香港城市大學
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Isolation and characterization of novel antimicrobial peptides from Japanese medaka plasma

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Fishery is the fastest growing food-producing sector and nearly 50 % of the world's fish is used for food

Food and Agriculture Organization of the United Nations

The Asian aquaculture sector nowadays accounts for nearly 90% of the global aquaculture production

http://www.fao.org/aquaculture/en/

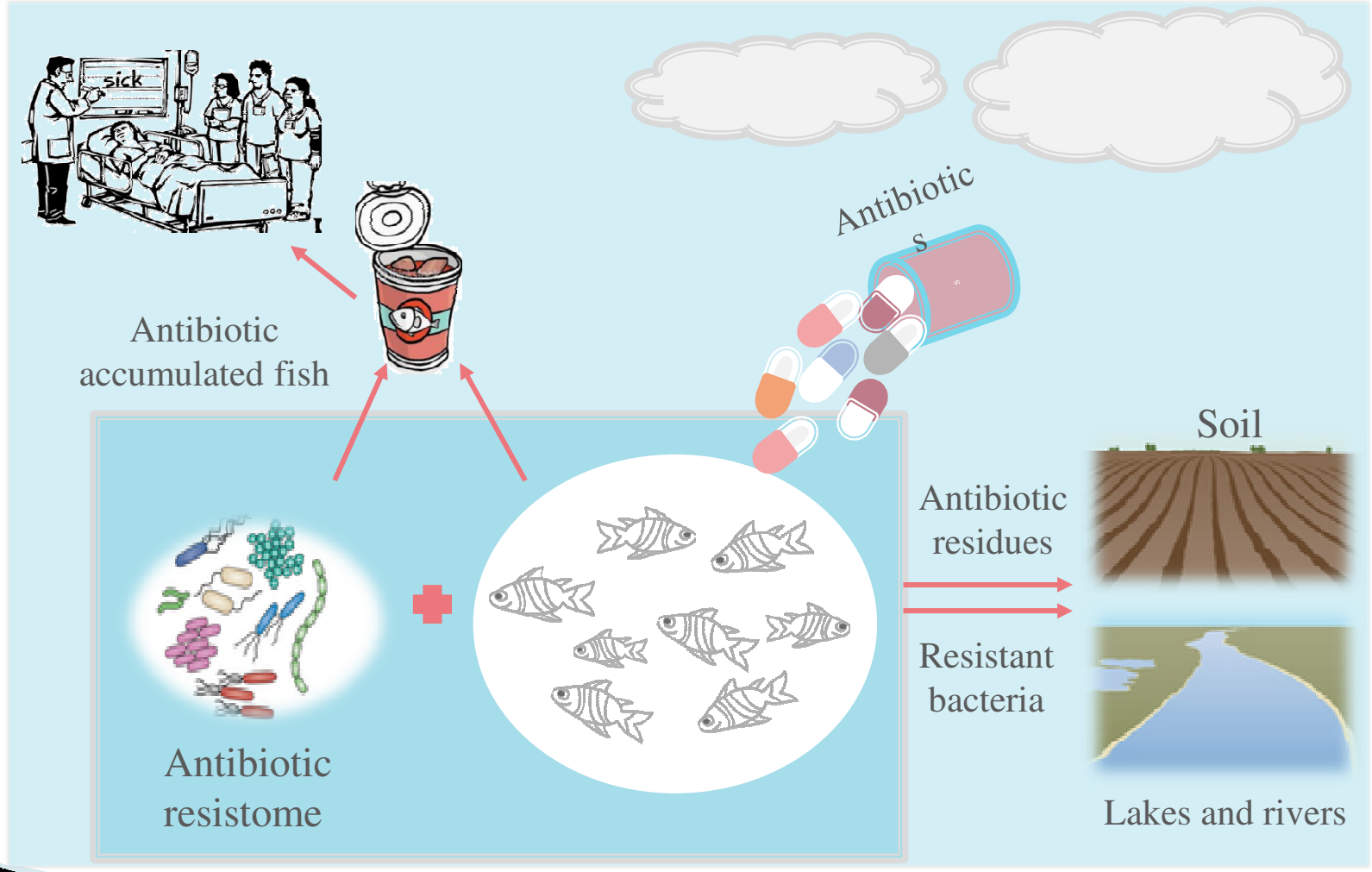
Antibiotics are used to improve the growth and quality of aquatic products

Antibiotics authorized for use in aquaculture

Antibiotic	Treatment of	Reference
Oxytetracycline [1]	Furunculosis in salmonids (salmon or trout) caused by *Aeromonas salmonicida*	USP, 2000g
(for medicated feed)	Gafkemia in lobsters (caused by *Aerococcus viridans).*	
	Hemorrhagic septicaemia due to *Aeromonas hydrophila, A. sobria* and *Pseudomonas.*	
	Cold water disease in salmonids, caused by *Cytophaga psychrophilia.*	
	Columnaris disease in salmonids, caused by susceptible *Chondrococcus (Flexibacter) columnaris.*	
	Enteric redmouth disease, caused by susceptible *Yersinia ruckeri.*	
	Indicated for the control of *Pseudomonas* disease in catfish and salmonids.	
	Indicated for the control of ulcer disease caused by susceptible *Haemophilus piscium* in salmonids (salmon, trout).	
Florfenicol [2] Premix	Indicated in the treatment of furunculosis caused by susceptible strains of *Aeromonas salmonicida.*	USP, 2000f
Sarafloxacin	Indicated in the treatment of furunculosis, vibriosis and enteric redmouth in Salmonidae.	EMEA, 1997
Erythromycin	In the treatment of bacterial kidney disease (*Renibacterium salmoninarum*) and streptococcosis in yellowtail in Japan.	GESAMP, 1997
Sulphonamides potentiated with trimethoprim or ormethoprim	Against furunculosis, enteric redmouth disease and vibriosis.	GESAMP, 1997

Responsible use of antibiotics in aquaculture. 2005 by FAO

The applications of antibiotics to fish is harmful to environment and human health



Alternative antibacterial agents-antimicrobial peptides （AMPs）

Properties	
Net charge	+
Residues length	4–100 aa
Hydrophobic ratio	30%–70%
Secondary structures	β –sheet, α –helical, loop, and extended peptides
Amphipathicity	Contain hydrophobic and hydrophilic regions
Isoelectric point (pI)	~10

AMPs facts summarized from on line AMP database

Time of AMP discovery (1970-2012)

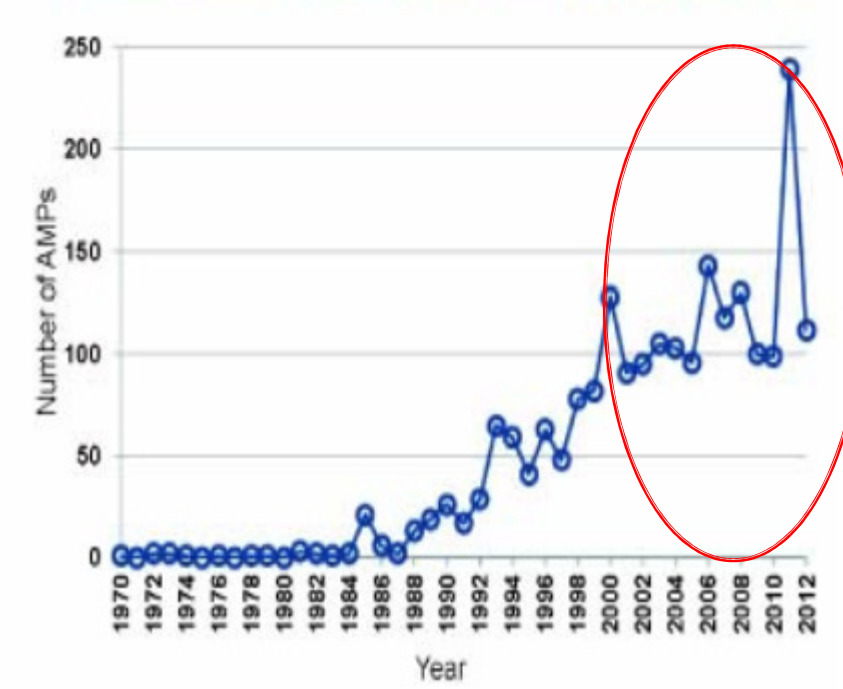


Table 1. Top 10 sources of the natural AMPs in LAMP.

Rank	Producer organisms	Numbers of AMPs
1	*Mus musculus*	135
2	*Homo sapiens*	119
3	*Bos taurus*	86
4	*Rattus norvegicus*	62
5	*Apis cerana*	48
6	*Viola odorata*	43
7	*Macaca mulatta*	40
8	*Bombina maxima*	40
9	*Gallus gallus*	38
10	*Odorrana grahami*	36

http://aps.unmc.edu/AP/facts.php

Zhao X, Wu H, Lu H, Li G, Huang Q (2013) LAMP: A Database Linking Antimicrobial Peptides. PLoS ONE 8(6)

Fish specific AMPs are relatively less

LAMP: A database linking antimicrobial peptide.

Antimicrobial peptides (AMPs) are genetically ancient members of innate defense systems. AMPs are attractive moleculars for clinical development: they can be sythesizes easily, kill drug-resistant bacteria and have a rapid antimicrobial mechanism.

