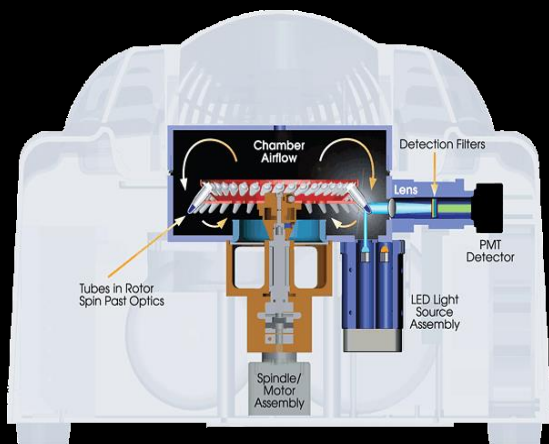




Detection of Efflux Activity by Real-time Fluorometry

- Detection of efflux activity on a real-time basis
- Separate detection of accumulation and efflux of ethidium bromide (EB)
- Identification of compounds with efflux pump inhibitory activity – efflux pump inhibitors (EPIs)

Rotor-Gene 3000™ (Corbett Research)





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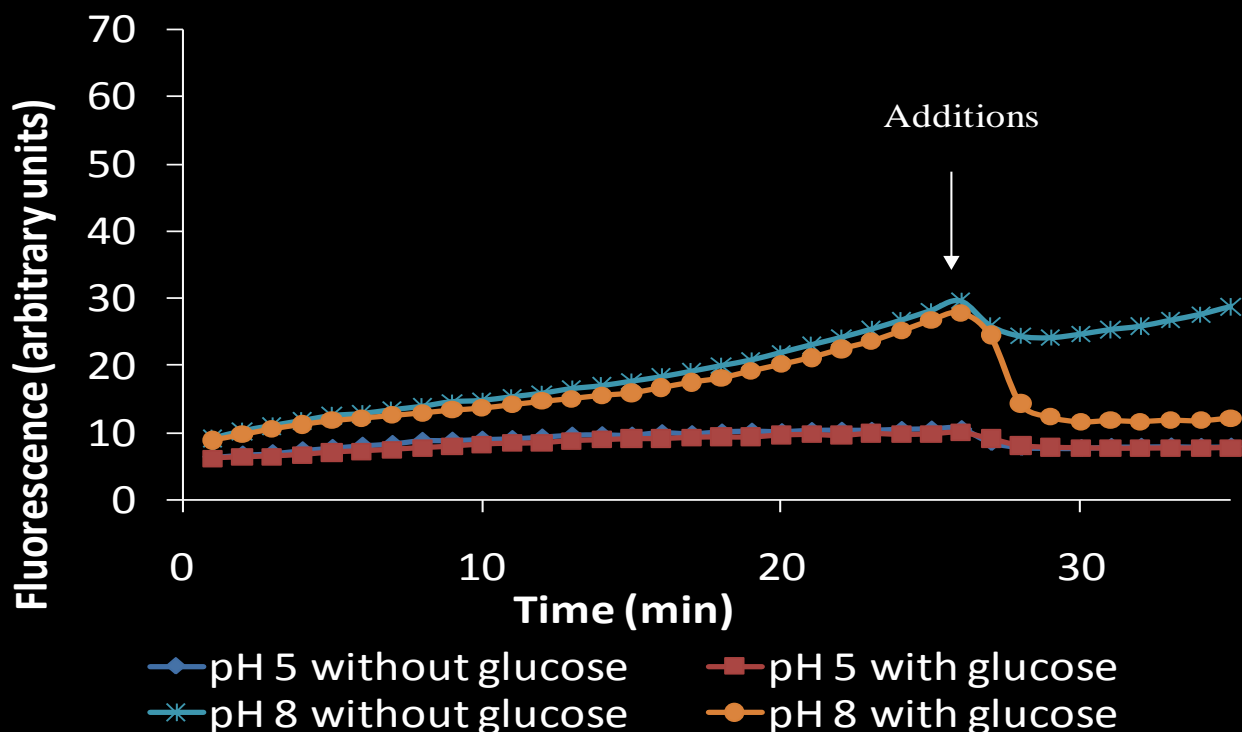


ROLE of pH in EFFLUX?

L Amaral, August 2014



EFFECT of pH on EFFLUX of EB





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Effect of pH and Glucose on the Accumulation and Efflux of EB

✓ **Mycobacteria**

Rodrigues L, Sampaio D, Couto I, Machado D, Wagner D, Kern WV, Amaral L and Viveiros M. The role played by efflux pumps in Intrinsic drug resistance of *Mycobacterium avium* complex to macrolides. *Int J Antimicrob Chemother* 2009;34:529-533.

✓ **E. Coli**

Martins A Spengler G, Rodrigues L, Viveiros M, Ramos J, Martins M, Couto I, Fanning S, Pagès JM, Bolla JM, Molnar J and Amaral L. pH Modulation of Efflux Pump Activity of Multi-Drug Resistant *E. coli*: Protection During its Passage and Eventual Colonization of the Colon. *PLoS One* 2009; 4:e6656.

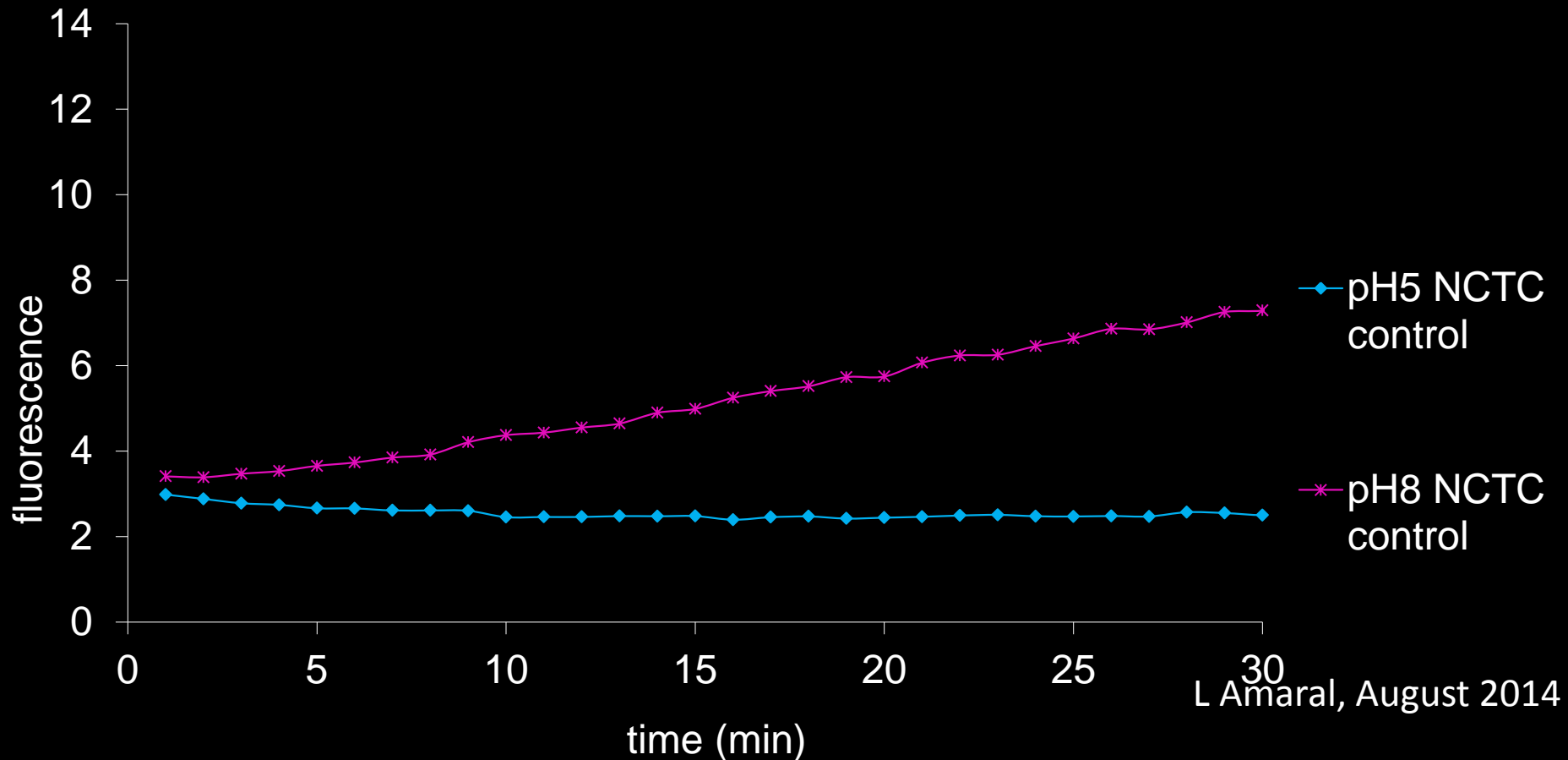
✓ **Salmonella:**

Amaral L, Cerca P, Spengler G, Machado L, Martins A, Couto I, Viveiros M, Fanning S and Jean-Marie Pagès. Ethidium Bromide Efflux by Salmonella: Modulation by metabolic energy, pH, ions and phenothiazines. *Int J Antimicrob Agents* 2011. In Press.

