Exploiting the cold environments: psychrophilic bacteria as a promising source of novel bioactive compounds

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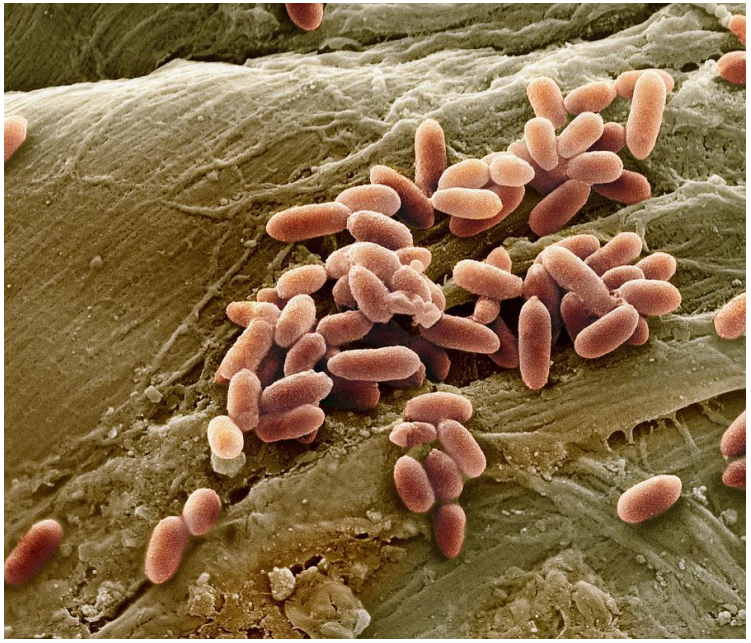
Multidrug resistance bacteria (MDR)

These bacteria are a worldwide concern and a major challenge for modern medicine.



They belong to an elite class of microorganisms: the so-called "superbugs", the causative agents of recalcitrant infections immune to the effects of the most common antimicrobials.

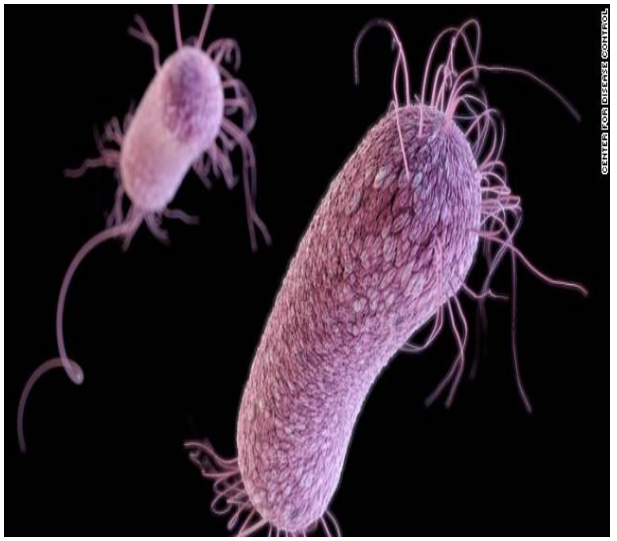
The effect on MDR bacteria on public health

Annual Cost / Per Patient	(Infections / Deaths) per Year USA
$3-4 Billion / $14,000 USD	90,000 – 100,000 / ~10,000