LAMP a database linking AMPs, is an integrated open-access database. It has been created with an objective to provide a useful resource and tools for AMP studies. AMPs in LAMP are short, of less than 100 amino acid residues and include natural, synthetic and predicted AMPs. AMPs in LAMP had been grouped into three catalogs by data source: experimental, predicted, and patent. This manually curated database currently holds **5547** AMP sequences, including **3904** natural and **1643** synthetic AMPs. To ensure data quality, we limited the source of information to authoritative public databases and published scientific literature, as well as patents. LAMP also supplies a user-friendly web interface, so that users can easily query and retrieve information on AMPs. A concise navigational interface that contains the database browse, search, tools, statistical information,

133 fish AMPs

- 119 homology with other species
- 14 fish specific

Ali Adem Bahar. et al., Pharmaceuticals 2013, 6, 1543-1575
J. a Masso-Silva and G. Diamond, "Antimicrobial peptides from fish.," *Pharmaceuticals (Basel).*2014

Existing problems on the practical use of AMPs

- Overlook of fish blood (a great source of AMPs)

- Shortage of high throughput technique by which large amounts of AMPs can be collected

A. Falco. et al., Fish & Shellfish Immunology (2008) 24, 102-112

Objectives

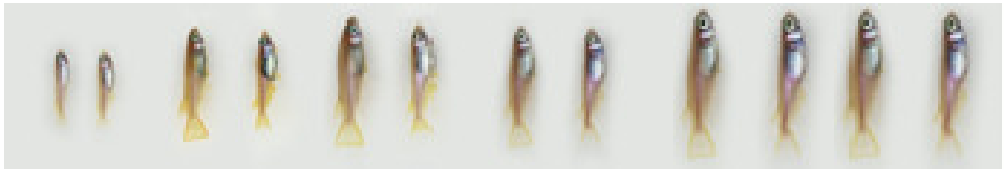
- Collecting and identifying of peptides isolated from fish plasma by using proteomic approach

- Prediction and validation of potential AMPs

Fish source-Japanese medaka *(O. Latipes)*

- Natural properties: small size, short generation times, high fecundity
- Well known fish model in fresh water

Plasma protein “supermix” isolation

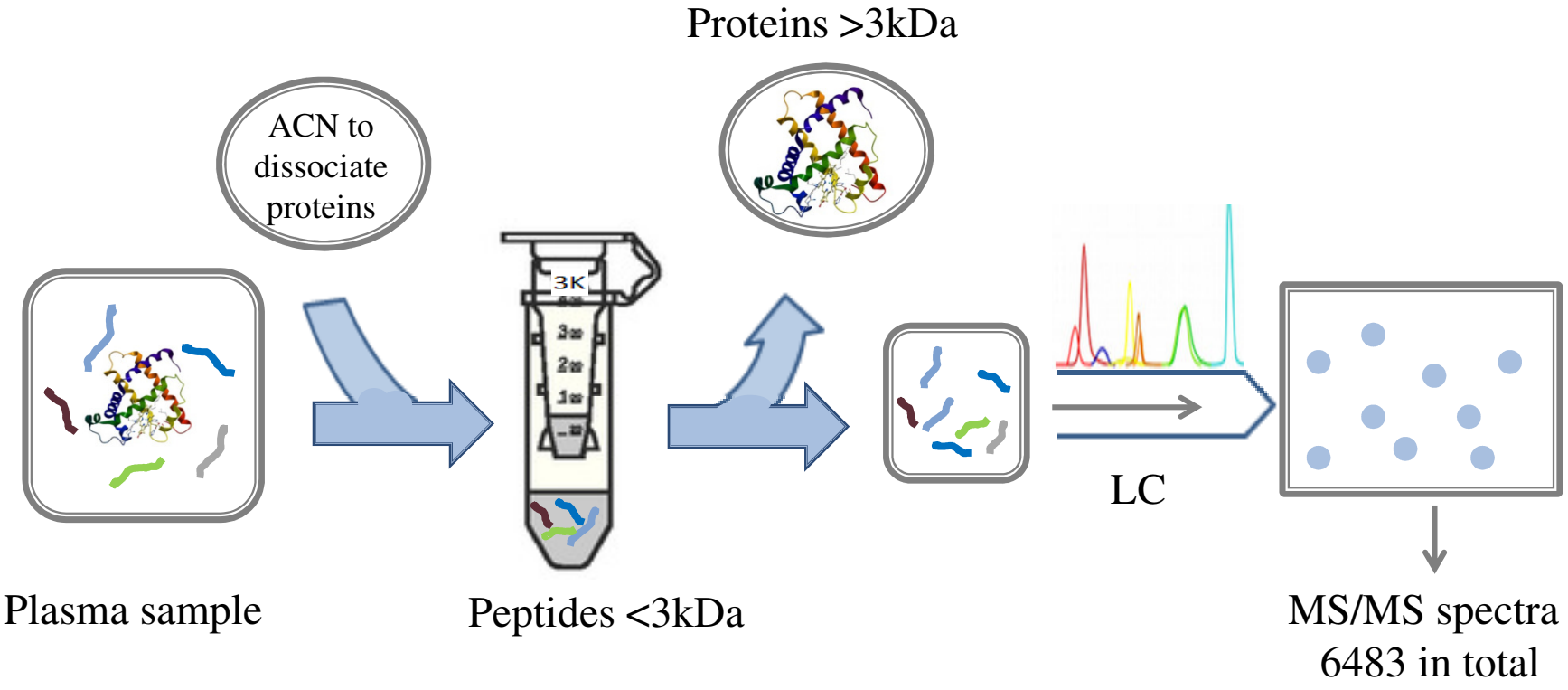


	healthy		3 day post infection	
fish age	female	male	female	male
4 months	2	2	2	2
8 months	1	1	1	1
11 months	1	1	1	1
14 month	1	1	1	1
15 months	1	1	1	1
19 months	1	1	1	1
22 months	1	1	1	1

32 in total

Done by Dr. Joseph L. Humble

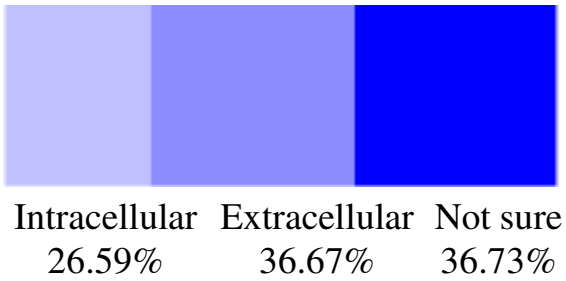
Peptide collection by using ultracentrifugation combining with proteomic approach



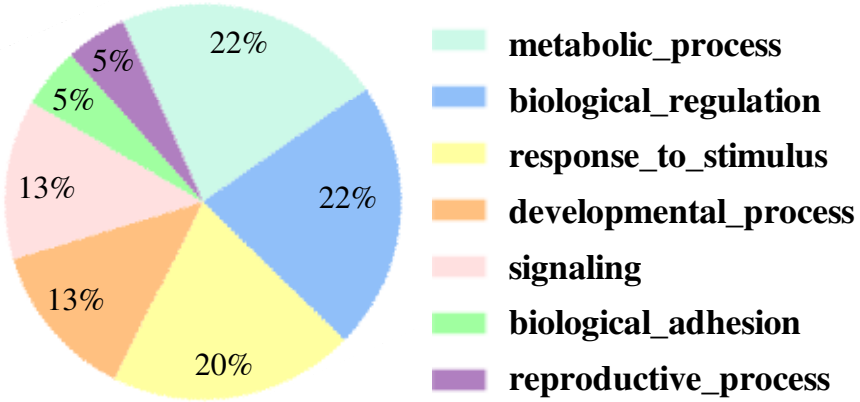
Done by Dr. Joseph L. Humble and Yimin Liang