L. Amaral, August 2014

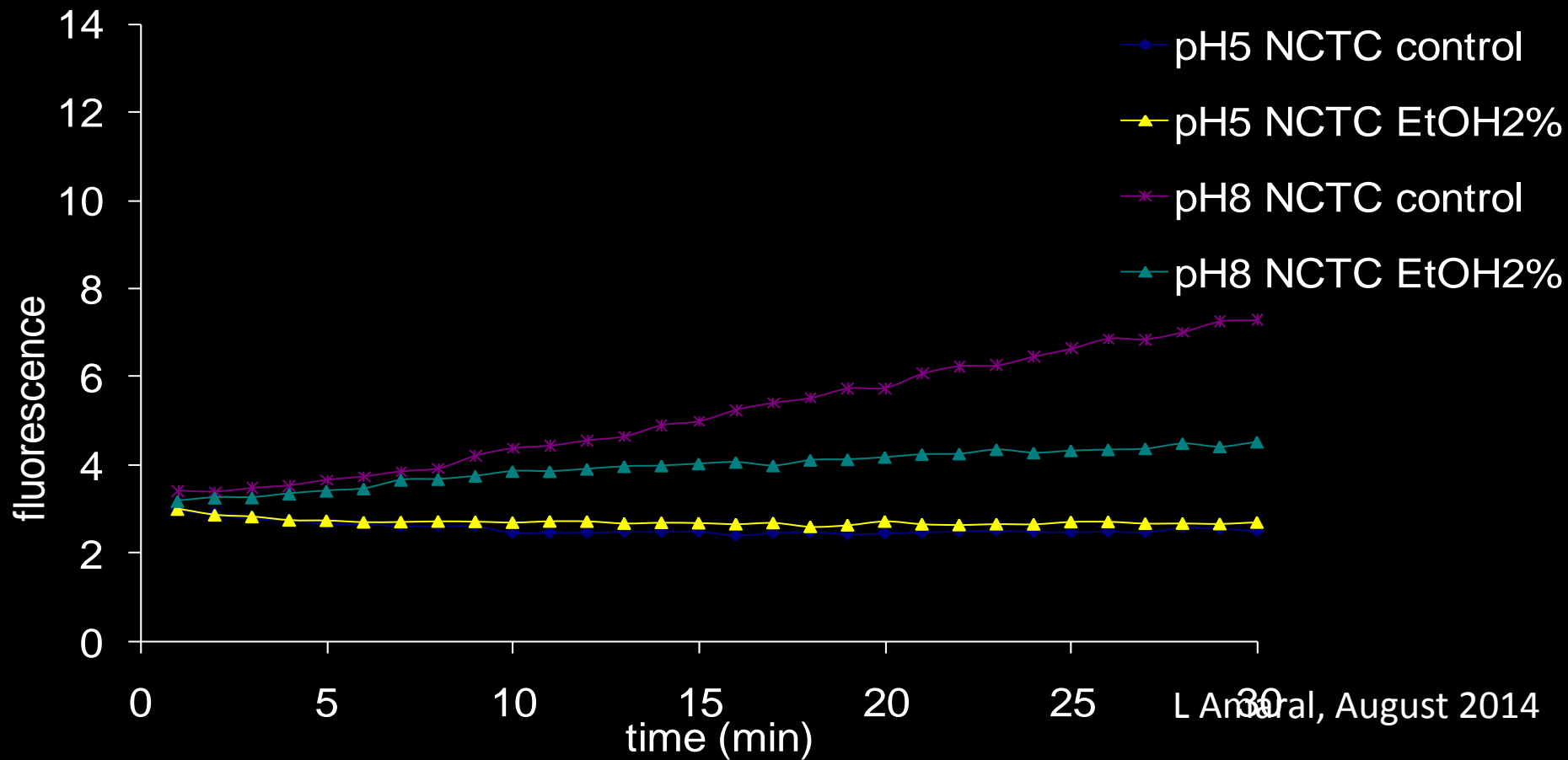


Accumulation of EB in dH₂O is modulated by pH, metabolic energy but not by Na⁺



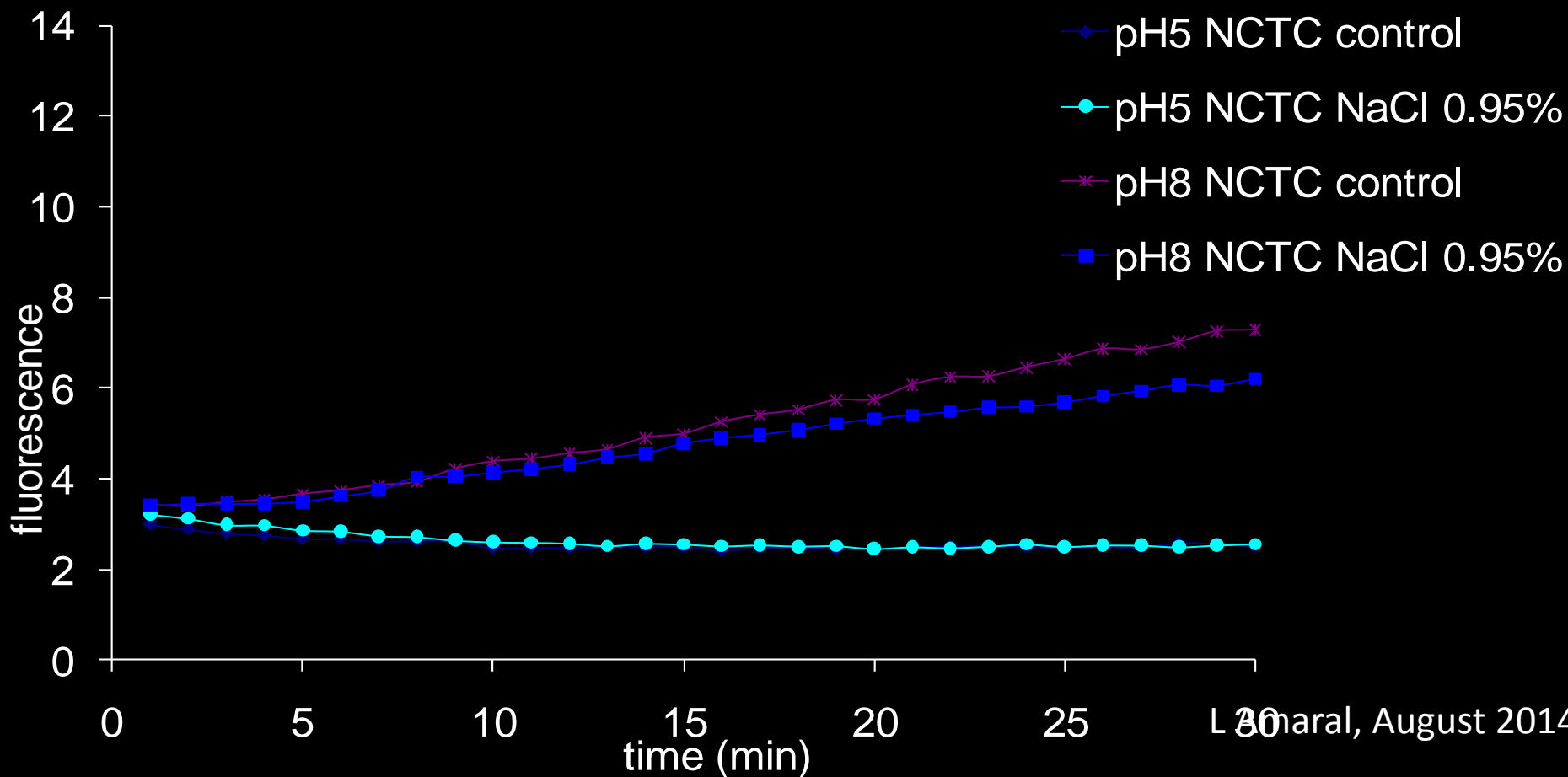


Accumulation of EB in dH₂O is modulated by pH, metabolic energy but not by Na⁺





Accumulation of EB in dH₂O is modulated by pH, metabolic energy but not by Na⁺

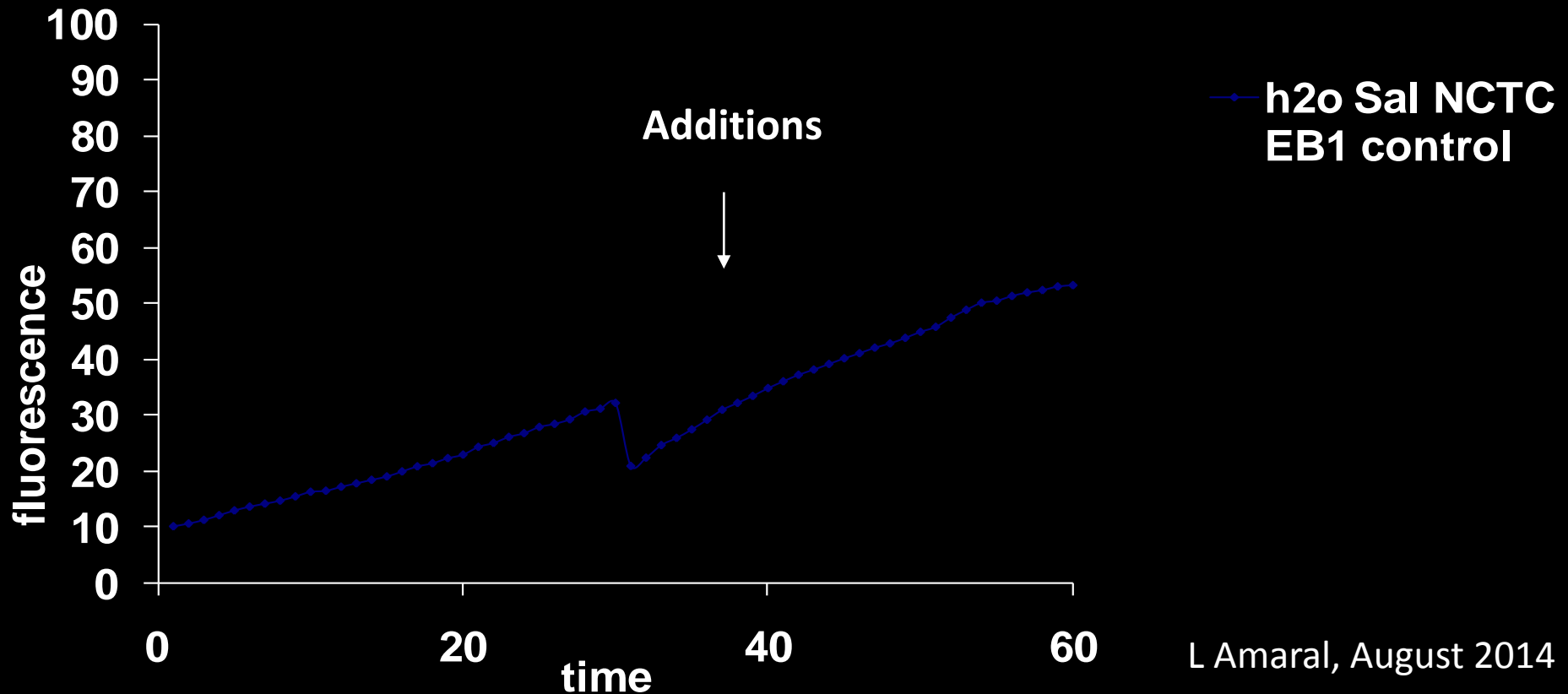




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Efflux of EB in dH₂O pH 5.5 is modulated by metabolic energy but not by Na⁺

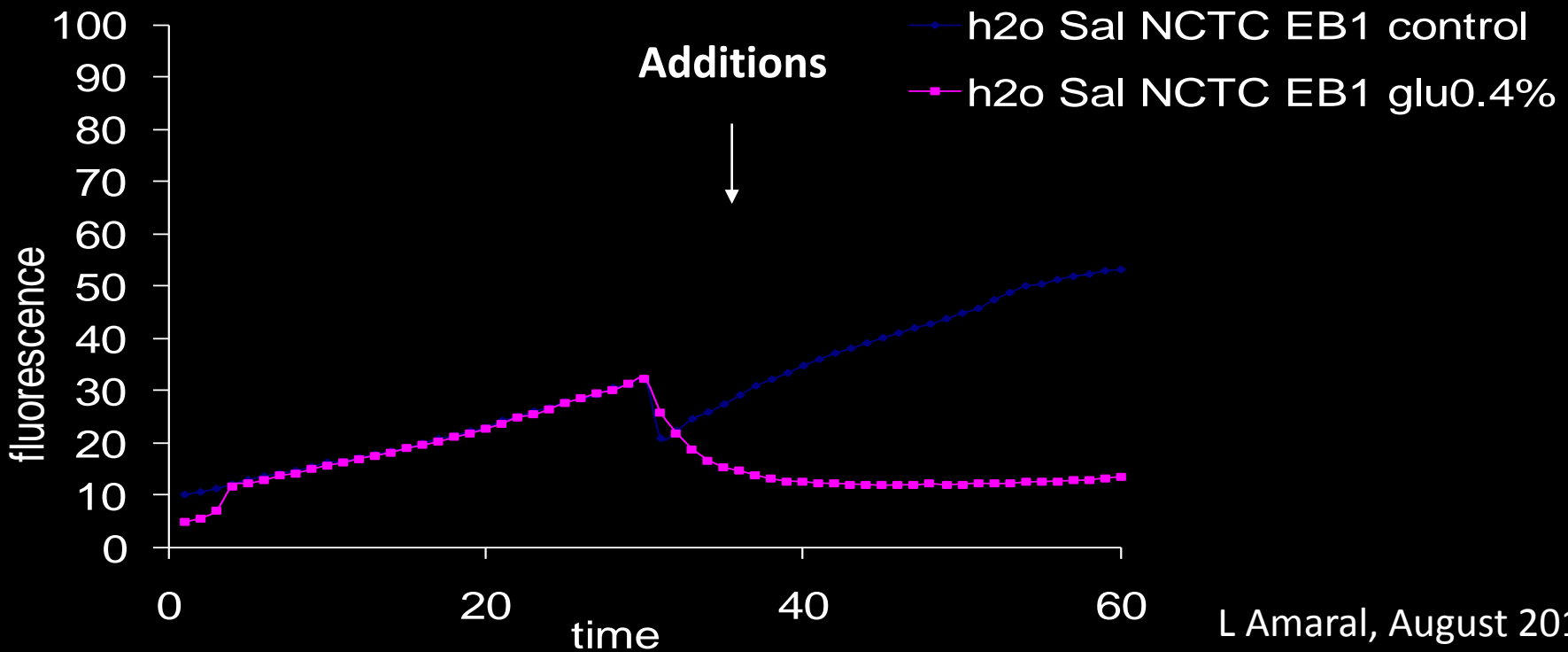




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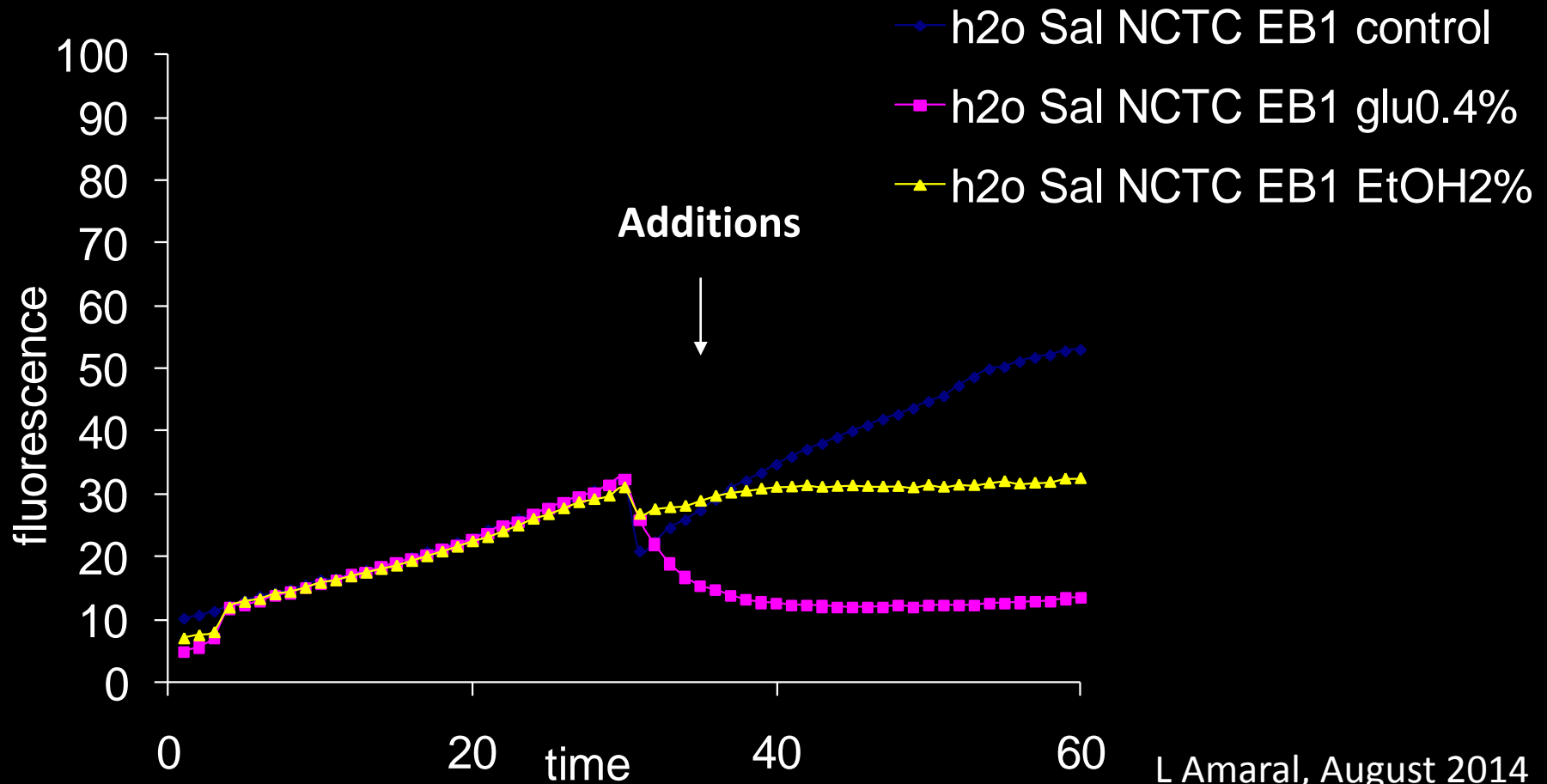