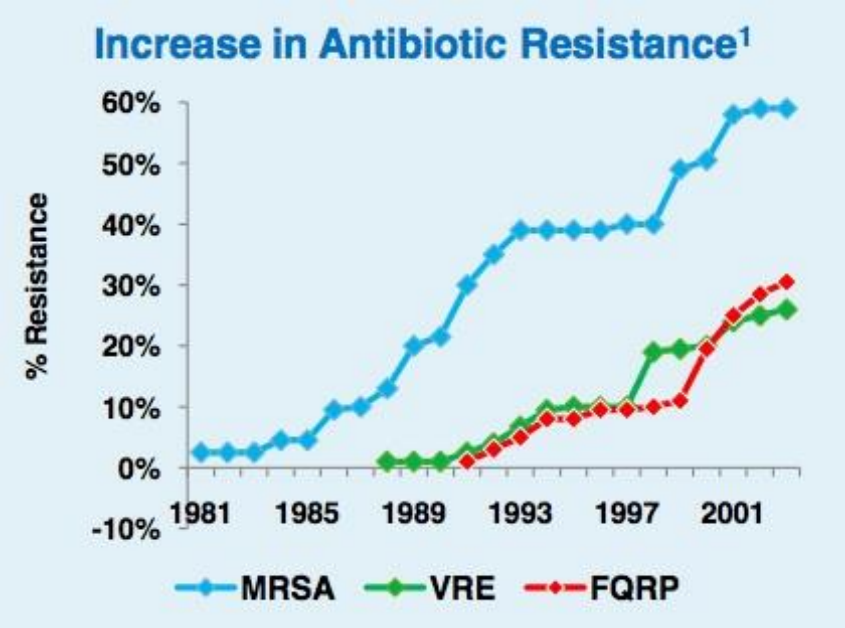
Methicillin-resistant *Staphylococcus aureus* (MRSA) is the most prevalent antibiotic resistant bacterial worldwide infection

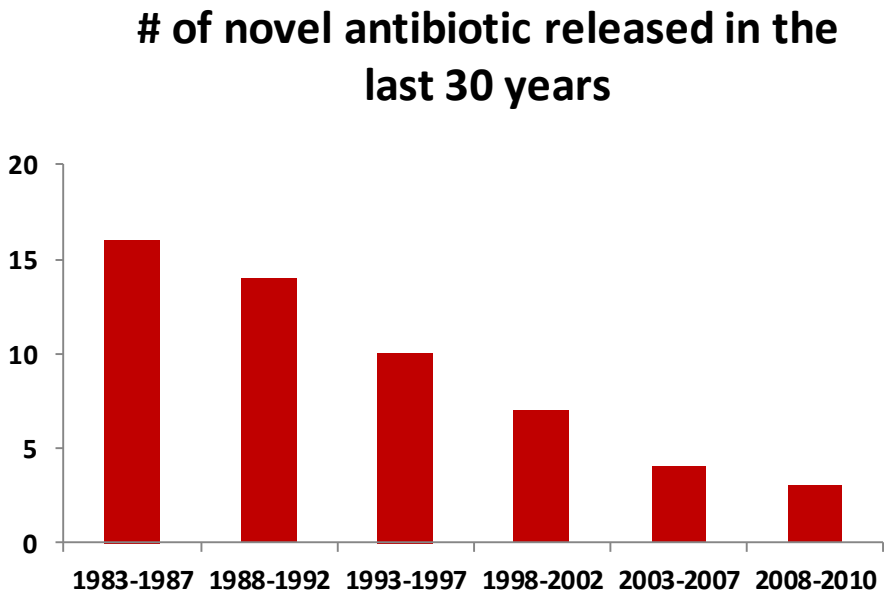
Emerging MDR strains

- Drug-resistant *Streptococcus pneumoniae*
- Multidrug-resistant *Burkhokderia cenocepacia*
- Quinolone-resistant *Neisseriaa gonorrheae*
- Penicillin-resistant *Neisseria meningitidis*