Summary of collected peptides and their parent proteins

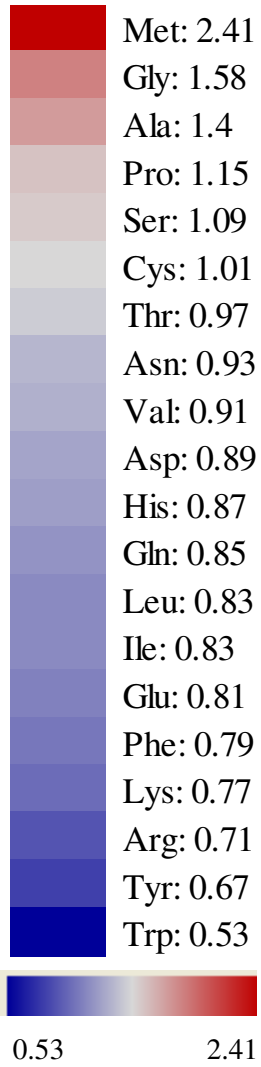
A. Cellular component



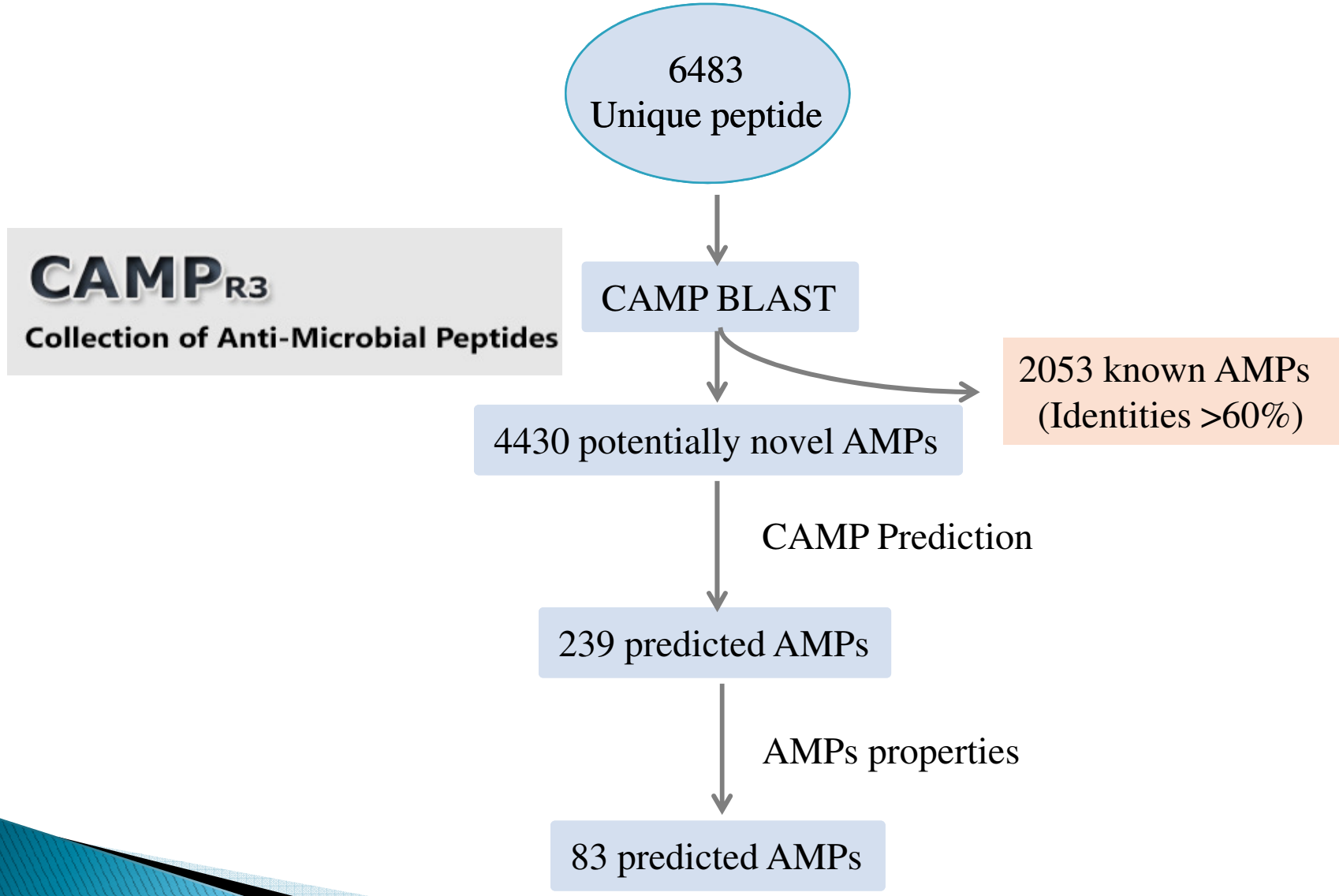
B. Biological process



C. Amino acid usage

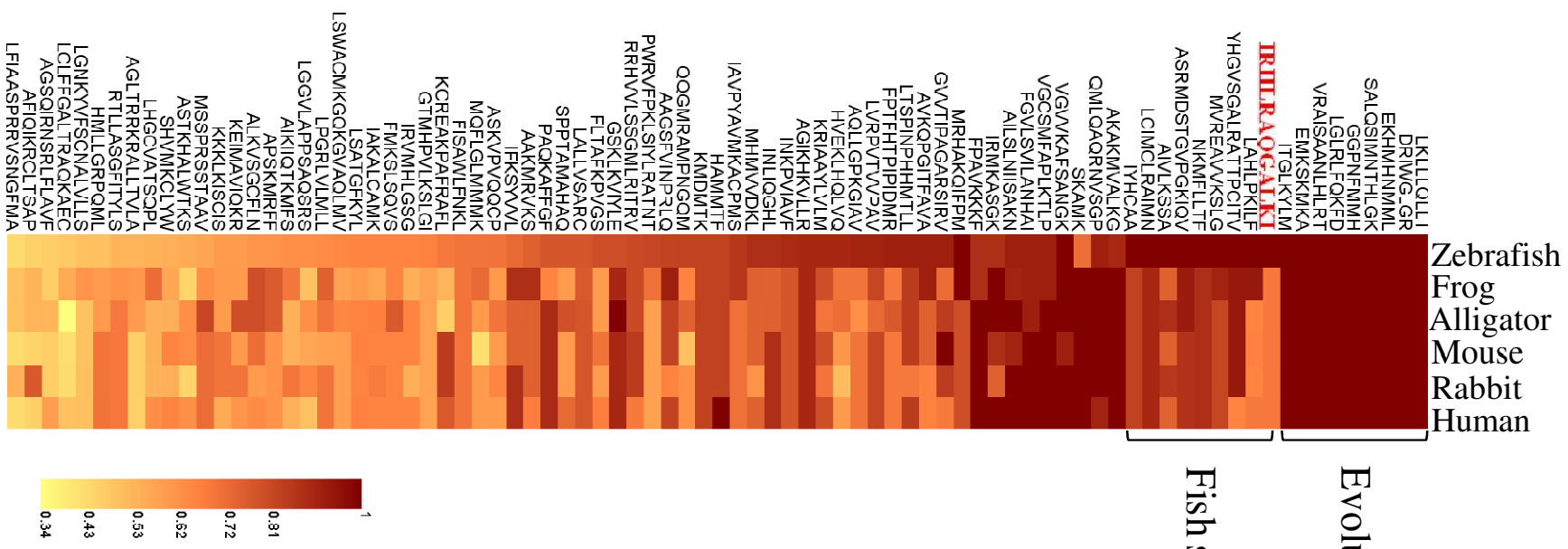


AMPs prediction



http://www.camp.bicnirrh.res.in/

Homology analysis



- Nine of 83 are evolutionary conserved
- Nine of 83 are fish specific

One (out of 83) predicted AMP- BING

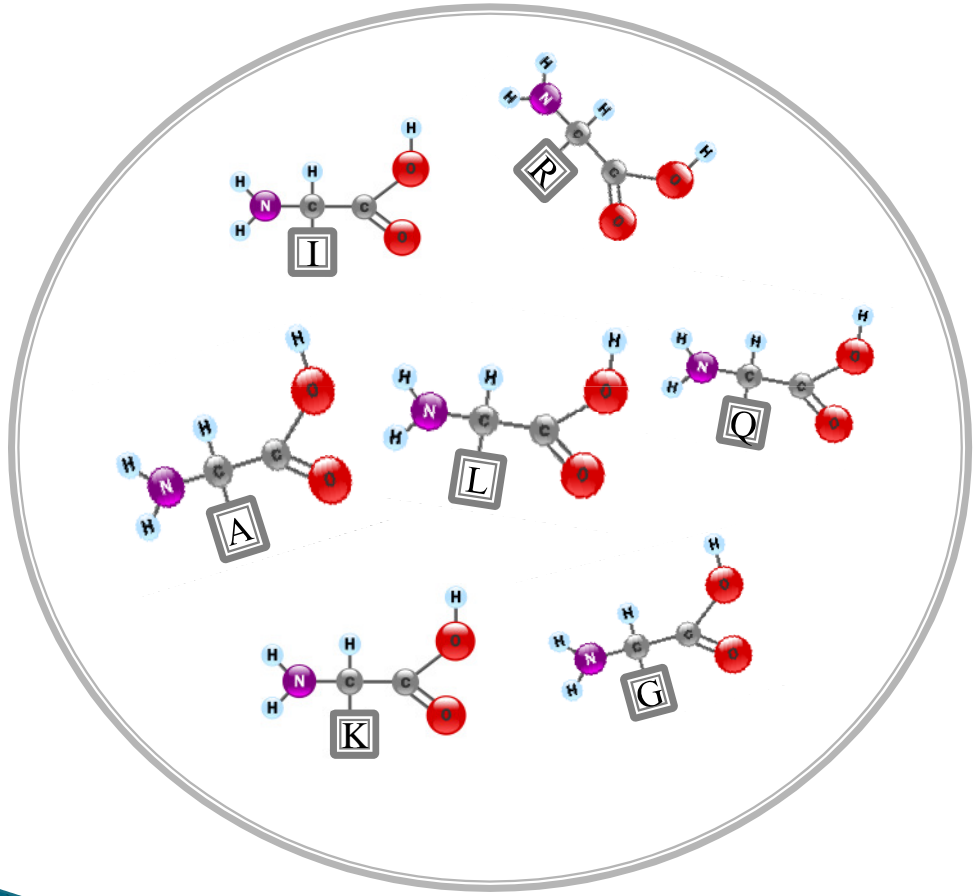
BING: Blocker of Intermembrane stress responses in Gram negative bacteria

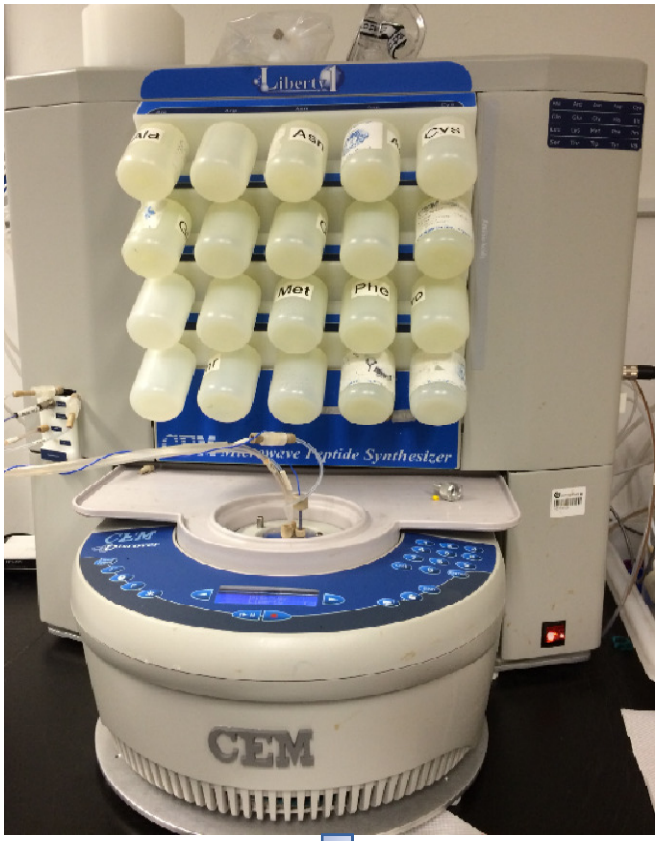
Sequence	Accession	Origin	MW	CAMP prediction						ADP2 prediction
				SVM		RF		DA		
IRIILRAQGALKI	gil432960258	vacuolar protein sorting-associated protein 13D-like	1464.85	AMP	88.90%	AMP	96.75%	AMP	93.70%	AMP