Efflux of EB in dH₂O pH 5.5 is modulated by metabolic energy but not by Na⁺



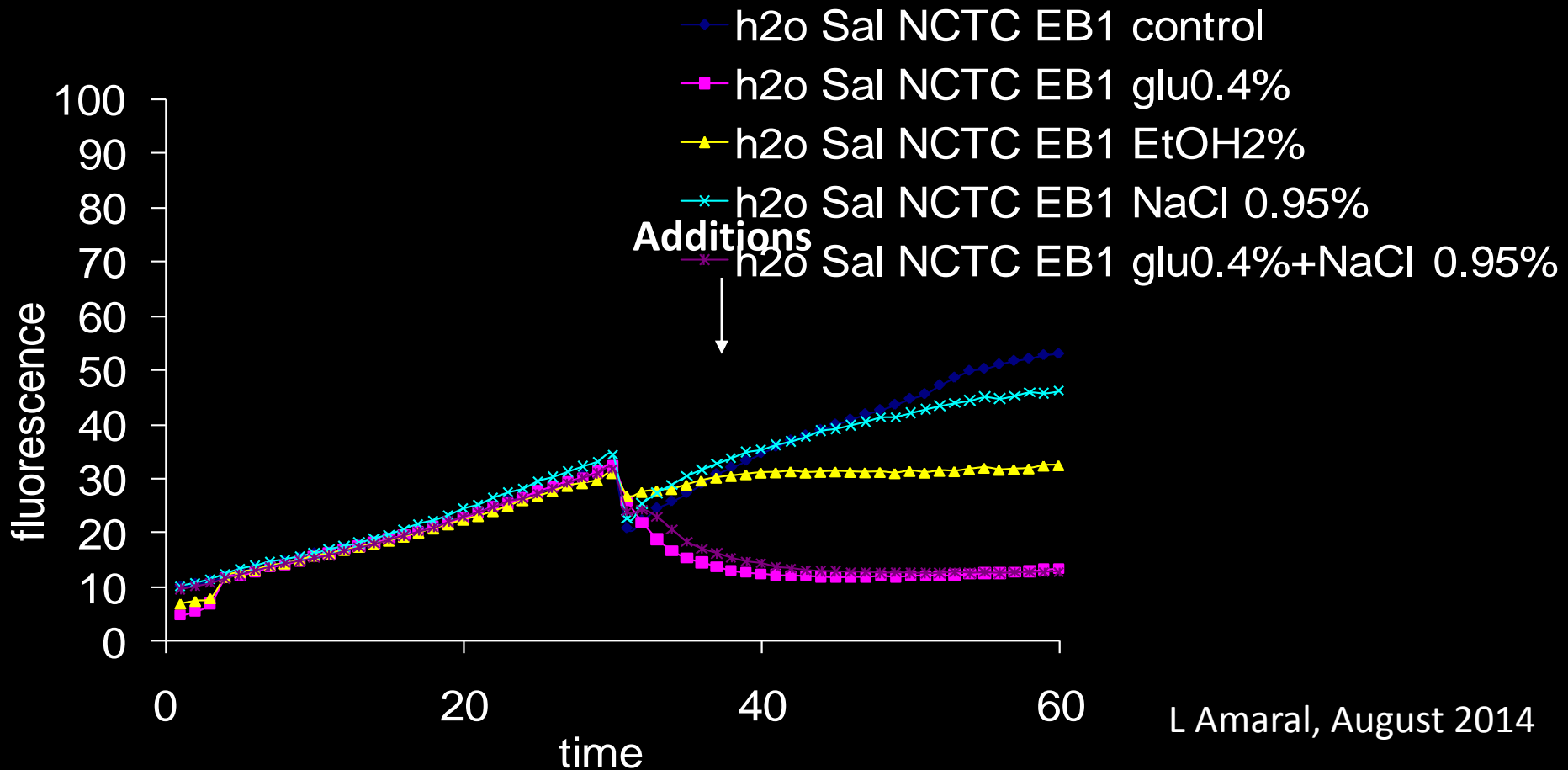


Efflux of EB in dH₂O pH 5.5 is modulated by metabolic energy but not by Na⁺





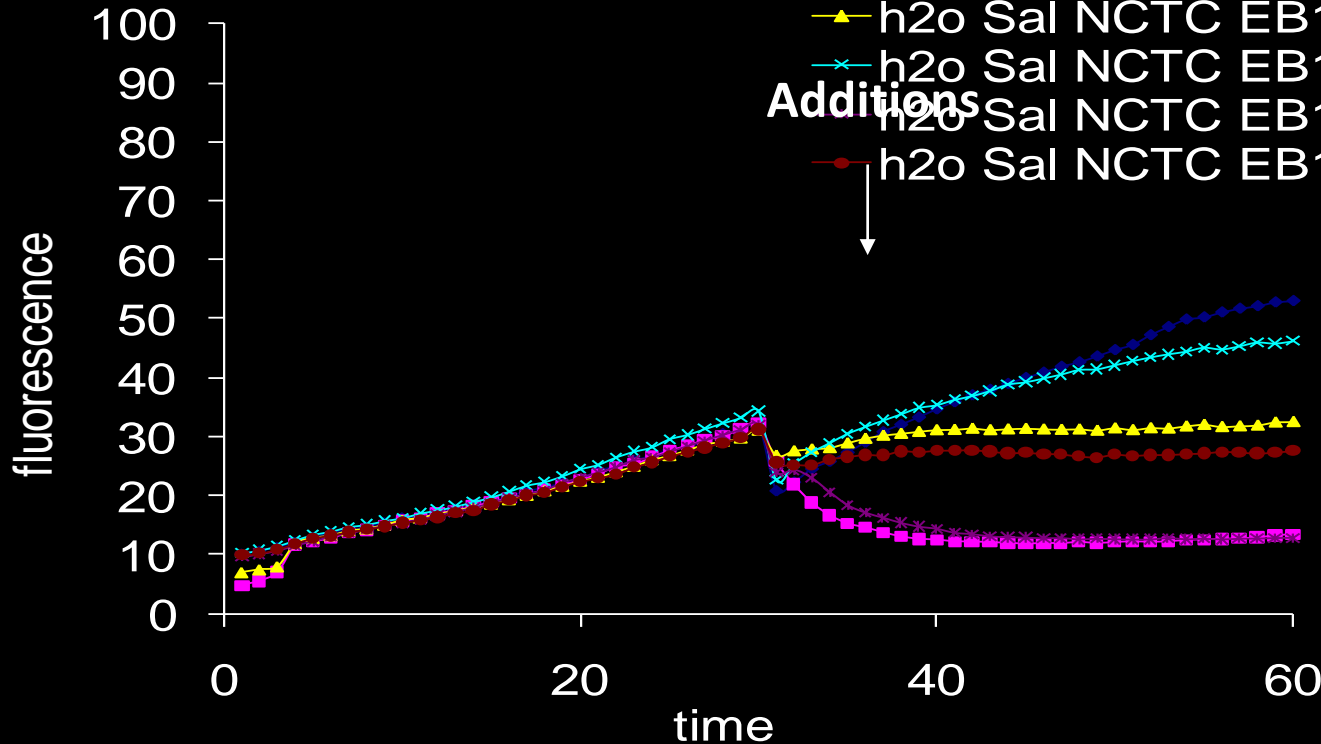
Efflux of EB in dH₂O pH 5.5 is modulated by metabolic energy but not by Na⁺





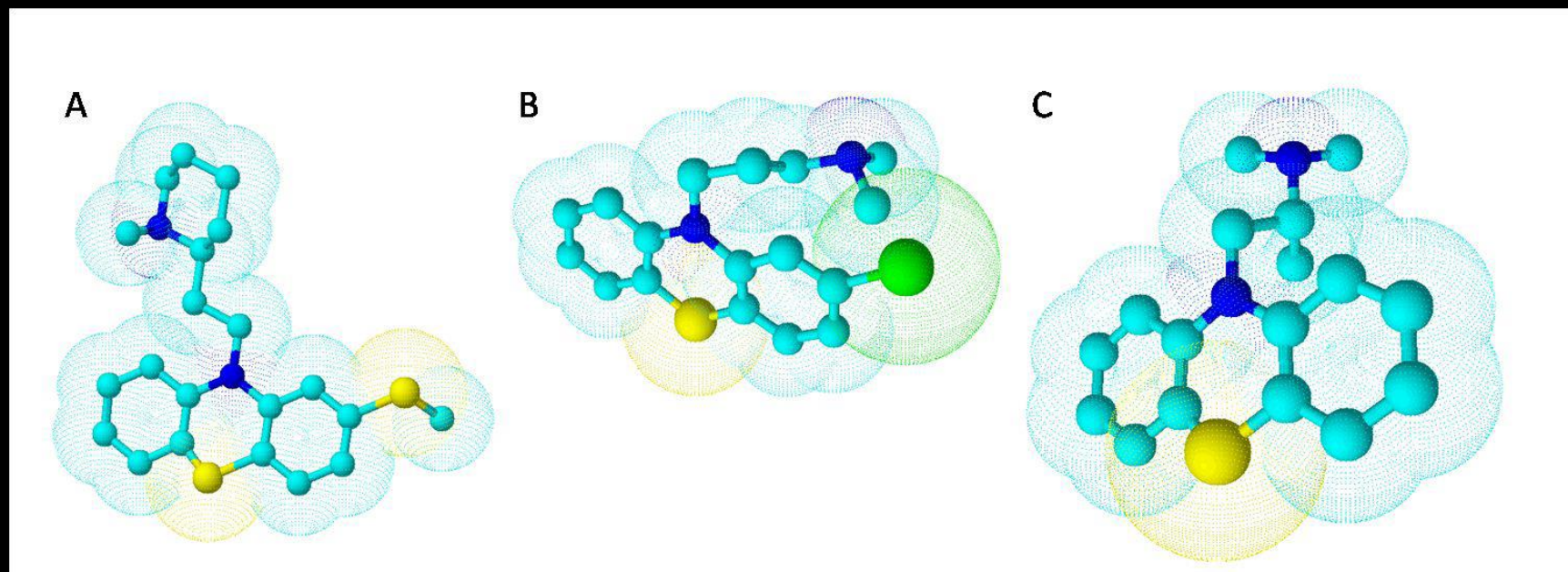
Efflux of EB in dH₂O pH 5.5 is modulated by metabolic energy but not by Na⁺

- h2o Sal NCTC EB1 control
- h2o Sal NCTC EB1 glu0.4%
- ▲— h2o Sal NCTC EB1 EtOH2%
- ×— h2o Sal NCTC EB1 NaCl 0.95%
- h2o Sal NCTC EB1 glu0.4%+NaCl 0.95%
- h2o Sal NCTC EB1 EtOH2%+NaCl 0.95%





Phenothiazines



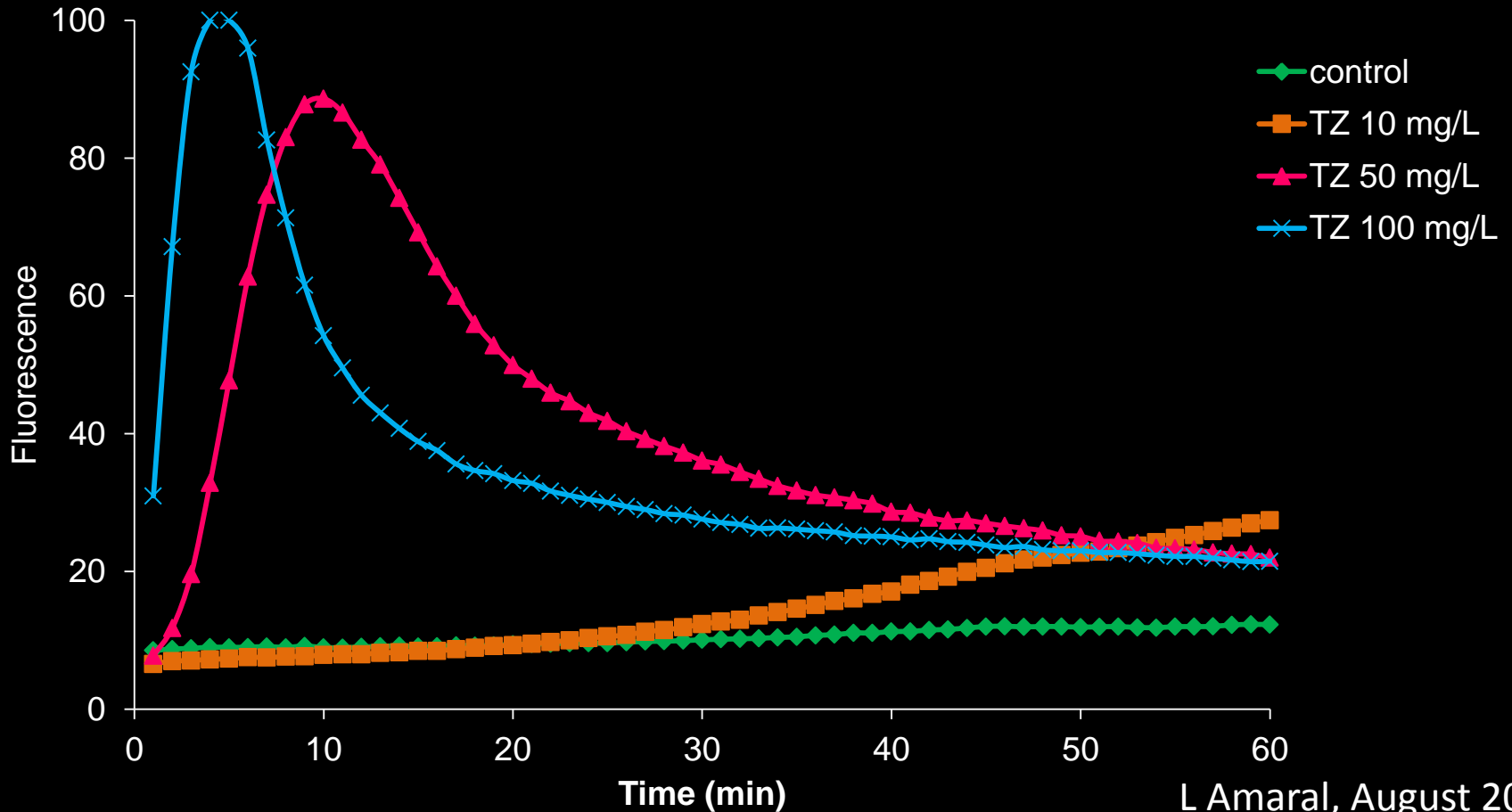
A: Thioridazine;

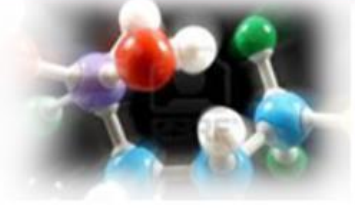
B: Chlorpromazine;