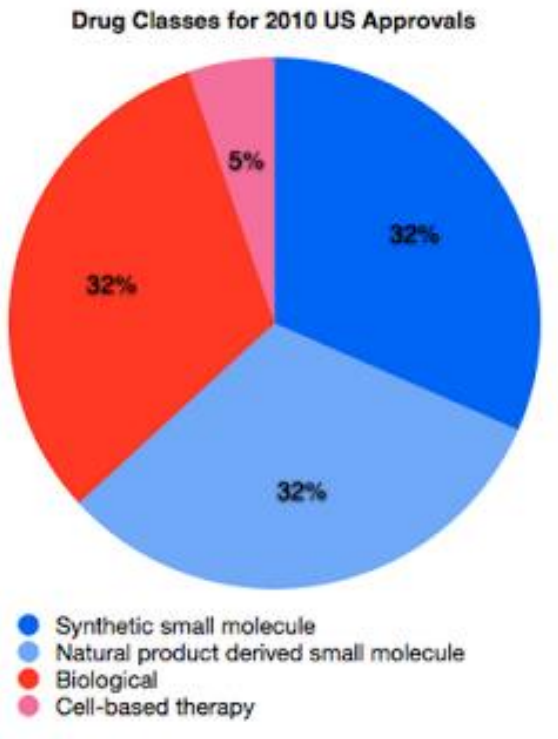
The antibiotic crisis





There is a pressing need to discover novel antibiotics

Natural Products



Natural products and their analogs continue to play a prominent role in medicine, accounting for two-thirds of new antibacterial therapies approved from 1980 to 2010

Promising Natural Products in the Pipeline

Agent	Source	Status
Bryostatin 1	Bryozoan (marine)	Phase I/II trials for AML, CLL, NHL, ovarian, prostate
ET-743	Tunicate (marine)	Phase II trials for various sarcomas
Aplidin	Tunicate (marine)	Phase II trials for ALL, myeloma, prostate
Dolastatin 10	Microbe (marine)	Phase I/II trials for CLL, CML, pancreatic, renal
Epothilones	Microbe (terrestrial)	Phase I/II/III trials for breast, colon, lung, renal
Combretastatin	Plant (terrestrial)	Phase II trials for thyroid

Discovery of novel natural products

BIOPROSPECTING FROM EXTREME ENVIRONMENTS

Extreme and unexplored environments may hide of new classes of natural products

Psychrophilic bacteria

These bacteria live in extreme conditions: low temperature, shortage of food

They adopt peculiar survival strategies in order to thrive and face adverse environmental conditions.

These bacteria may produce interesting compounds with antimicrobial activity

Aim of the work

Discovery of **novel bioactive compounds** from **psychrophilic bacteria**

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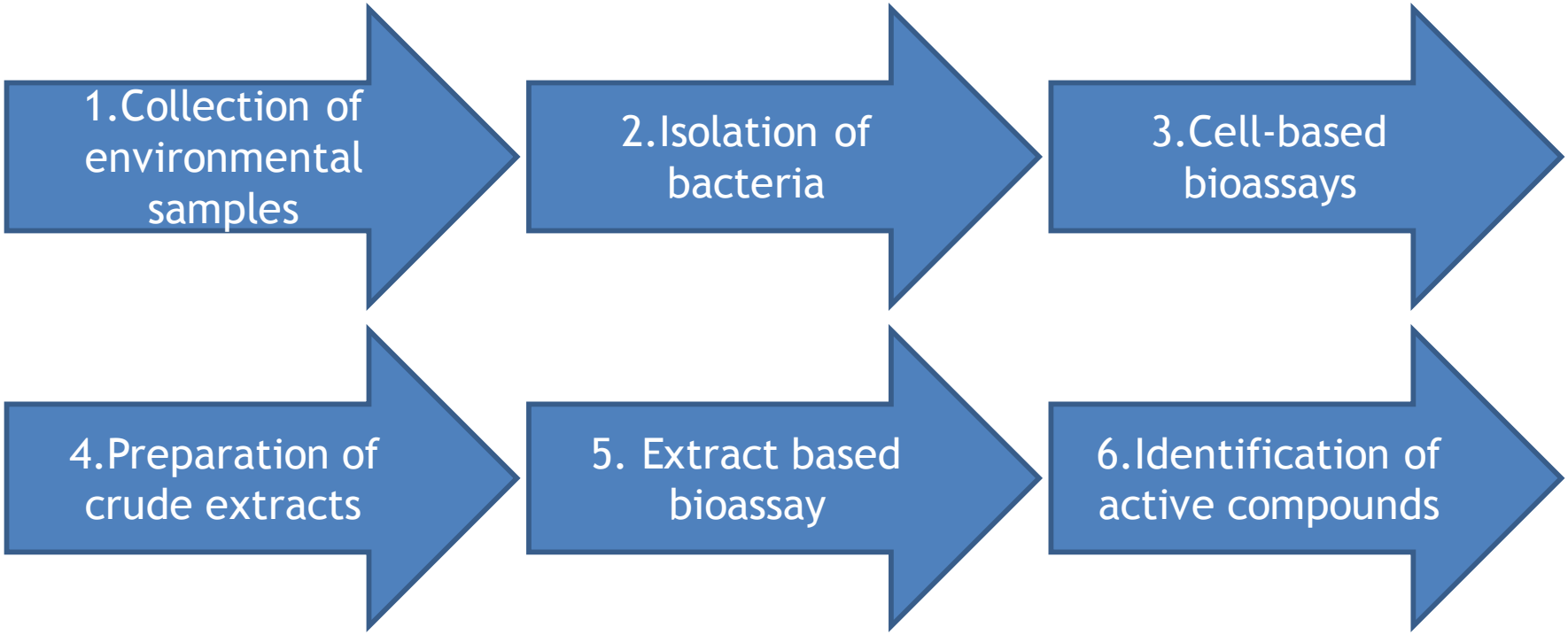
COOPERATION