	AMPs Properties	**Selected AMP property**
Net charge	+	+3
Residues length	4-100 aa	13 aa
Hydrophobic ratio	30%-70%	61 %
Secondary structures	β -sheet, α -helical, loop, and extended peptides	β -sheet
Amphipathicity	Contain hydrophobic and hydrophilic regions	Yes
Isoelectric point (pI)	~10	12.52

Peptide synthesis

Fmoc-based solid-phase peptide synthesis

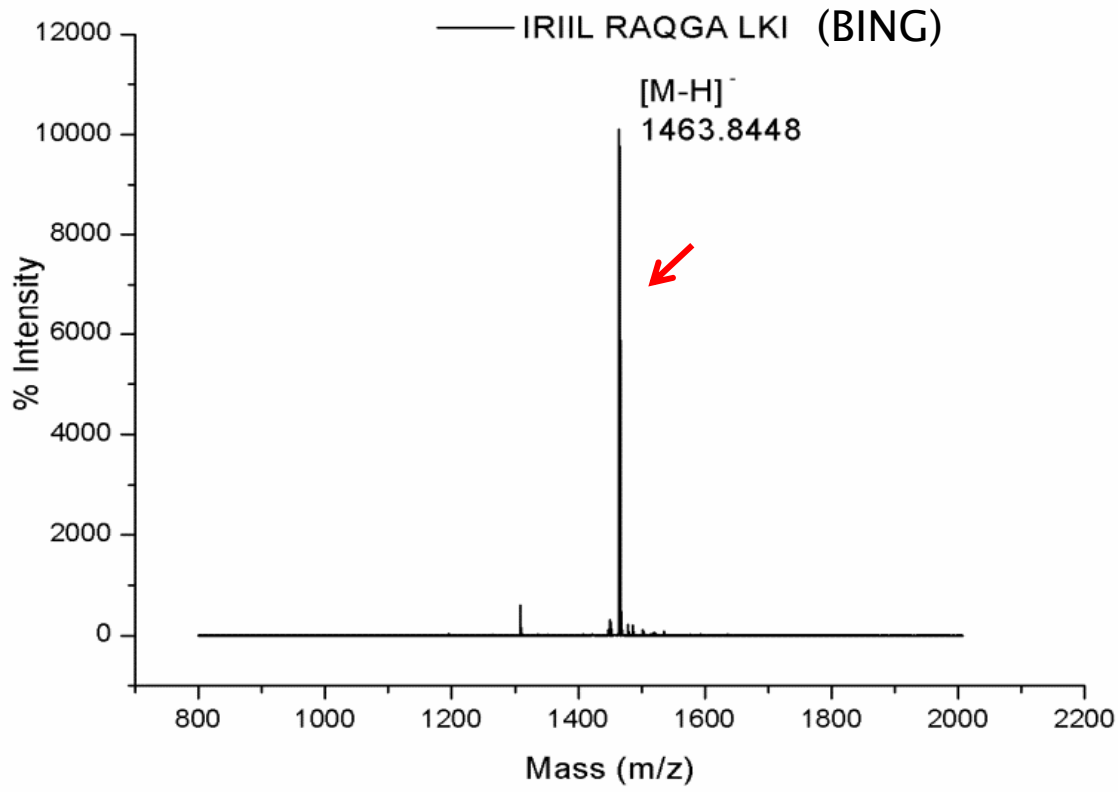




IRIILRAQGALKI

Done by Haipeng Lei

MALDI-TOF-MS analysis



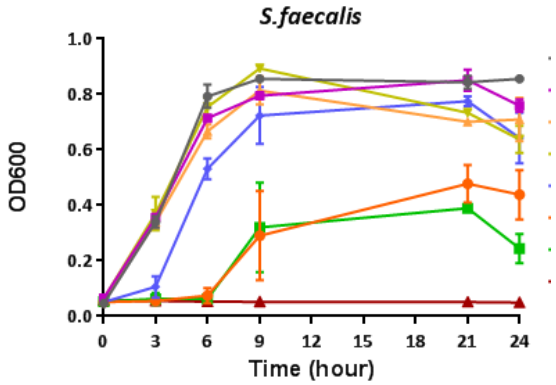
Done by Haipeng Lei

Antibacterial activity detection of Vpsin

Series concentrations of peptide

Bacteria (10^6 cfu/ml)