C: Promethazine



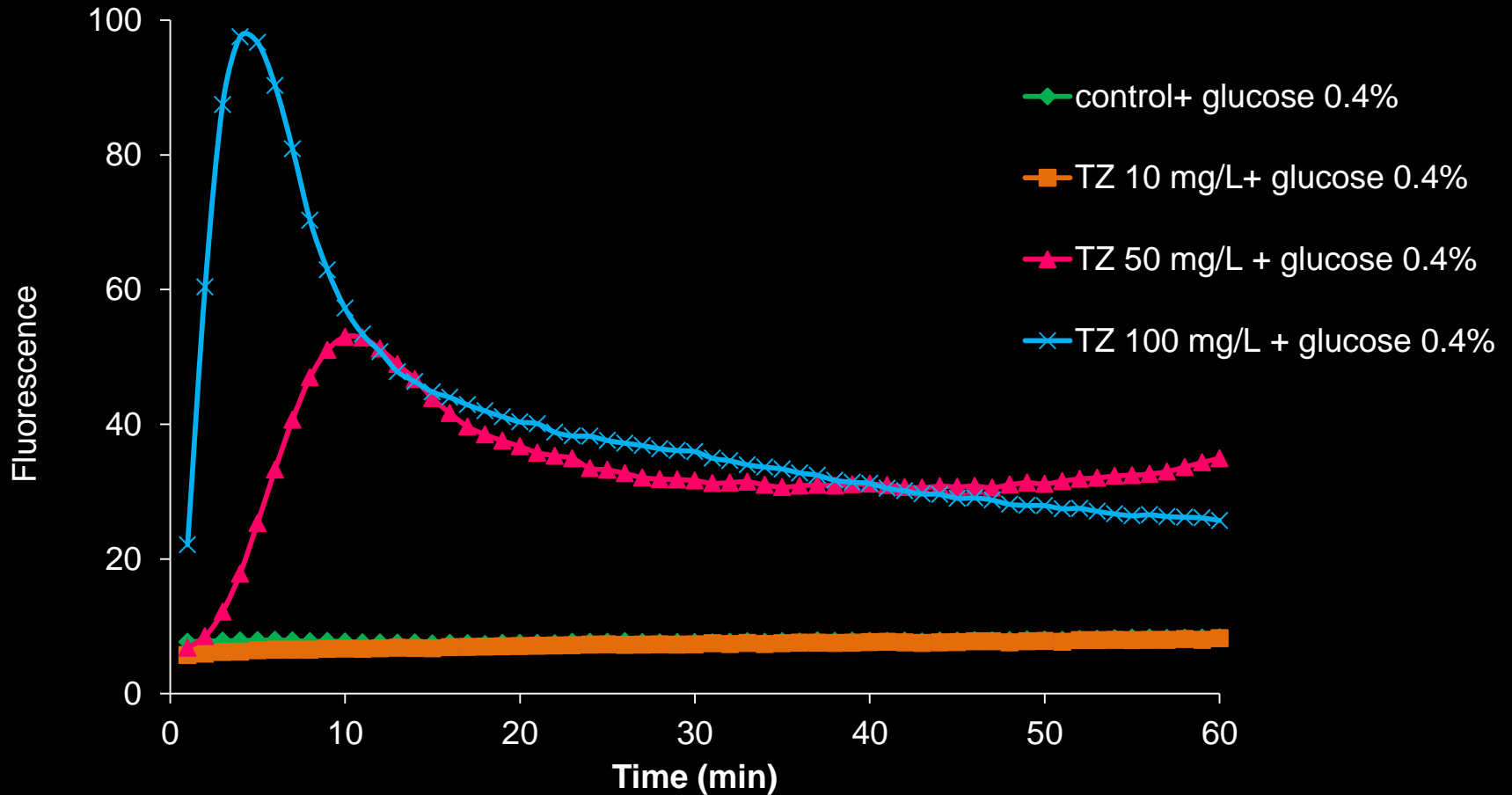
Efflux/accumulation of EB mediated by TZ at pH 7.4 (PBS) no glucose.





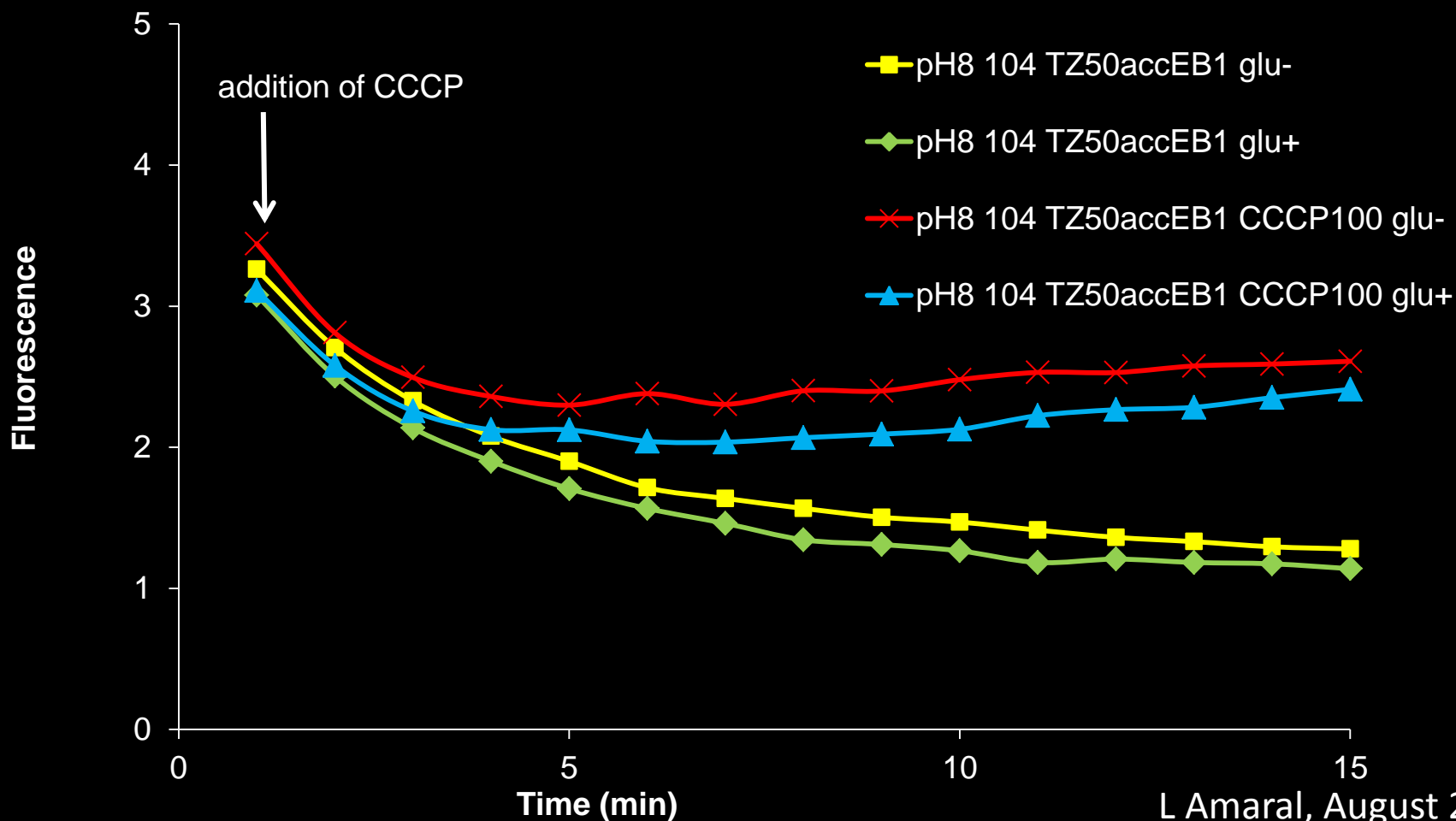
TROPICAL

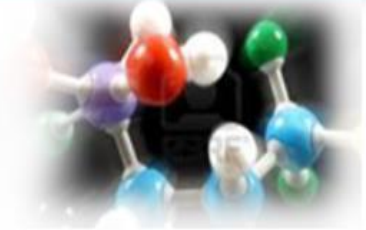
Efflux/accumulation of EB mediated by TZ at pH 7.4 plus glucose.



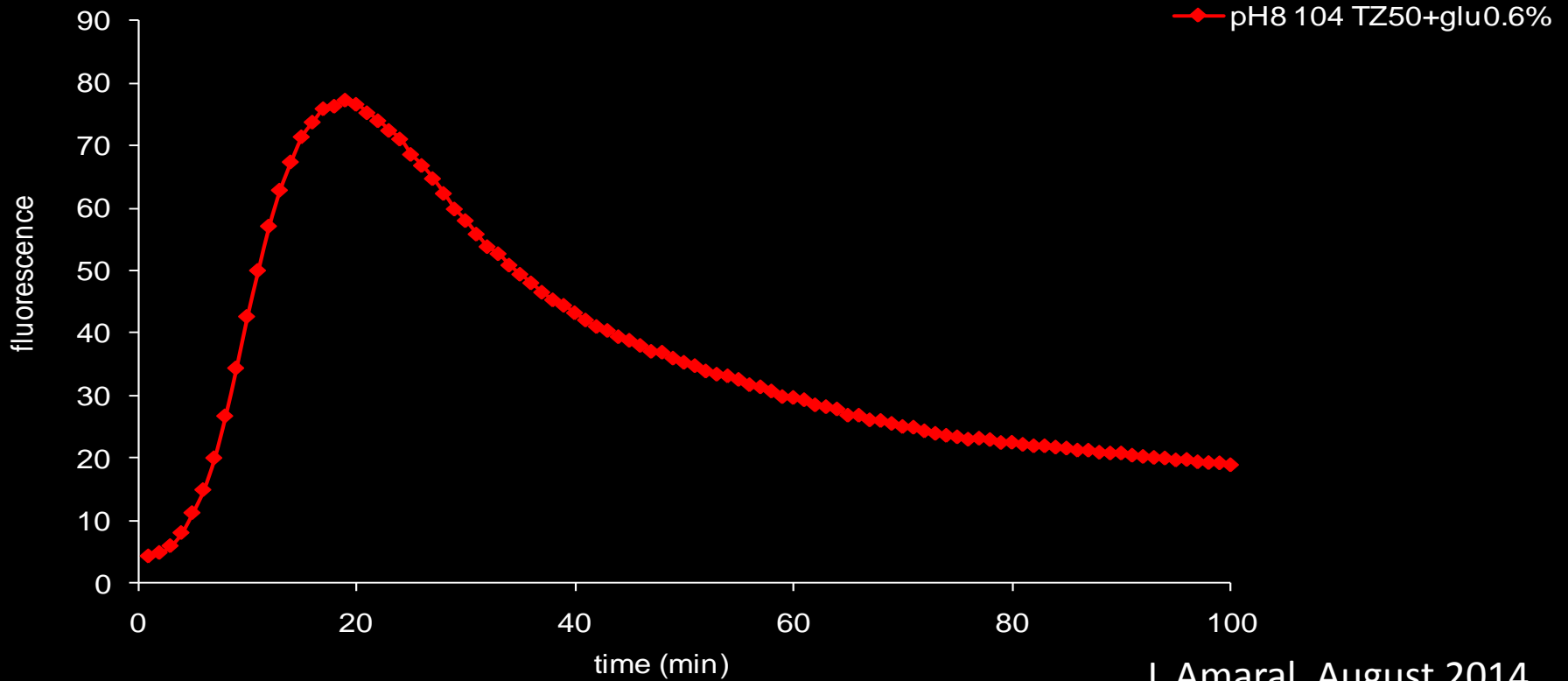


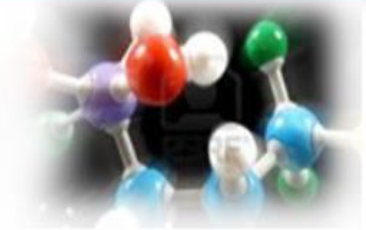
Efflux/accumulation of EB mediated by TZ at pH 8.



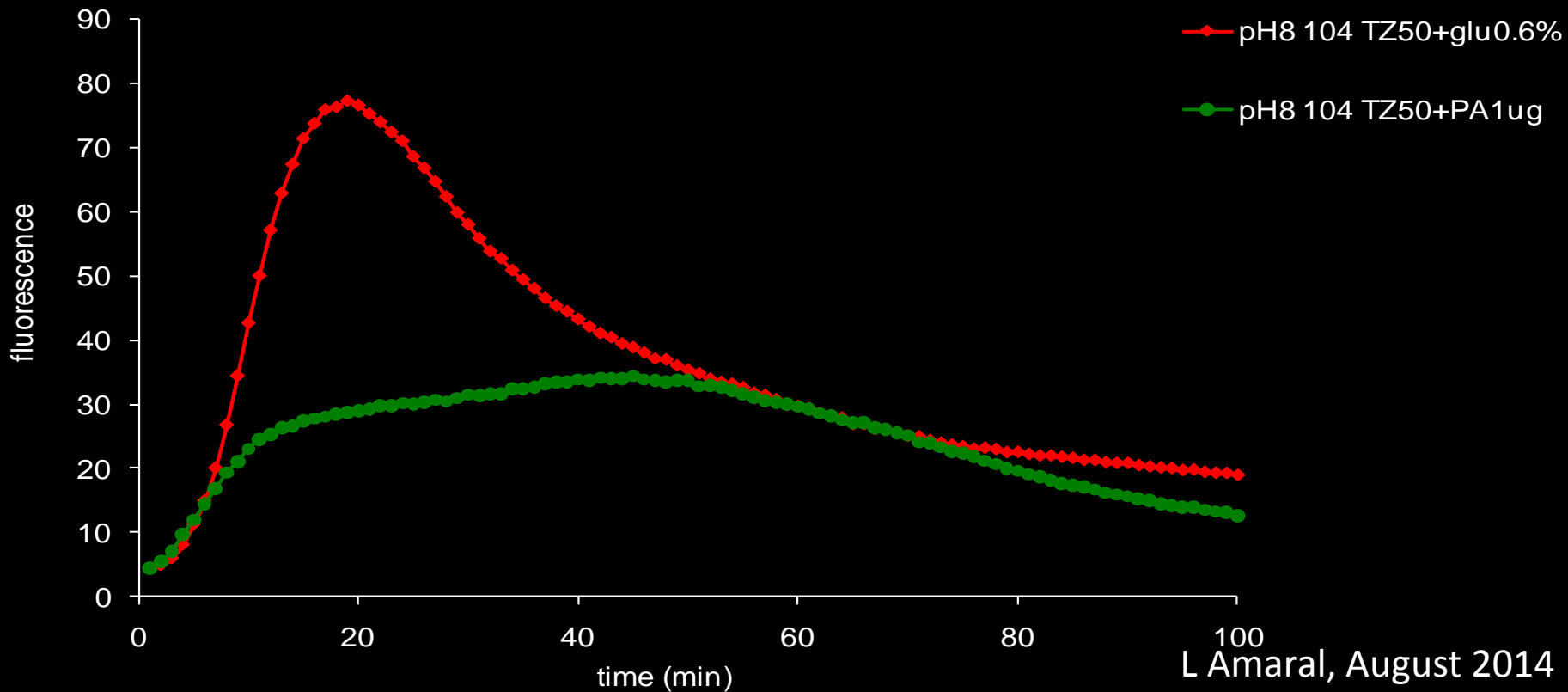


Effect of TZ on accumulation of EB in PBS pH 8, absence of metabolic energy and modulation by a fatty acid