Increasing Value and Flow in the Marine Biodiscovery Pipeline

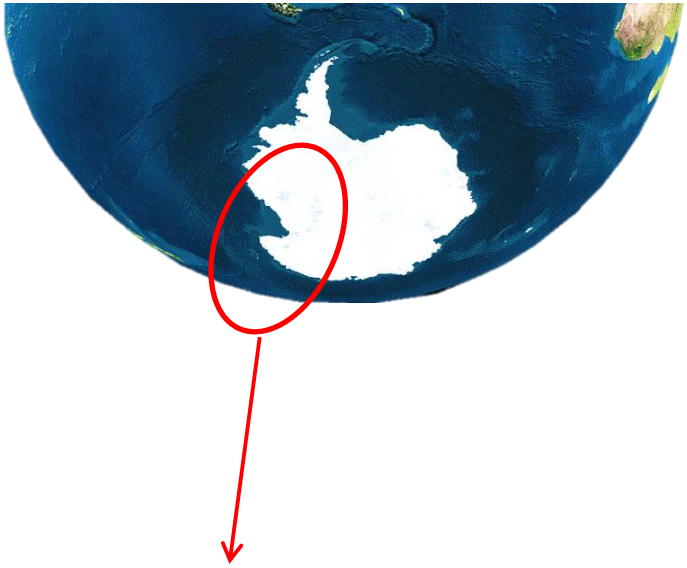
"To improve the quality, volume and value of active agents discovered in the marine environment and increase the speed at which they can be delivered to the marketplace, by addressing bottlenecks and restrictions and adding technical booster-pumps"

KBBE.2012.3.2-01: Innovative marine biodiscovery pipelines for novel industrial products.
Grant agreement for: Collaborative project no: 3121184, 2012-2016

Development of a biodiscovery pipeline



1. Collection of environmental samples → Isolation of bacteria → Cell-based bioassays → Preparation of crude extracts → Extract based bioassay → Identification of active compounds