OD600

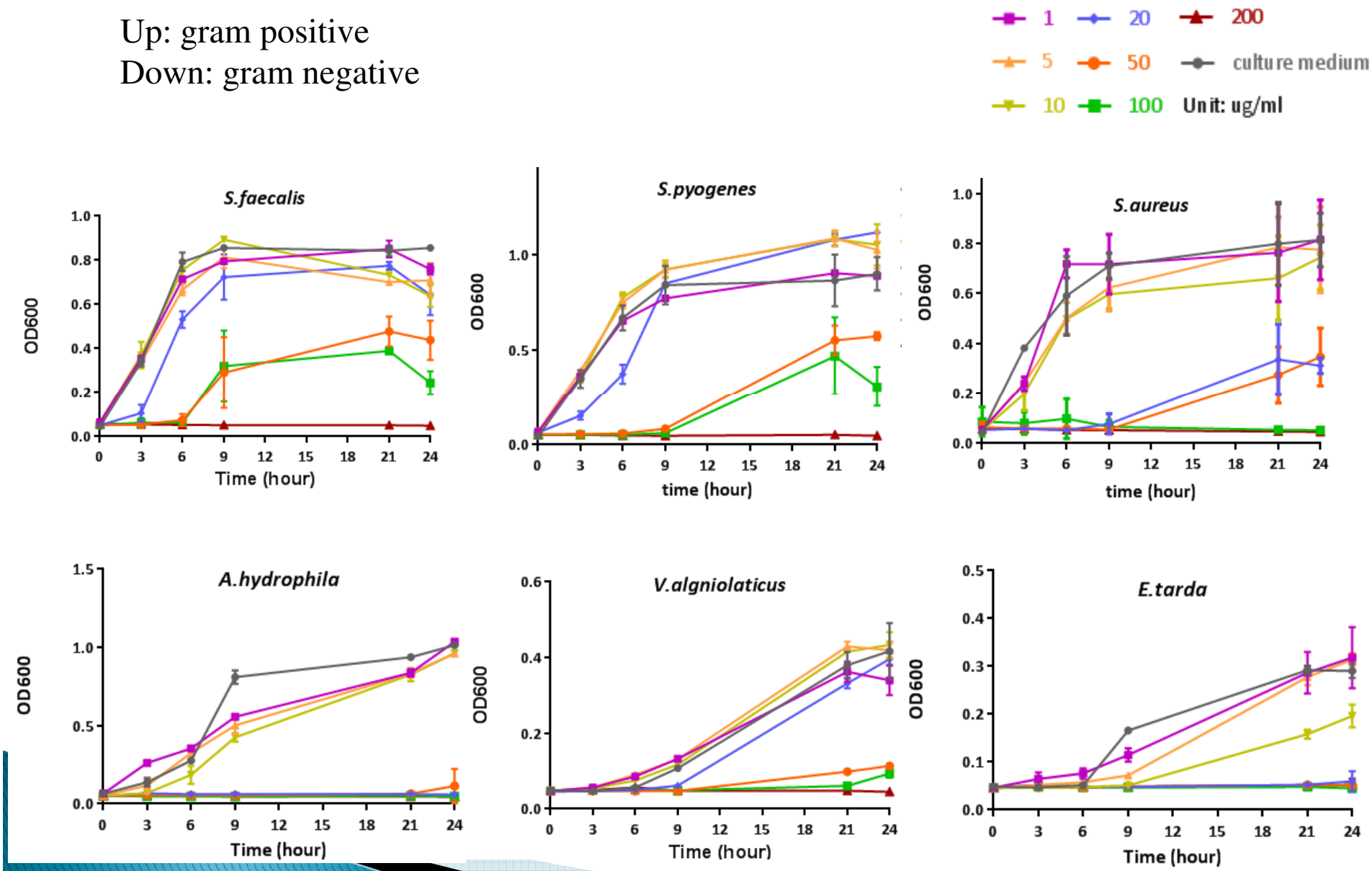


Bacteria involved in the antibaricidal assay

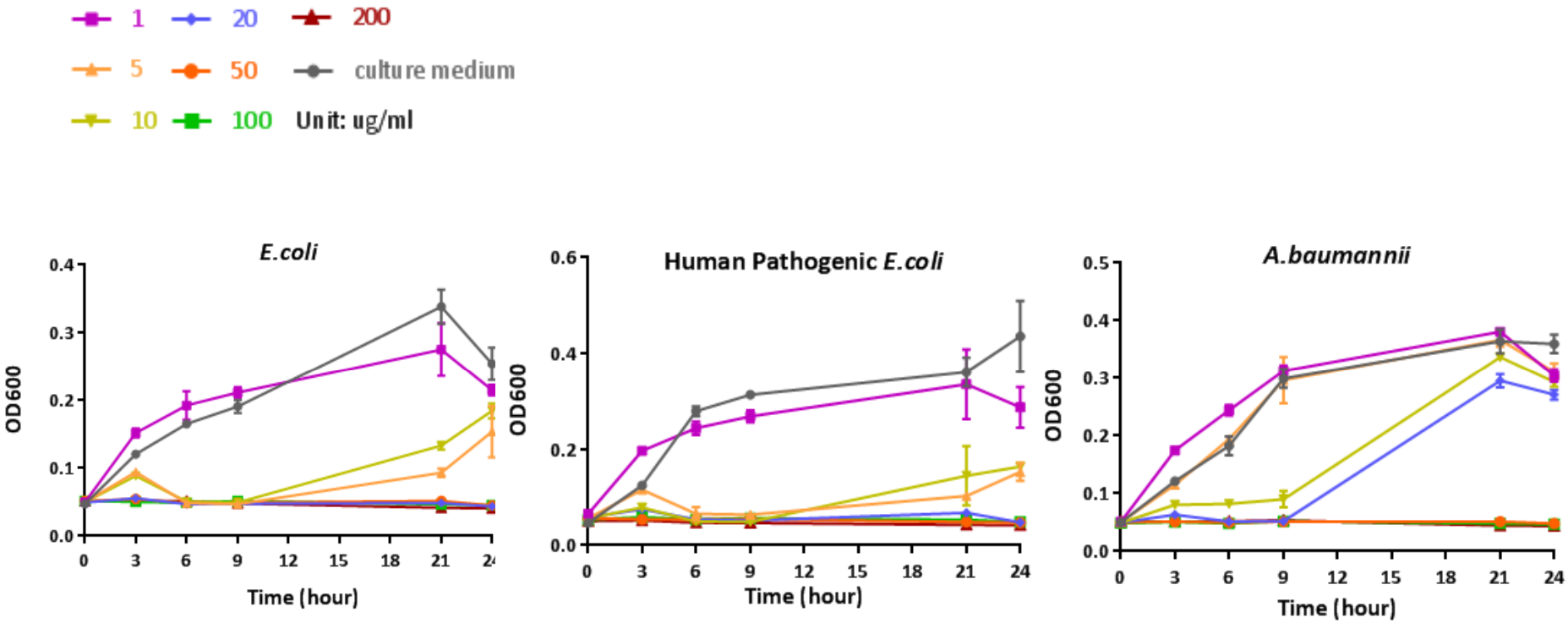
Bacteria	Ref. No.	Source	Gram(+/-)	
Streptococcus faecalis	N/A	HKP	+	fish
Streptococcus pyogenes	14289	ATCC	+	
Staphylococcus aureus	6538	ATCC	+	
Aeromonas hydrophila	49140	ATCC	-	
Vibrio alginolyticus	33840	ATCC	-	
Edwardsiella tarda	PE210	Japan	-	
Escherichia coli	10536	ATCC	-	Human
Escherichia coli (pathogenic)	N/A	Hospital	-	
Acinetobacter baumannii	19606	ATCC	-	

Antibacterial activity on fish pathogenic bacteria

Up: gram positive
Down: gram negative



Antibacterial activity on human bacteria



Normal bacteria

Human pathogenic bacteria

BING shows antibacterial ability on different kinds of pathogenic bacteria

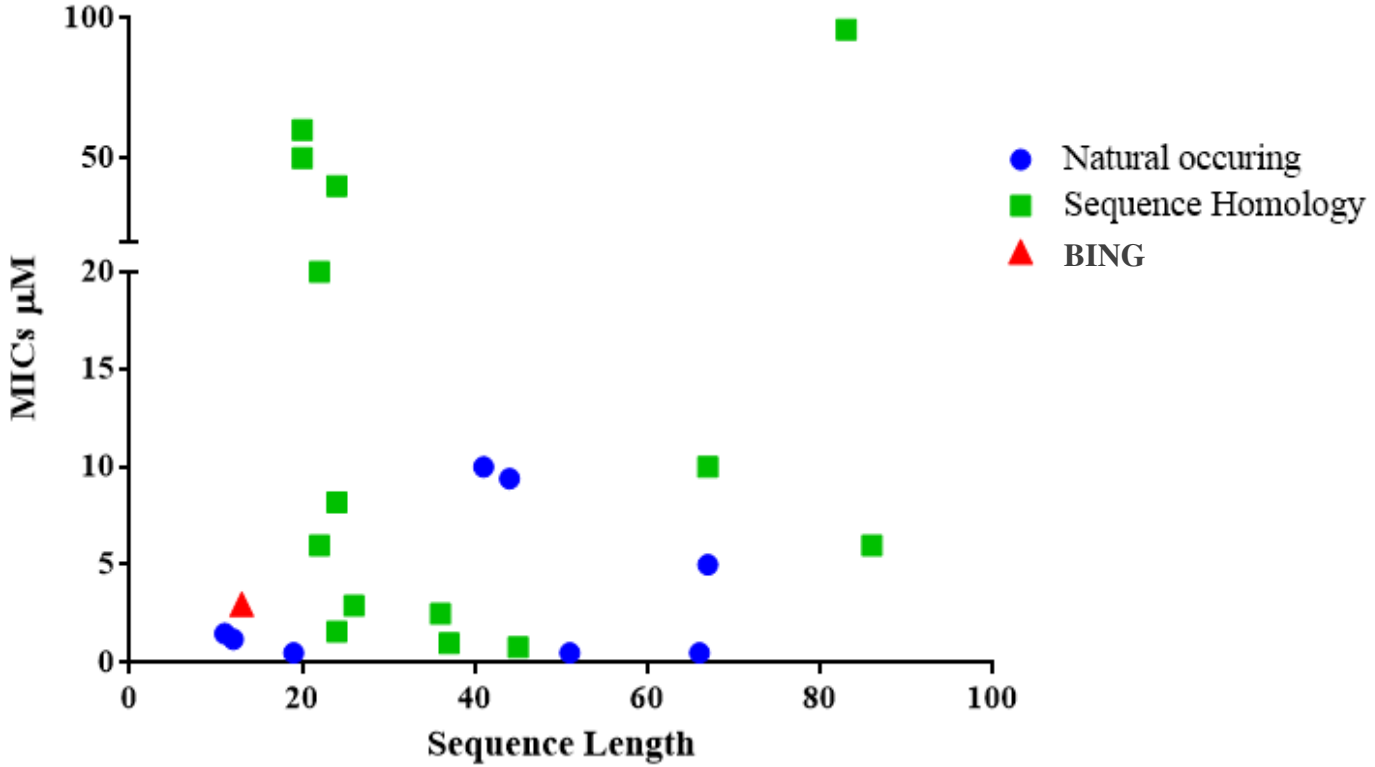
Bacteria	Gram(+/-)	Suspected MICs (ug/ml)	Suspected MICs (uM)
S. faecalis	+	50	34.1
S. pyogenes	+	50	34.1
S. aureus	+	20	13.7
A. hydrophila	-	20	13.7
V. alginolyticus	-	50	34.1
E. tarda	-	10	6.8
E. coli	-	5	3.4
E. coli (pathogenic)	-	5	3.4
A. baumannii	-	10	6.8

Minimum inhibitory concentrations (MICs) are defined as the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation

BING shows antibacterial ability on drug resistant bacteria

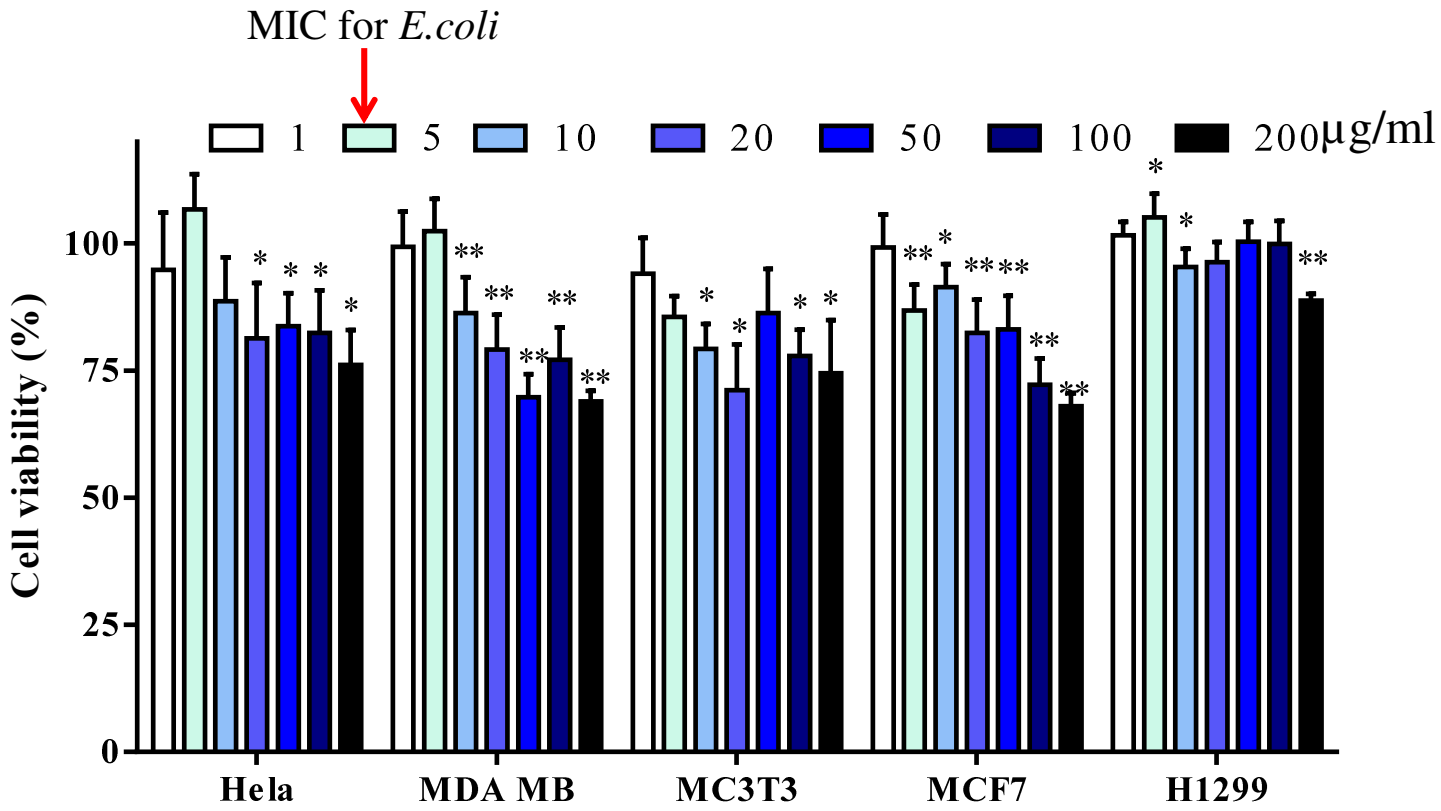
	Bacteria	Gram(+/-)	Suspected MICs (ug/ml)
	E. coli BL21	-	8
	K. pneunmoniae NDM-1	-	32
	E. coli NDM-1	-	16
Beta-lactam	NDM-1/BL21	-	4
Beta-lactam	SHV-1/BL21	-	4
Beta-lactam	TEM-1/BL21	-	8
Colistin resistance	MCR-1/BL21	-	16
	S. aureus 29213	+	64
	Methicillin-resistant *S. aureus* 41	+	32
	Multidrug-resistant *S. aureus* 44	+	32