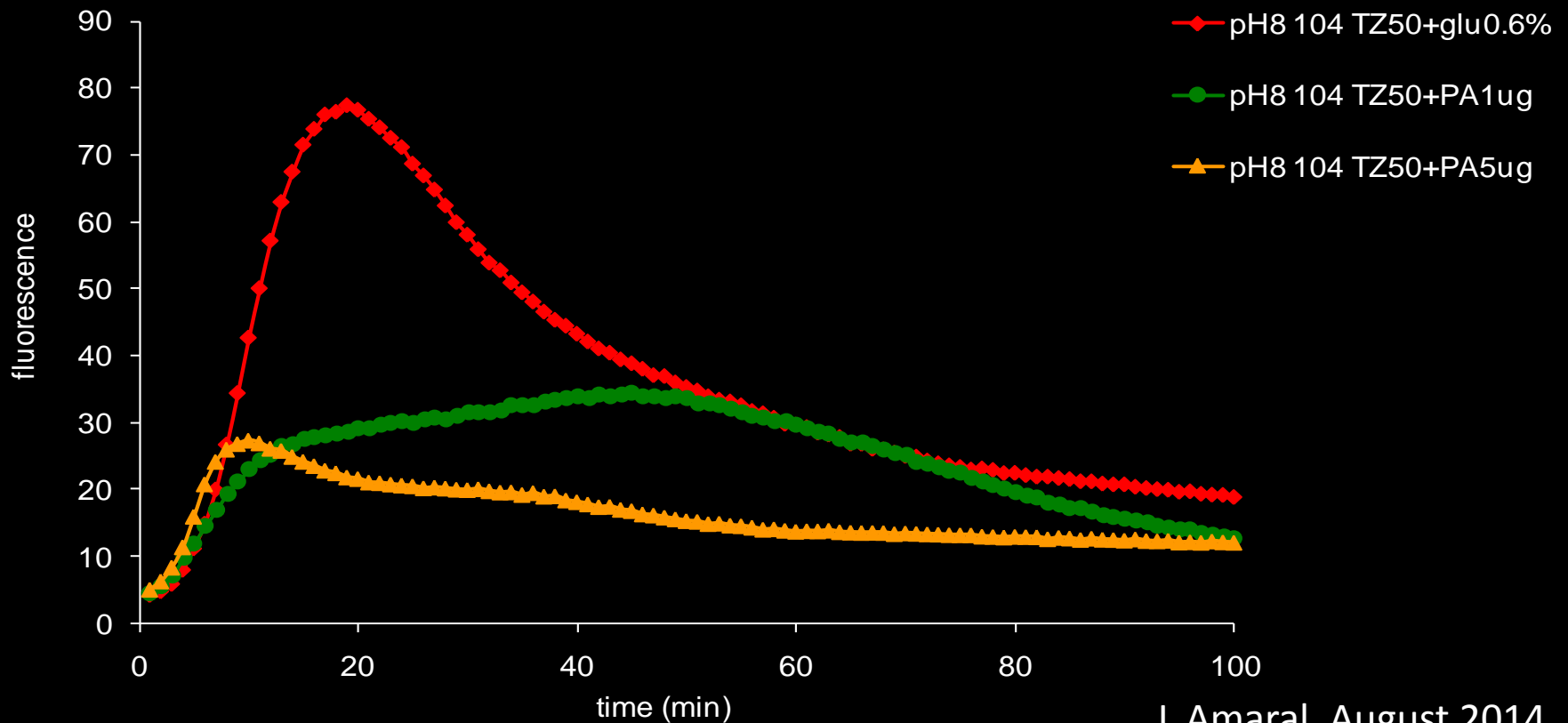


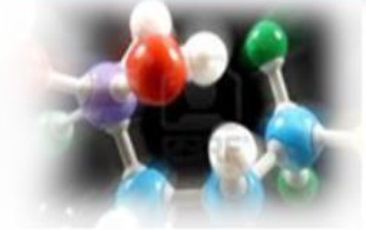
Effect of TZ on accumulation of EB in PBS pH8, metabolic energy and modulation by a fatty acid



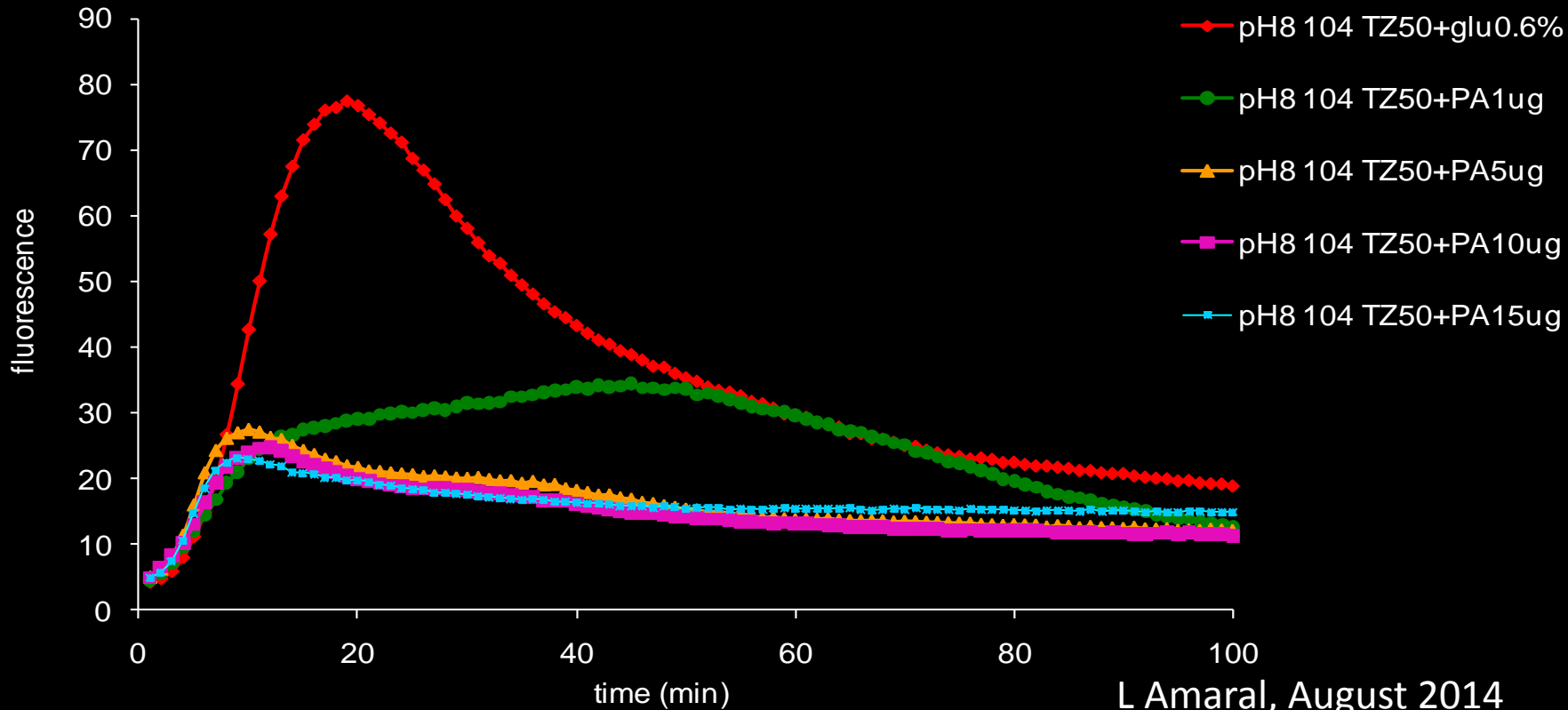


Effect of TZ on accumulation of EB in PBS pH8, metabolic energy and modulation by a fatty acid





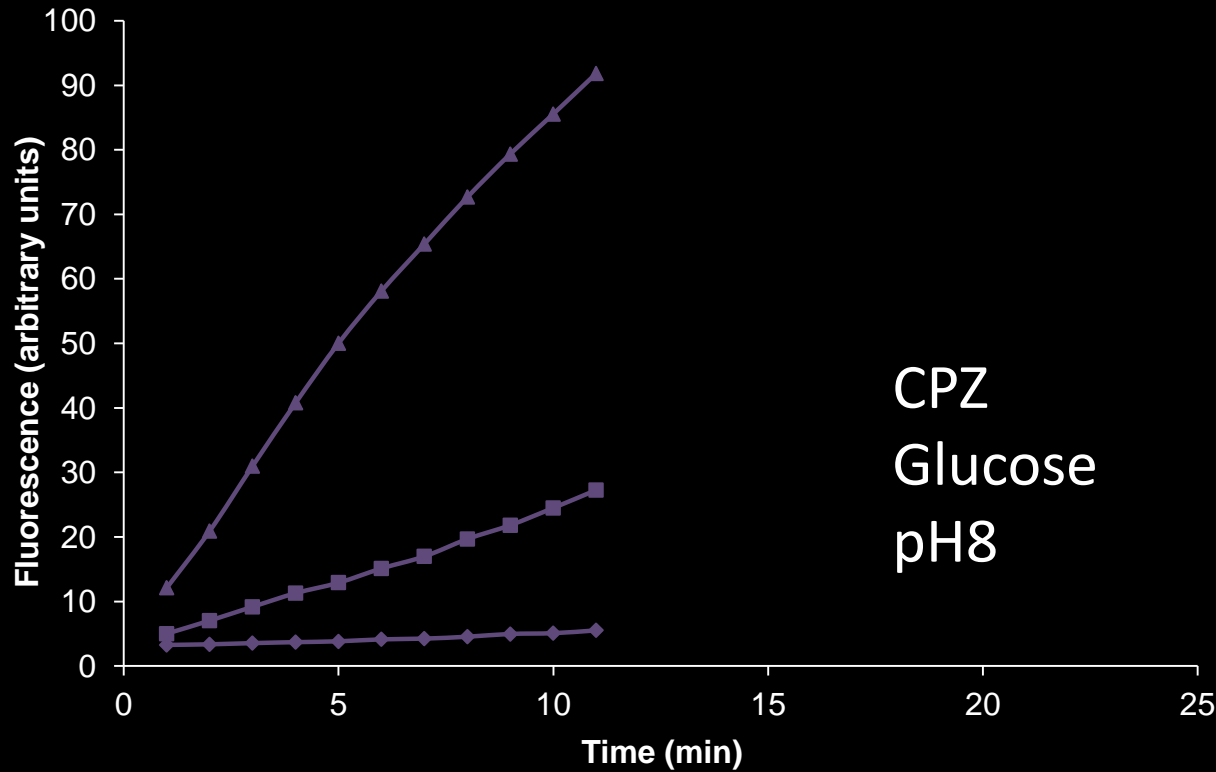
Effect of TZ on accumulation of EB in PBS pH8, metabolic energy and modulation by a fatty acid





Influence of calcium on efflux of *E. coli* at pH 8

Calcium



CPZ
Glucose
pH8

At pH 8, CPZ enhances the retention of EB, especially in the absence of glucose.

The simultaneous presence of CPZ and EDTA synergistically increases accumulation of EB.