Antarctic sub-sea sediments

Mario Zucchelli Station, Baia Terranova Ross sea, Antarctica

Karuola glacier

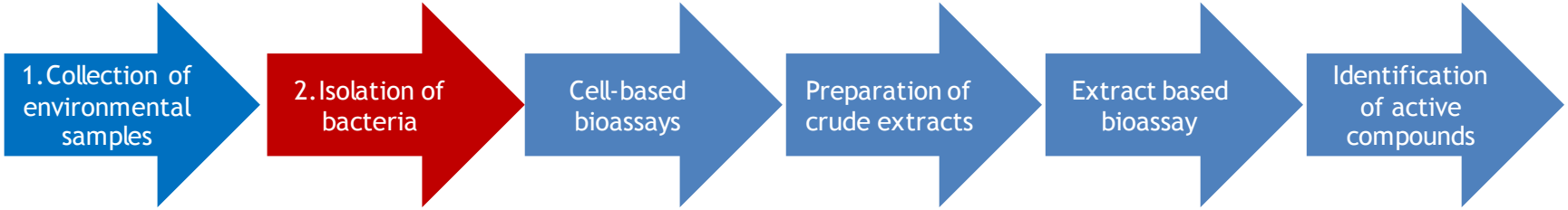
Midui glacier

Glacier samples in Tibet

Samples collected by Dr Lei Zhai

CNR-IMCAS Cooperation agreement 2011-2013

Dr. Zhu Cheng- Dr. de Pascale



PYG medium (g/L)

Polypeptone	5.0
Tryptone	5.0
Yeast extract	10.0
Glucose	10.0
Salt solution	40 ml

salt solution, pH 7.2,contained:
0.2 g/L $CaCl_2$, 0.4 g/L $MgSO_4.7H_2O$,
1.0 g/L K_2HPO_4, 1.0 g/L KH_2PO4,
10.0 g/L $NaHCO_3$ and 2.0 g/L NaCl.

15 days at 4°C

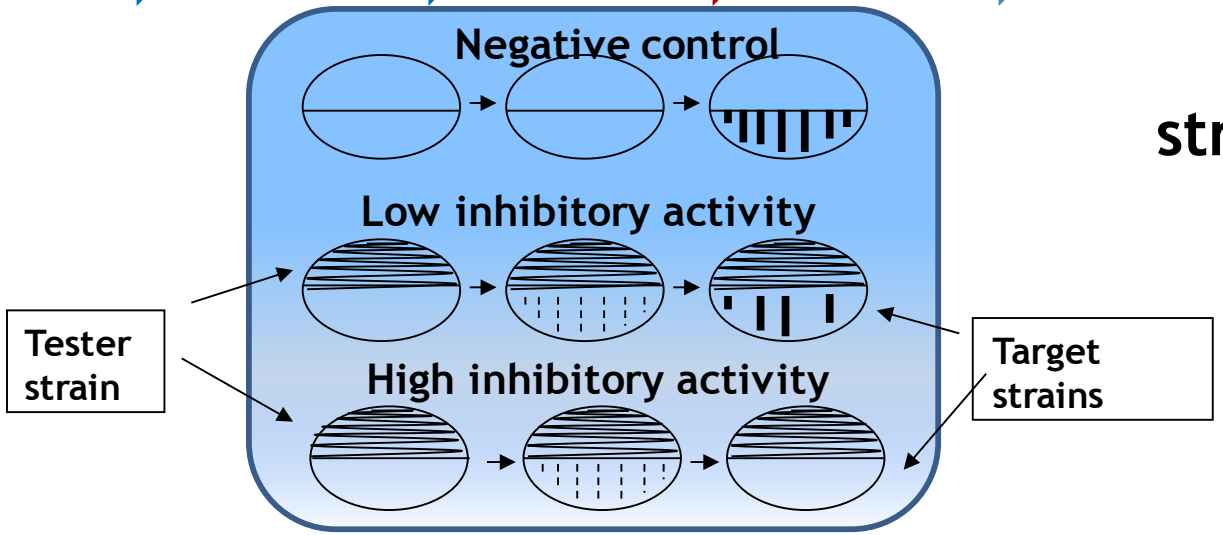
We obtained

24 isolates from Antarctic sub-seasediments

11 isolates from Tibetan glaciers

1. Tibetan Isolates

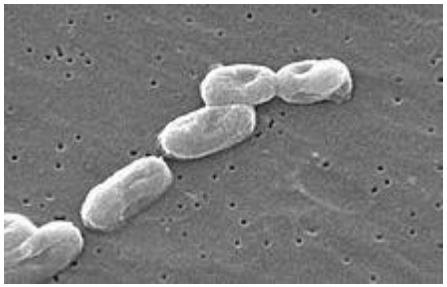
1. Collection of environmental samples → 2. Isolation of bacteria → 3. Cell-based bioassays → Preparation of crude extracts → Extract based bioassay → Identification of active compounds