BING is shorter and more effective on *E.coli* killing comparing with other Fish AMPs



V. Rajanbabu, J.Y. Chen / Peptides 32 (2011) 415–420
Jorge A. Masso-Silva and Gill Diamond/ Pharmaceuticals (2014) 265-310

BING has no observable cell and fish toxicity



14 days post injection of BING into medaka fish

Amount	Survival rate %
1 μg	100

Done by Wei Shen

Summary

- BING has broad-spectra toxicity on fish and human pathogenic bacteria
- BING shows stronger antibacterial activity on gram negative than positive bacteria
- BING is non-toxic to mammalian cells and fish

Fish efficacy study

(A) One injection

PBS alone

BING+E.tarda

E.tarda alone

BING: 1ug/ml
E.tarda:10^4CFU/fish

(B) Double injection

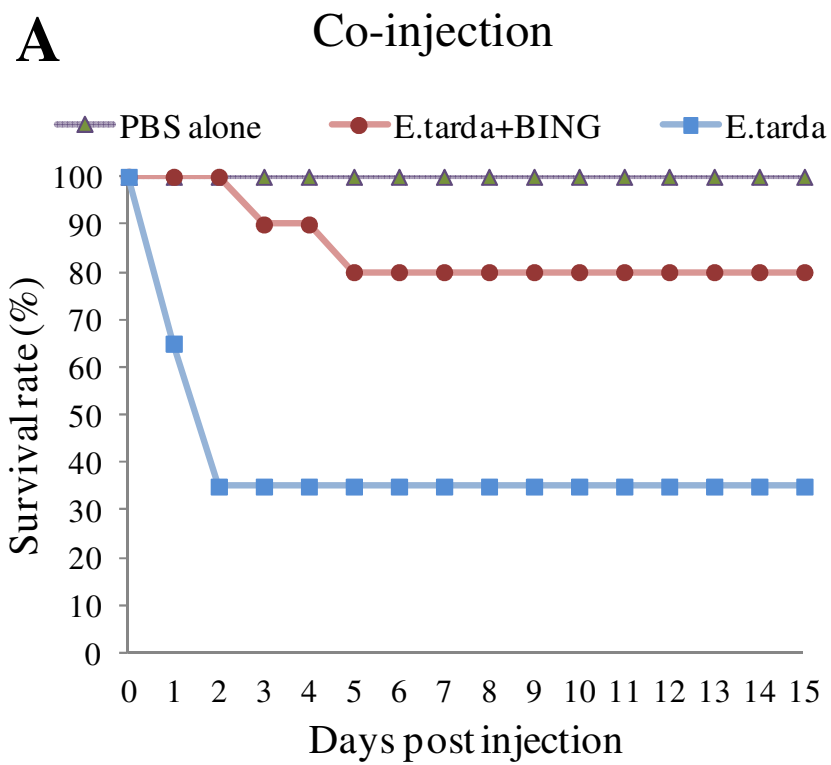
PBS+E.tarda

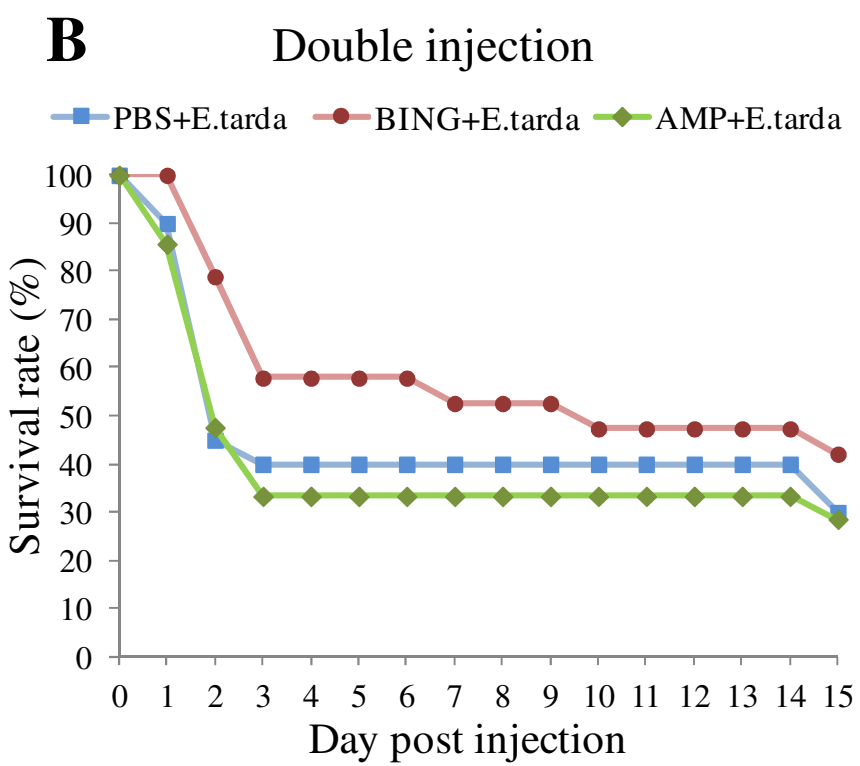
BING+E.tarda

Ampicillin+E. tarda

BING: 2ug/ml
AMP: Ampicillin 2ug/ml
E.tarda:10^4CFU/fish

Fish survival rate after different injections





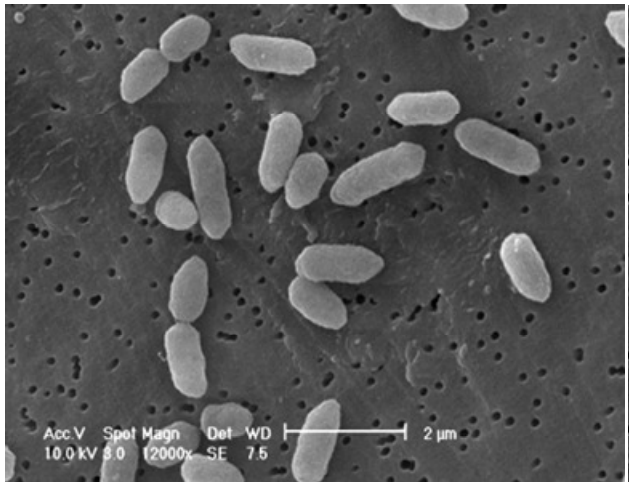
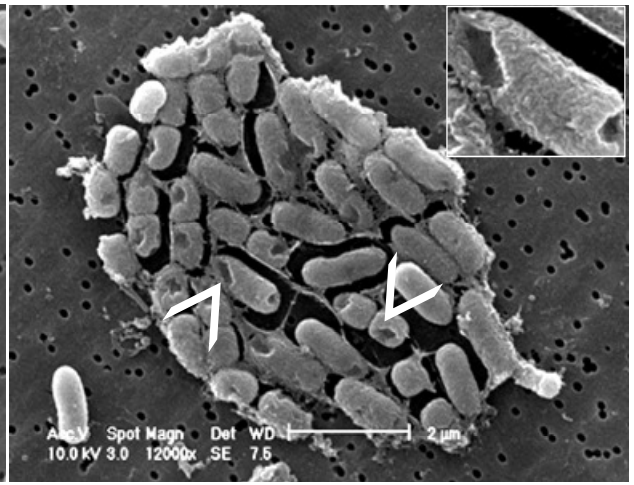
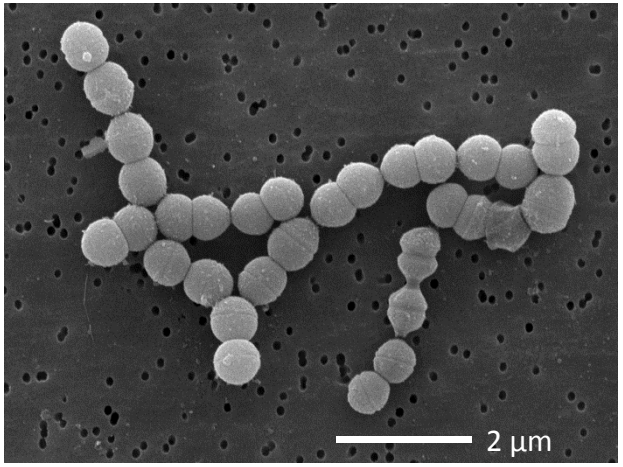
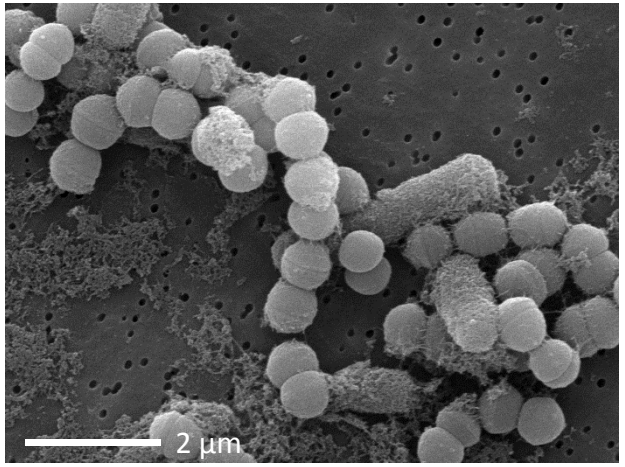
BING: 1ug/ml
E.tarda:10^4CFU/fish