Inhibition of Ca²⁺ binding to:

Calcium channels

Calcium dependent enzymes

▲ Accumulation with EDTA ■ Accumulation ◆ Accumulation with Ca

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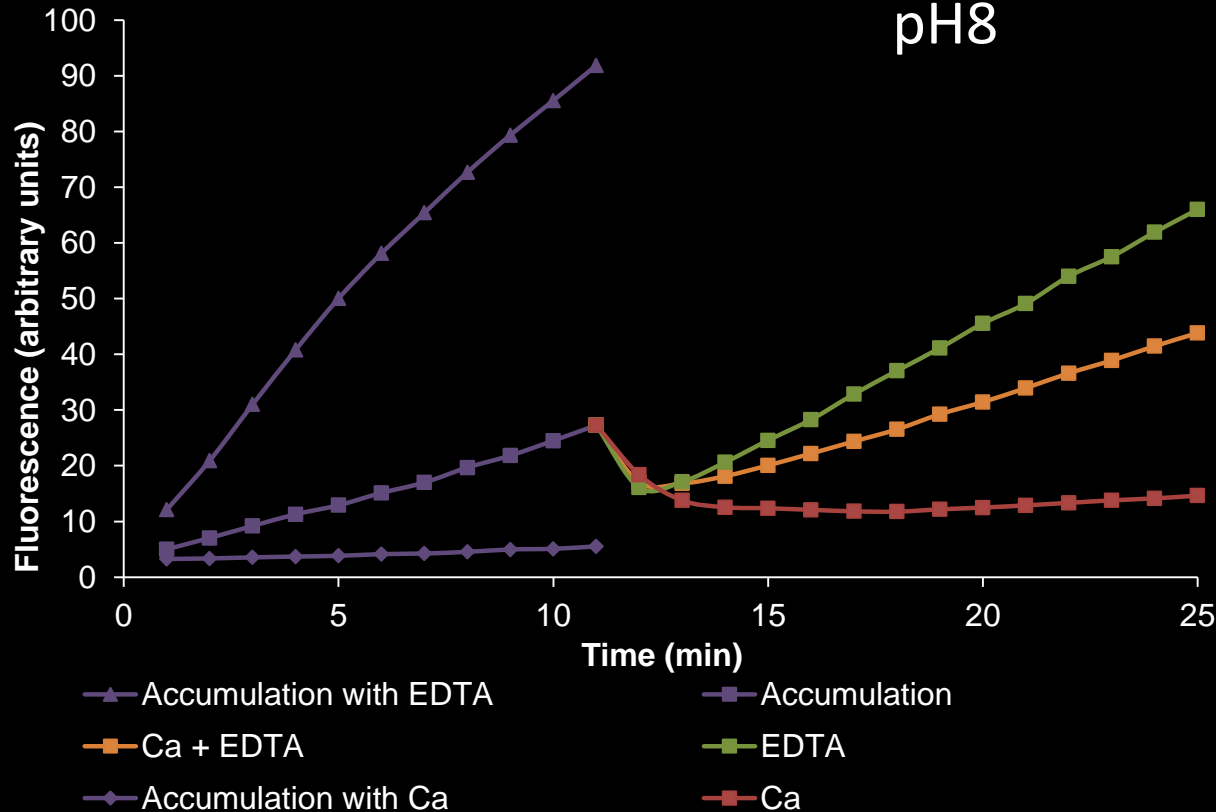


Influence of calcium on efflux of *E. coli* at pH 8

Calcium

CPZ
Glucose
pH8

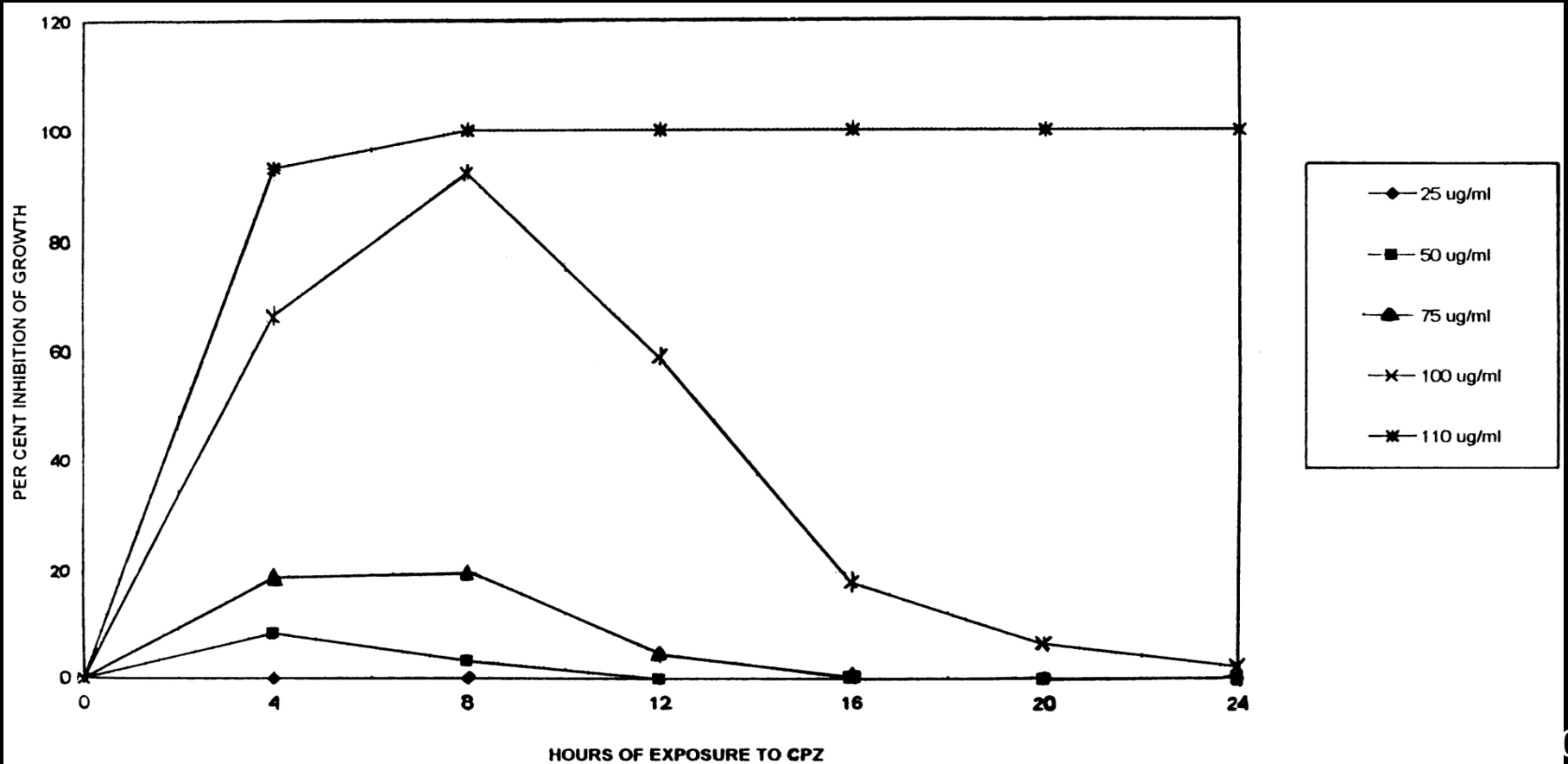
The CPZ promoted retention of EB at pH 8 can be nullified by the addition of calcium to the medium.



The addition of EDTA, which by binding the calcium that is present, promotes the increase of EB retained.



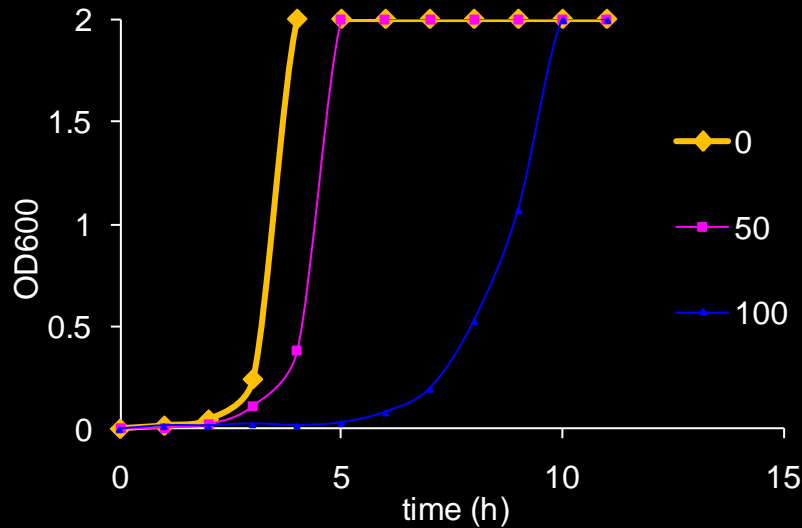
Effect of CPZ on the growth of Salmonella ATCC



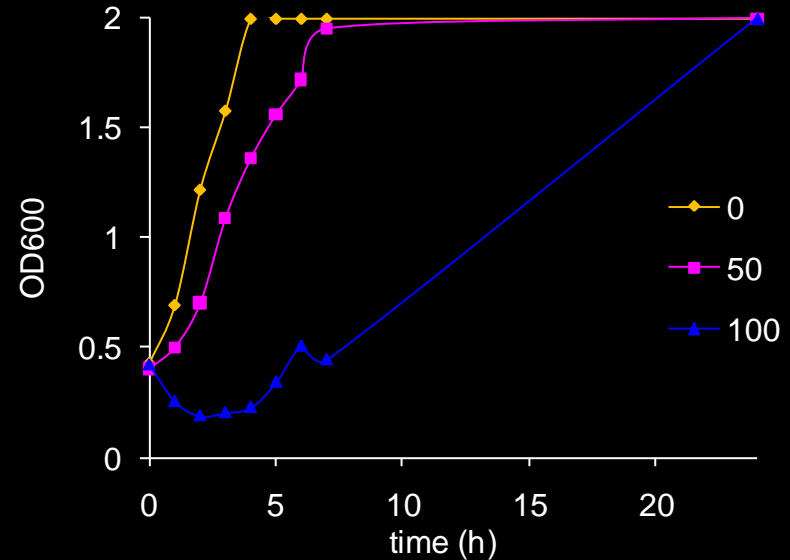


Effect of TZ on the growth of Salmonella 104

MIC inoculum



Fresh culture @ OD 600



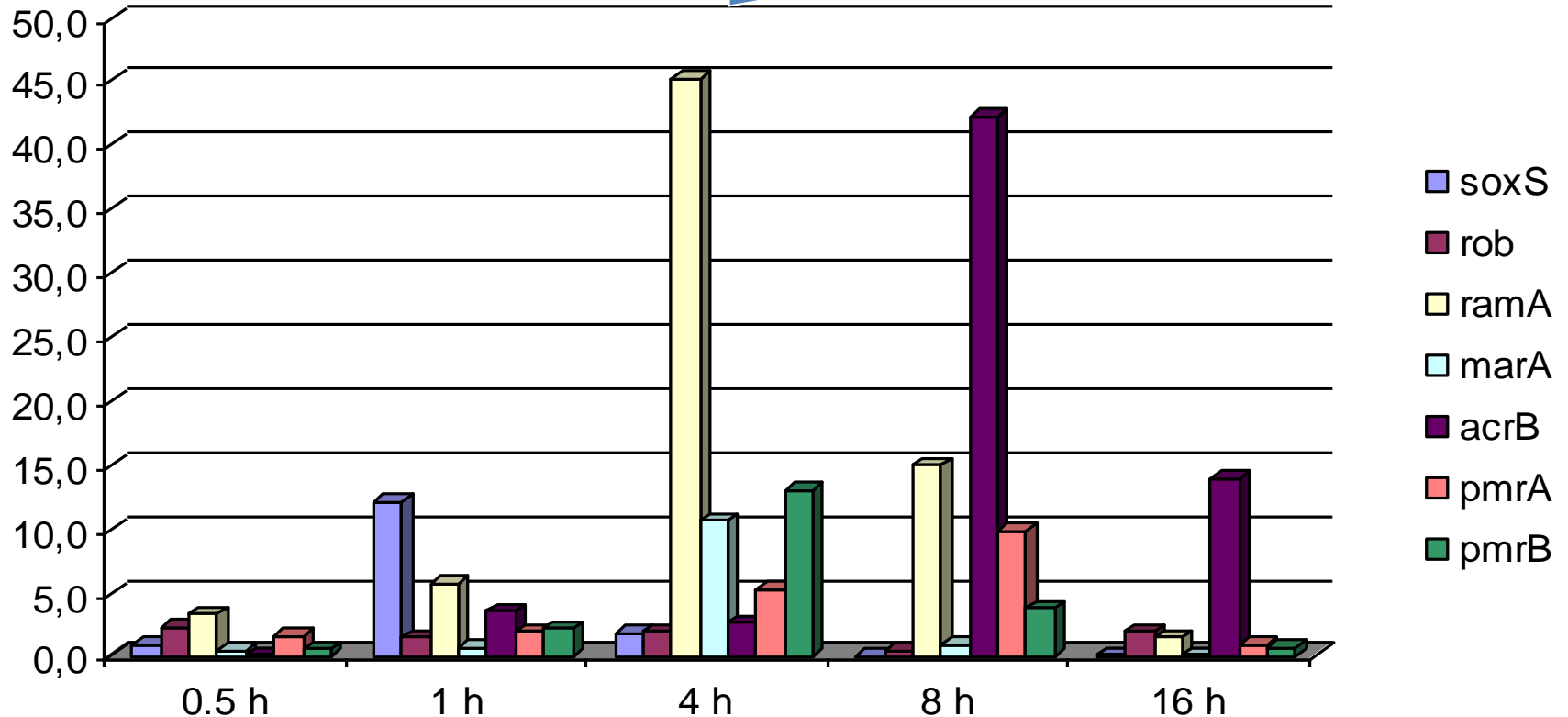
Sal 104, pH 7, TZ 0-100 mg/L



Activities of genes during transient inhibition of growth from exposure to 100mg/L of TZ

INHIBITION OF GROWTH FROM 0.5-8 HRS.

GROWTH FROM 8-16 HRS.