BING: 2ug/ml
AMP: Ampicillin 2ug/ml
E.tarda:10^4CFU/fish

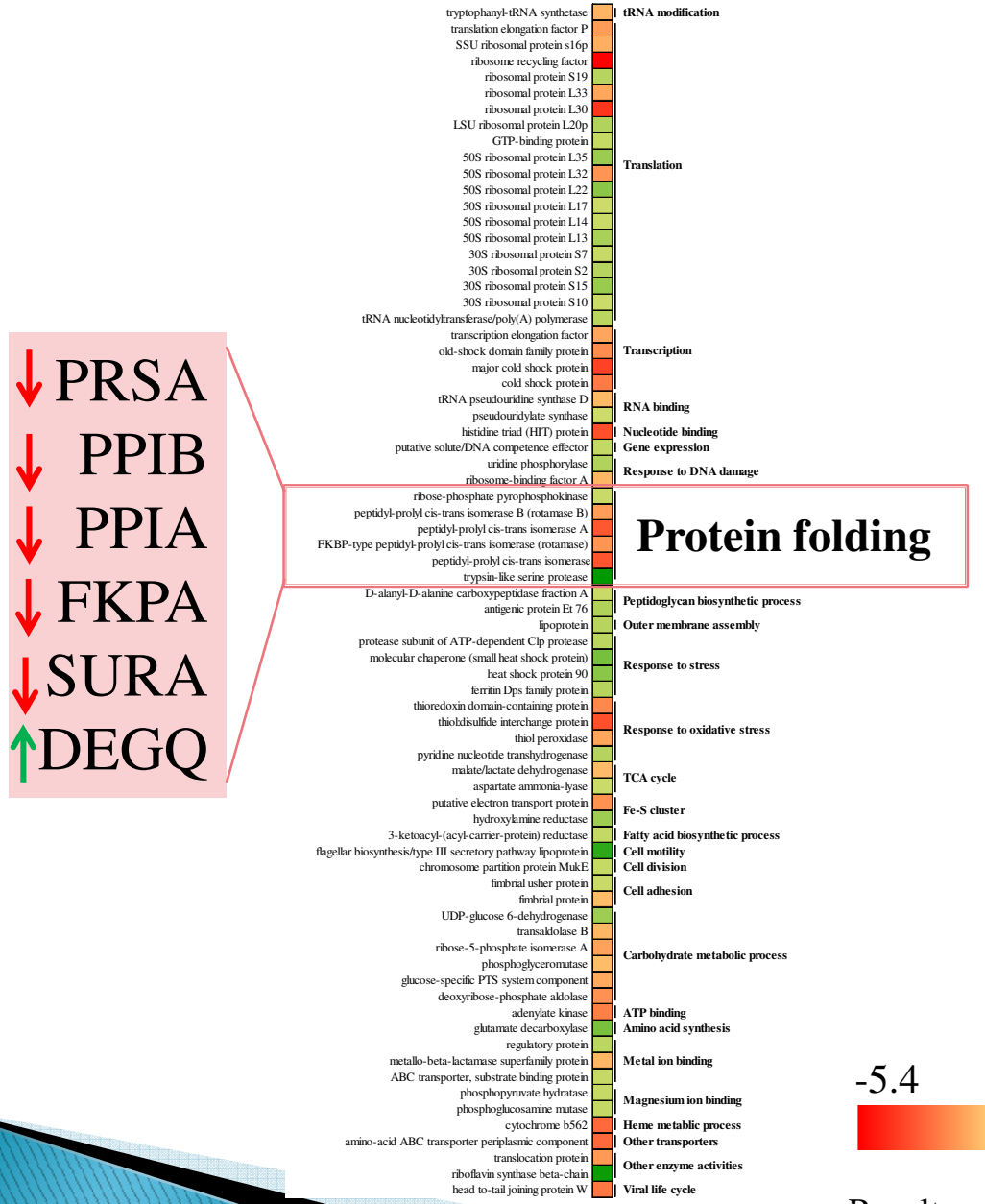
Summary

- BING can inhibit *E.tarda* infection in fish, suggesting that BING can be an effective chemotherapeutic agent for aquaculture in the future.

BING can disrupt bacteria cell membrane

	Cultural medium	BING:50 μg/ml
E.tarda		
S. pyogenes		

Antibacterial mechanism study-label free proteomic



↓PRSA
↓PPIB
↓PPIA
↓FKPA
↓SURA
↑DEGQ

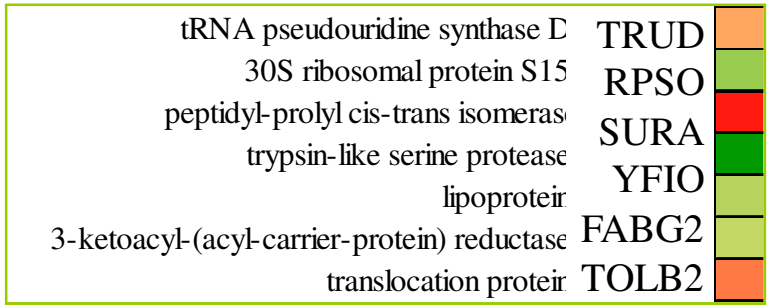
Tips:

➢BING can downregulate PPIASE which are responsible for protein folding

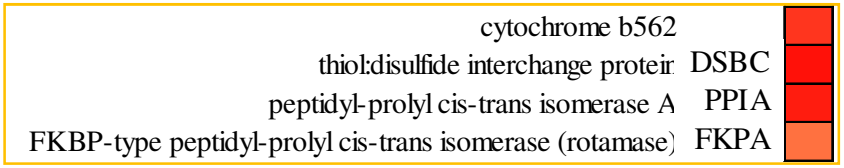
Result expressed as log2 fold change.
Only values >1 or <-1 are shown in treeview