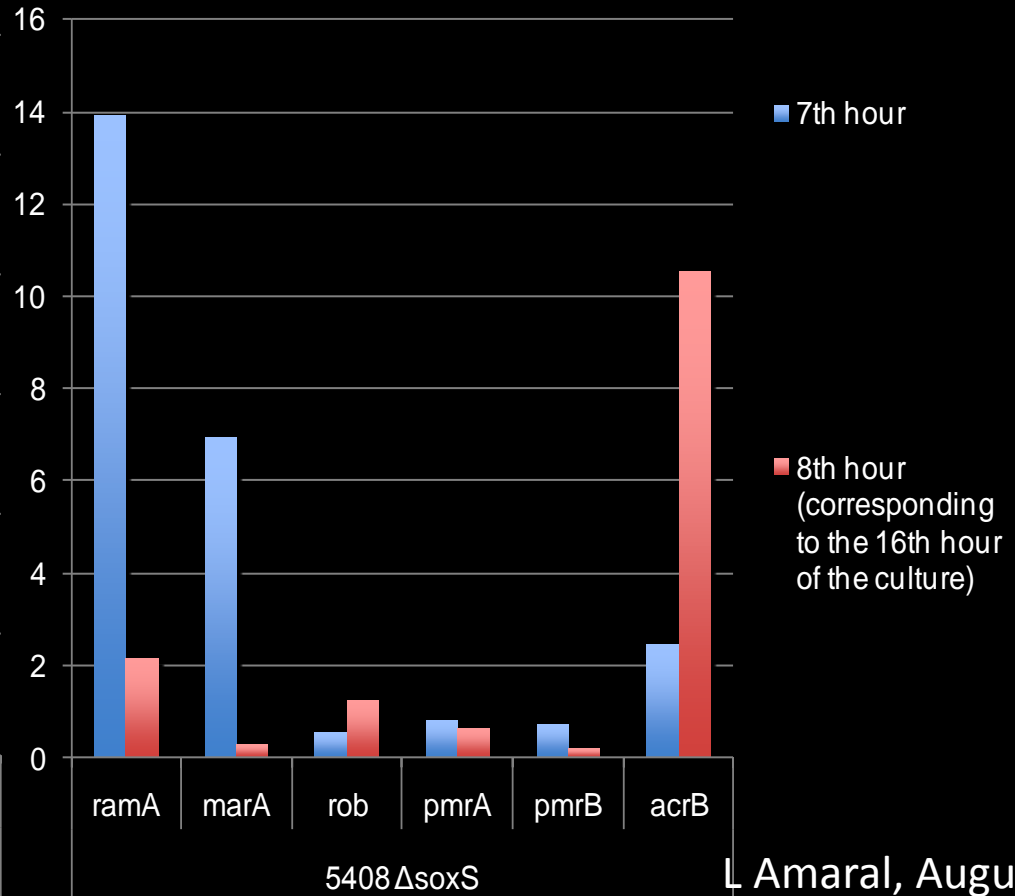
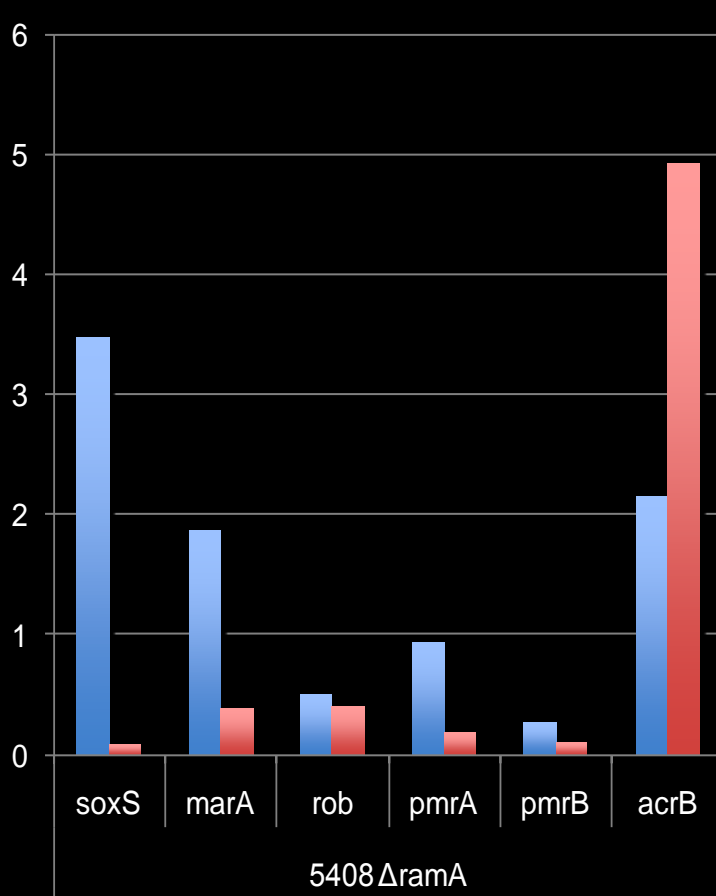




Activities of genes during transient inhibition of growth from exposure to 100mg/L of TZ

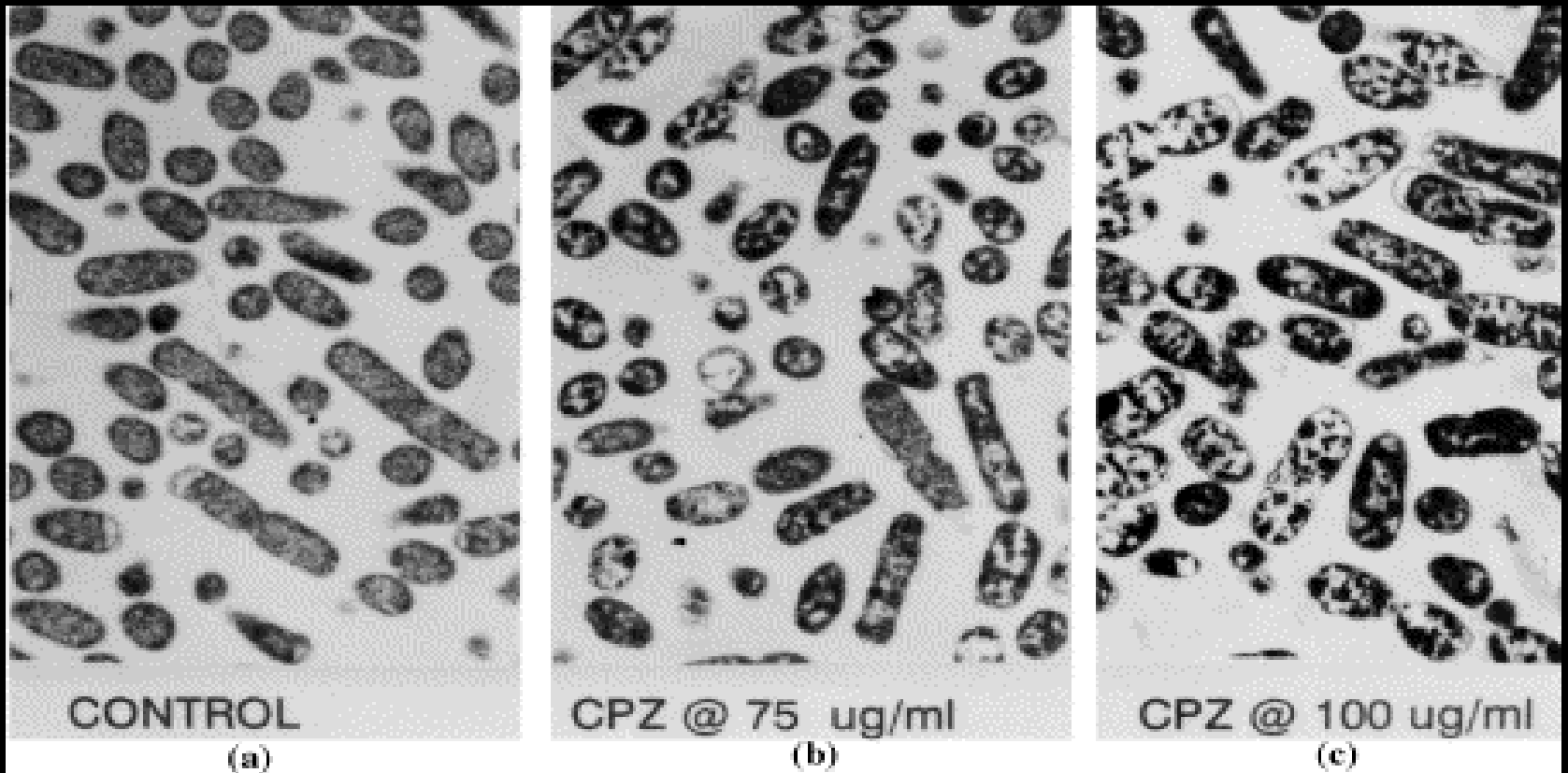
ramA deleted 5408 Salmonella

soxS deleted 5408 Salmonella





The effect of increasing concentrations of CPZ on the ultrastructure of *S. typhimurium*



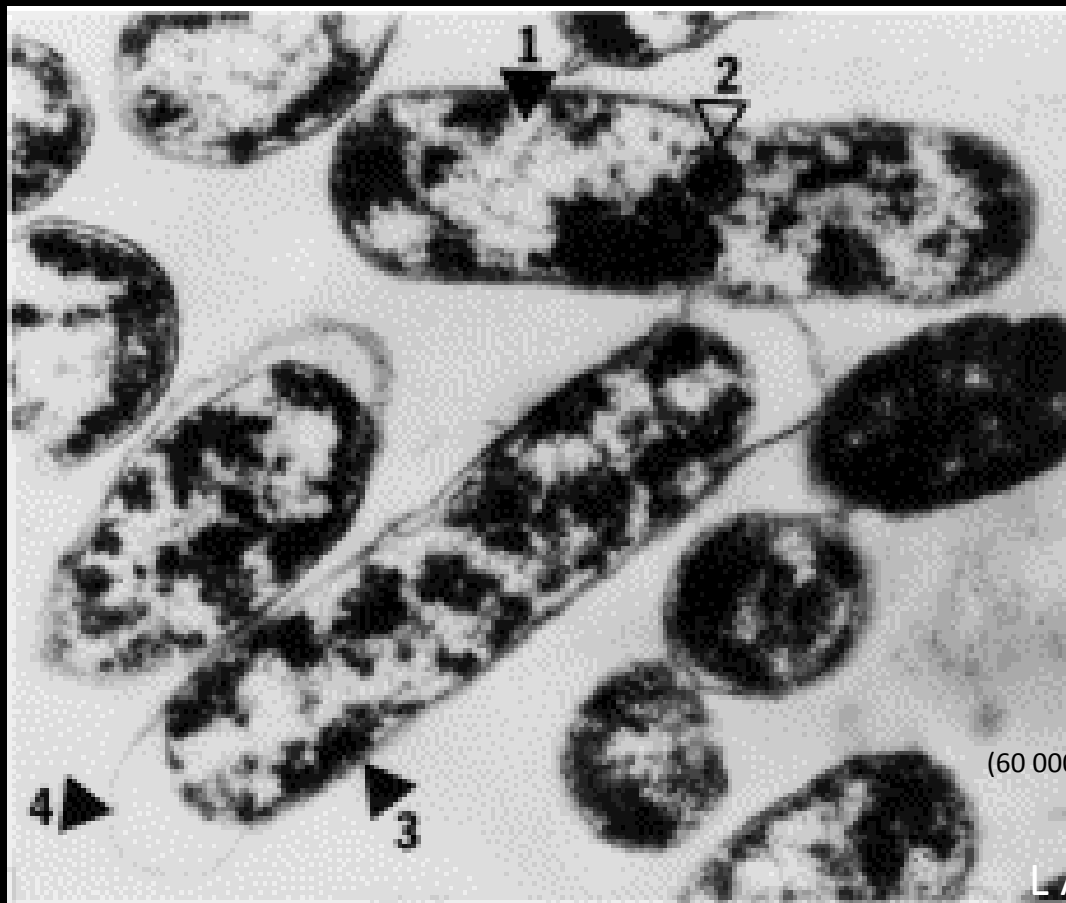
(a) Control

(b) with chlorprozamine at 75 mg/l;

(c) with chlorprozamine at 100 mg/l.



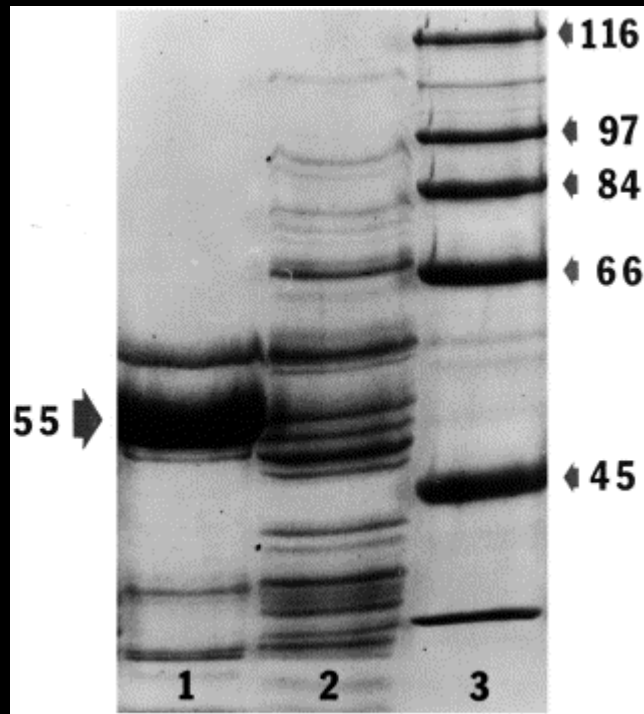
Ultrastructure of *Salmonella typhimurium* exposed to 100 mg/l of CPZ



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Electrophoretic pattern of outer cell wall proteins of control and CPZ exposed *S. typhimurium*



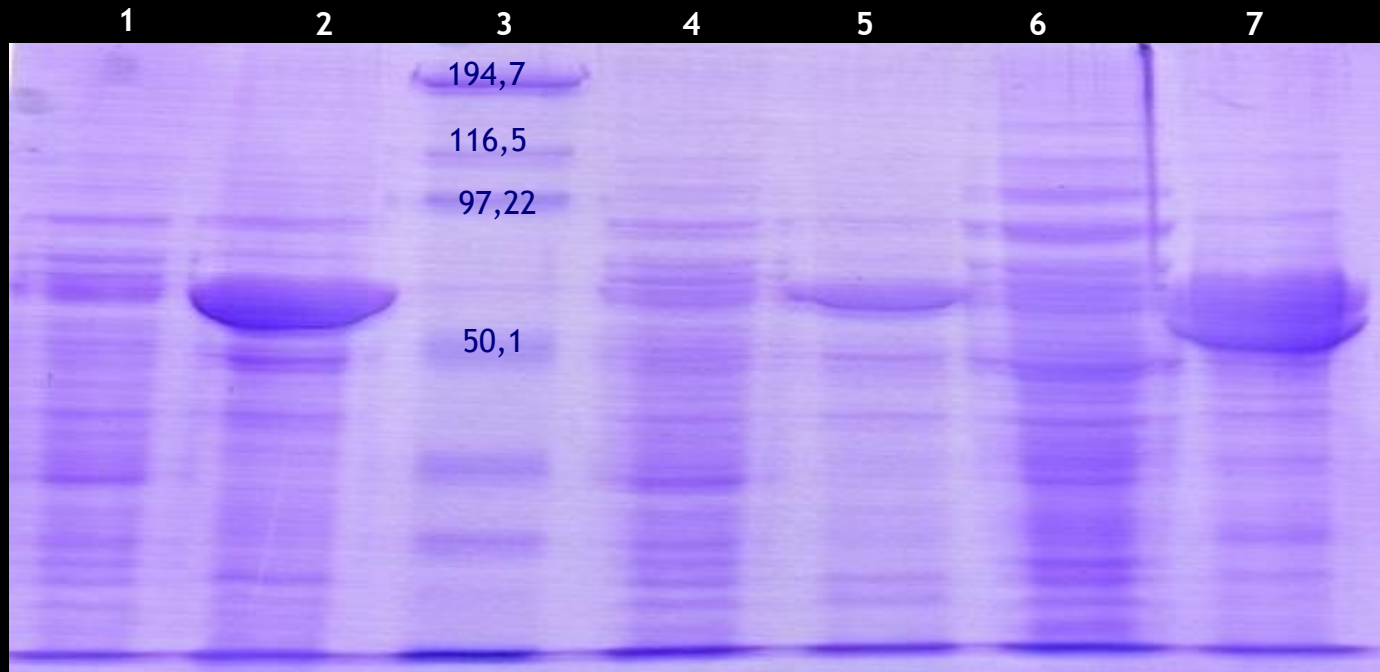
Lane 1 - Control (55 kDa protein);

Lane 2 - outer cell wall of exposed-*Salmonella* (100 mg/l of chlorpromazine) with 55 kDa protein greatly reduced;

Lane 3 - molecular weight markers.



Electrophoretic pattern of outer cell wall proteins of *Salmonella*: Agar vs Broth



Gel SDS-PAGE 8,5 %

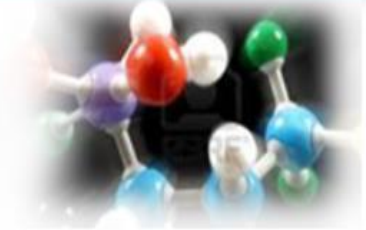
1. Strain 104 - growth in agar;
2. Strain 104 - growth in broth;
3. Marker - Prestained SDS-PAGE standards Broad Range, Bio-Rad;
4. Strain 5048 - growth in agar;
5. Strain 5048 - growth in broth;
6. Strain 1246 - growth in agar;
7. Strain 1246 - growth in broth



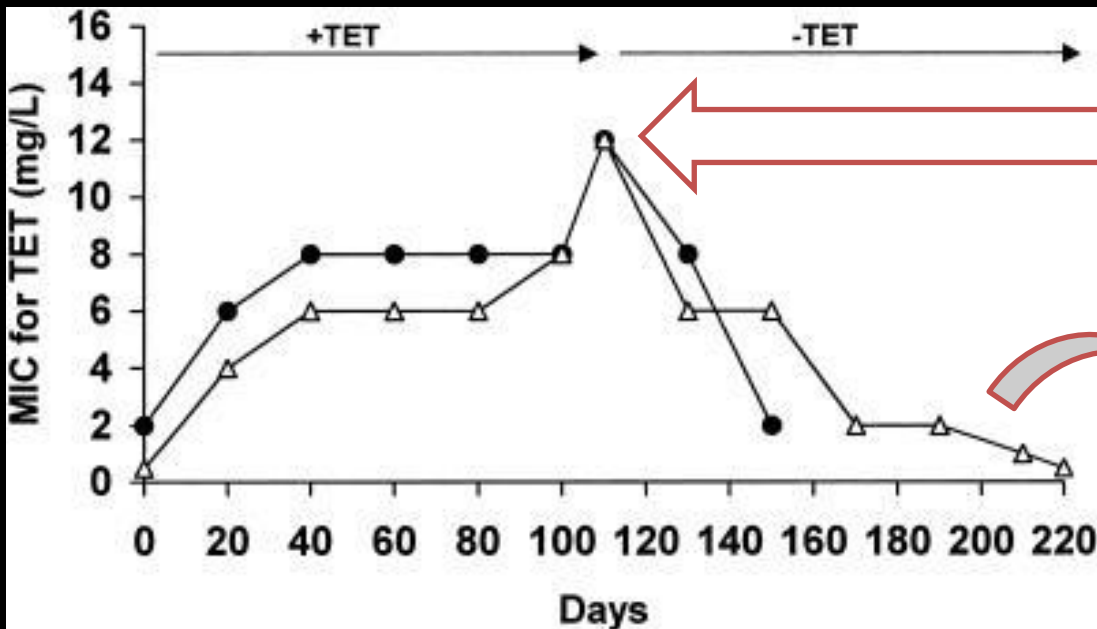
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Laboratory demonstrations of induced efflux activity by bacteria.



Time course of induced tetracycline resistance of *E. coli*

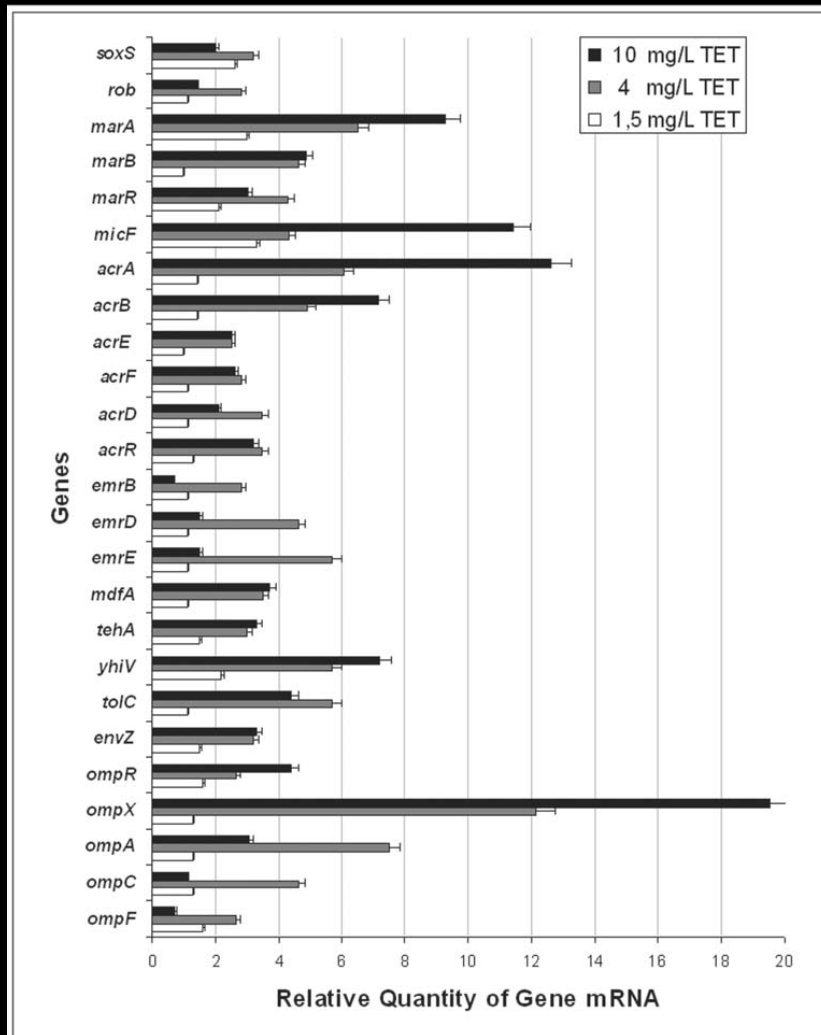


Addition of thioridazine immediately restores susceptibility to tetracycline.

Reversal of induced resistance by transfer to drug-free medium.

- *E. coli* AG100
- △ *E. coli* AG100A

Modulation of genes of *E. Coli* during prolonged exposure to concentrations of tetracyclin.



Data from total mRNA extractions of *E. coli* AG100 physiologically adapted to increasing concentrations of TET compared to its parental non-induced strain grown in the absence of TET.

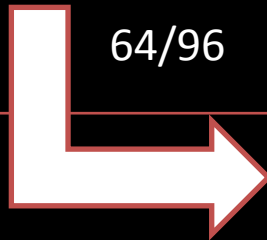
Ratio =1 No alterations in the expression.

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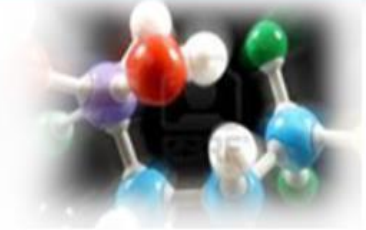


Effect of serial exposure of *Escherichia coli* AG100_{TET8} strain to 10 mg/L tetracycline on the MIC of tetracycline

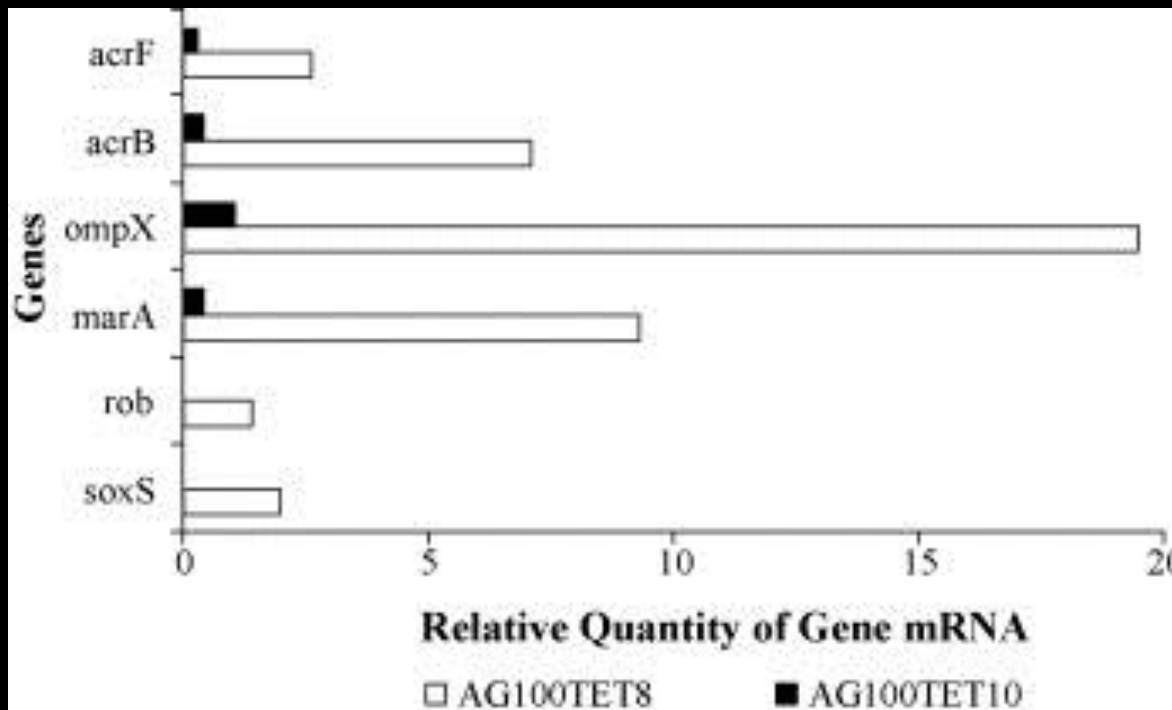
	AG100 _{TET8}		AG100 _{TET10}				
	Passage 1	Passage 10	Passage 20	Passage 30	Passage 40	Passage 50	Passage 60
MIC TET	10	64	64/96	96	96/128	128	256



From this point the strain cannot revert its resistance to tetracycline and to the antibiotics that contributed to its multidrug-resistant status, therefore the strain is named AG100_{TET10}.



Activities of the regulator, stress and transporter genes of Escherichia coli strains AG100_{TET8} and AG100_{TET10}





Test	Increase in TDRU*		Class of antibiotic and target
	TET8	TET10	
Enoxacin	19450	41154	fluoroquinolone, DNA unwinding (gyrase and topoisomerase)
Ofloxacin	40488	44797	
Norfloxacin	18895	21316	
Ciprofloxacin	20519	21240	
Nalidixic acid	21984	21352	quinolone, DNA unwinding (gyrase and topoisomerase)
Oxolinic acid	36125	41336	
Cinoxacin	19749	21048	
Pipemidic Acid	38867	40375	
Kanamycin	19522	21029	aminoglycoside, protein synthesis (30S ribosomal subunit)
Sisomicin	18700	20991	
Tobramycin	19532	21523	
Chlortetracycline	21158	22107	
Demeclocycline	18629	21997	tetracycline, protein synthesis (30S ribosomal subunit)
Penimepicycline	41074	45607	
Rolitetracycline	18013	20270	
Oxytetracycline	38366	40913	
Geneticin (G418)	20910	20558	aminoglycoside, protein synthesis
Doxycycline	20325	21749	
Cefazolin	19558	20840	tetracycline, protein synthesis
Cephalothin	19308	22623	
Cefuroxime	19623	23278	1st generation cephalosporin, cell wall
Cefotaxime	20110	22397	2nd generation cephalosporin, cell wall
Cefoperazone	58973	61403	3rd generation cephalosporin, cell wall
Amoxicillin	60936	60582	
Cloxacillin	19914	23548	β -lactam, cell wall
Nafcillin	40098	44947	
Oxacillin	19322	21372	
Carbenicillin	37677	41972	
Aztreonam	41948	45618	
	20157	20760	

[\[1\]](#) TDRU, tetrazolium dye reduction units, the increase in the area under the kinetic plot in comparison to the wild-type parent strain AG100 is given; an increase of $\geq 20,000$ TDRU is considered significant. The highlighted TDRUs are highly significant and suggest high resistance to the corresponding agents.



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Conclusions

- ✓ @ pH 5 metabolic energy not needed for efflux in PBS but required in deionised water of pH 5.5.
- ✓ @ pH 8 efflux metabolic energy is required regardless of sodium.
- ✓ Inhibition of efflux by a phenothiazine is mediated initially by inhibition of metabolic energy which with time is replaced by alternative sources (lipids).
- ✓ Prolonged exposure to the phenothiazine activates regulatory, transporter and two-step regulon genes.
- ✓ Increased activity of the transporter *acrB* gene to the phenothiazine takes place in absence of the global regulator *ramA* or stress *soxS* genes.

Because the two-step regulons PmrA/B and PhoP/PhoQ are not affected, there must be another manner by which *acrB* is regulated.



TROPICAL

EFFLUX PUMP SYSTEM OF SALMONELLA energetics; modulation; genes



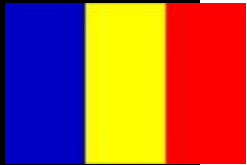
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M. McCusker



VW Kern



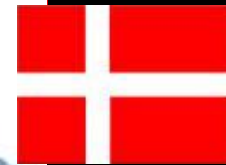
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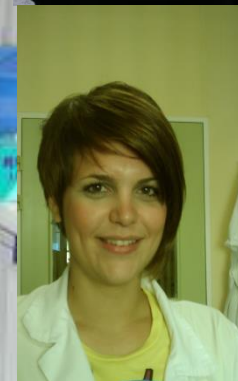




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