Cell envelope stress pathway

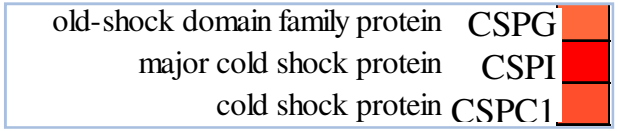
Positively regulated by σ^E



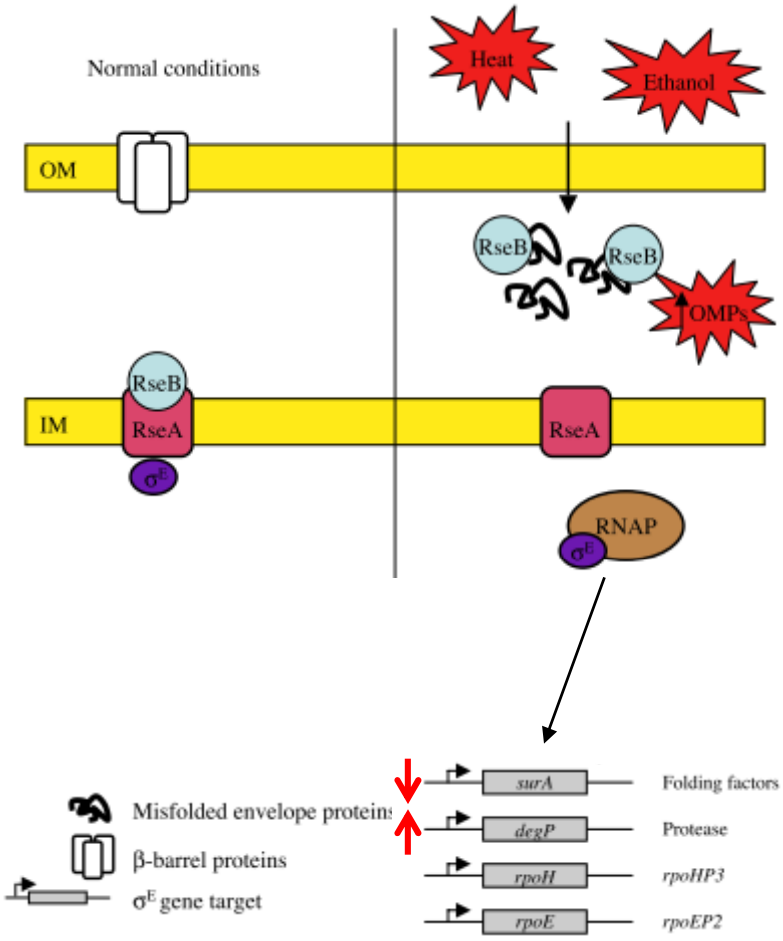
Positively regulated by CPX



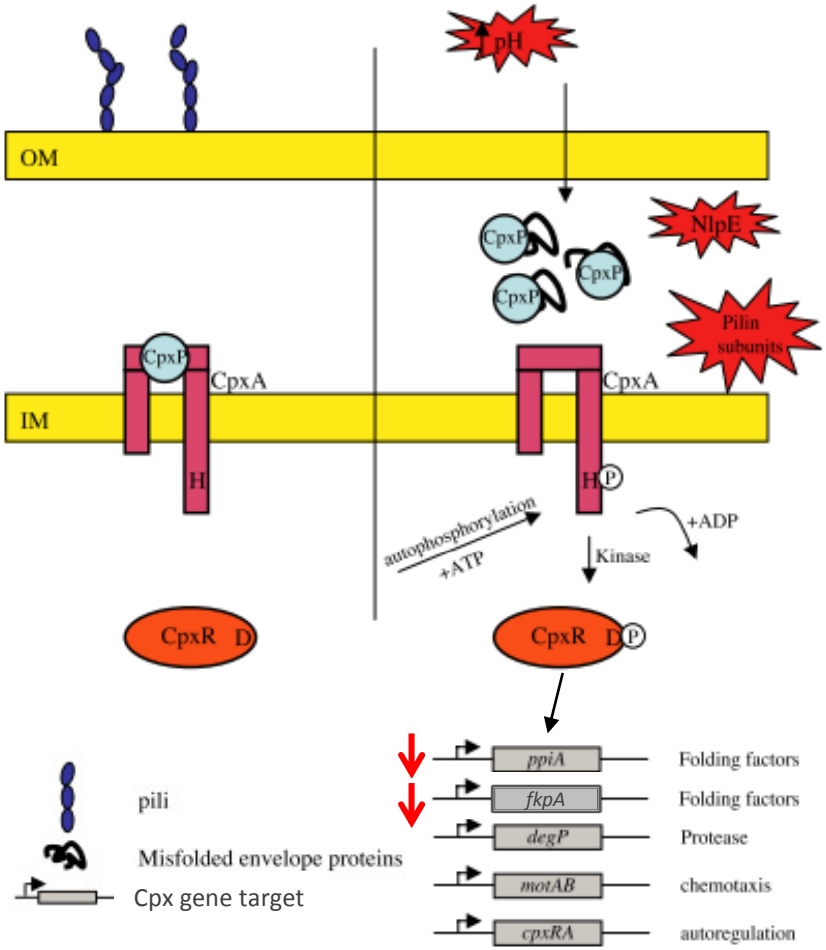
Negatively regulated by both σ^E and CPX



Model for the σ^E regulon

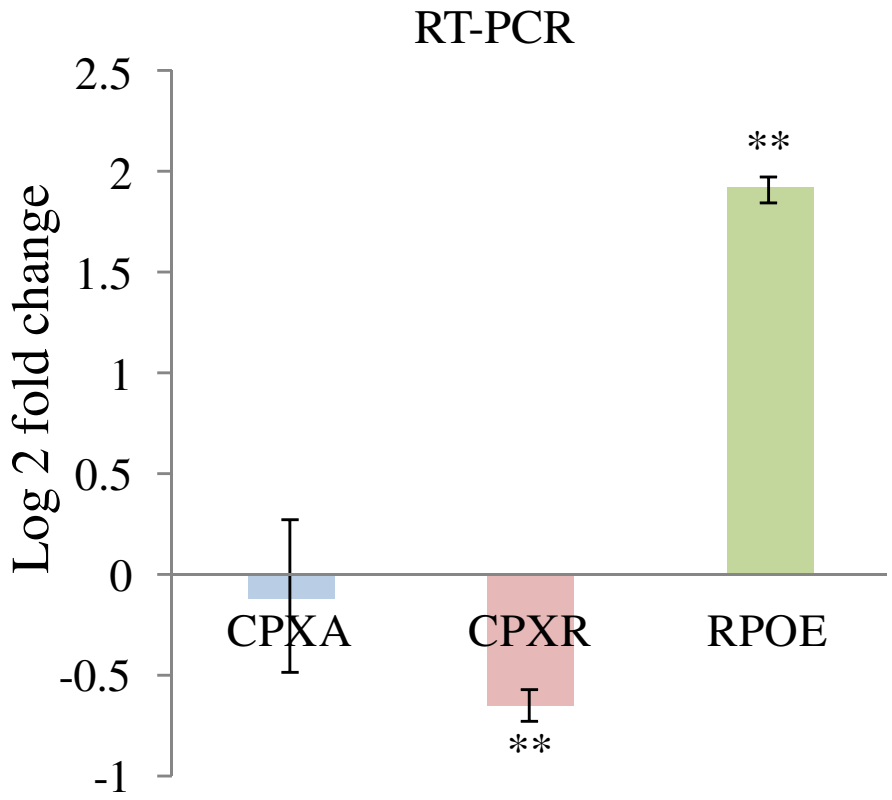


Model for the CPX regulon



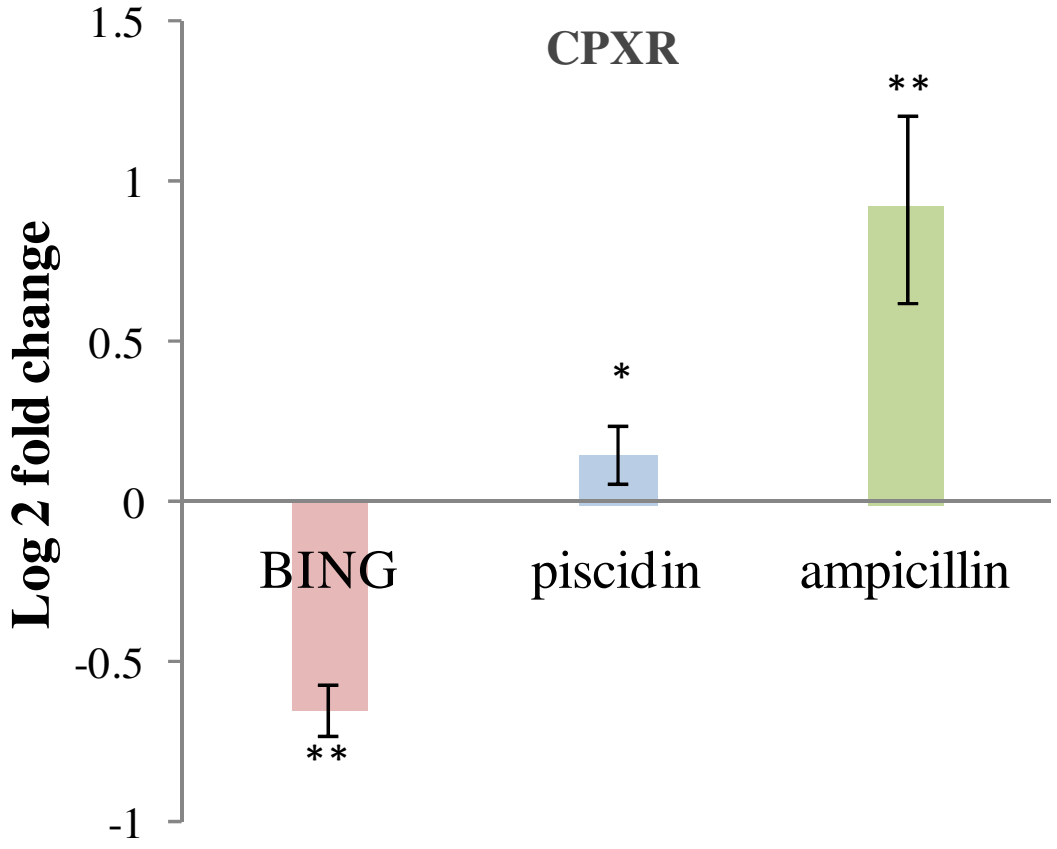
A. R. Duguay and T. J. Silhavy, "Quality control in the bacterial periplasm," *Biochim. Biophys. Acta - Mol. Cell Res.*, vol. 1694, [illegible] 121–134, 2004.

BING induces σ^E pathway but suppresses CPX pathway



Three replicates, result normalized with control group
Same concentration and incubation time as proteomic experiment

Other drugs can not knock down the expression of CPXR



Summary

- BING induce bacteria cell envelope stress by activating σ^E pathway, while specifically suppresses CPXR pathway can be suppressed

Conclusions

Identification

- By using proteomic approach we got 6483 unique peptides
- Combining with web-based prediction tools we got 83 potentially novel AMP candidates

Application

- BING has broad-spectrum antibacterial activity on fish and human pathogens including multi drug resistant bacteria, but non-toxic to mammalian cells and fish
- BING can inhibit *E.tarda* infection in fish, suggesting that BING can be an effective chemotherapeutic agent for aquaculture in the future
- This method can also apply on AMPs collection from other species

Mechanism

- BING can specifically suppress CPX pathway which is a resistance system to both AMPs and antibiotics.

Long term goal

- Antibacterial activity of modified BING (D-isomer, C-amidated and cyclic)
- Test the antibacterial activity of rest 82 candidates
- Utilize these fish AMPs, either alone or in combination, to combat bacterial infections in aquaculture

Acknowledgement

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Collaborators:
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Dr. Hongyan Sun lab, Haipeng Lei
Prof. Kwok yin Wong lab, Dr. Ann So Lok Yan

Thank you