To identify strains producing bioactive compounds

Methods to determine antagonist interaction against different bacterial isolates

Burkholderia cepacia complex



Many Bcc strains are MDR opportunistic human pathogens and represent a serious concern for Cystic Fibrosis (CF) patients and immune-compromised individuals

Several Tibetan strains were able to inhibit the growth of some Bcc strains

\+ growth
\- no growth

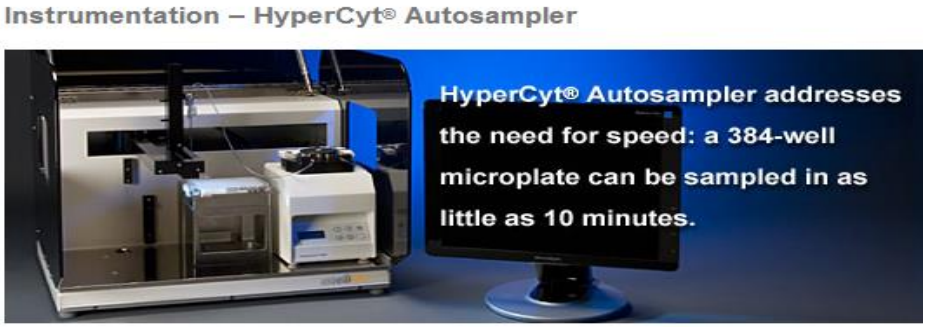
Species	Strain	MD1	MD2	MD3	MD4	MD5	KRL1	KRL2	KRL3	KRL4	KRL5	KRL6
B. ambifaria	LMG 19182	+	-	-	+	+	+	+	-	-	+	-
B. anthina	LMG 20980	-	+	+	+	+/-	+/-	+/-	+/-	+	+	+
B. arboris	LMG 24066	+	+	-	+	+	+	+	-	-	+	+
B. cenocepacia	LMG 16656	-	-	-	-	+	-	+	-	-	+	-
B. cepacia	LMG 1222	+	-	-	-	-	-	-	-	-	-	-
B. contaminas	LMG 23361	+	-	+	-	+	+	+	-	-	+	-
B. diffusa	LMG 24065	+	+	-	-	+/-	-	+	-	+	+	+
B. dolosa	LMG 18943	-	+	+	-	+	-	-	-	+	+	+
B. lata	LMG 22485	+	-	-	+	+	-	+	-	-	+	+
B. latens	LMG 24064	-	+	-	+	+	+	+	+	-	-	-
B. metallica	LMG 24068	-	-	-	+	+	-	+	-	-	+	-
B. multivorans	LMG 13010	-	+	-	+	+/-	+/-	+/-	+	+	+	+
B. pseudomultivorans	LMG 26883	+	+	-	+	+	-	+	-	-	+	+

We decided to increase the panel of target pathogens

Collaboration with Prof George Tegos of the UNM

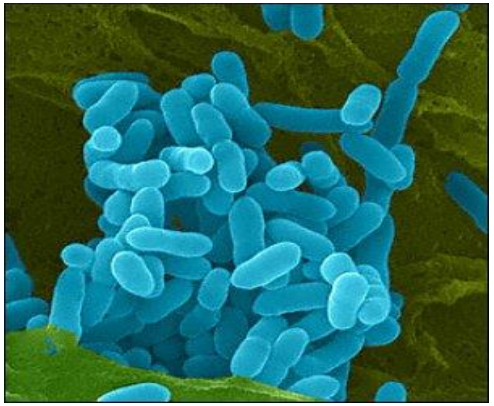
UNM HEALTH SCIENCES CENTER | *Center for Molecular Discovery*

- HTS instrumentation
- Bioinformatic facilities
- CDC-certified laboratory to conduct research on Category A select agents such as *Francisella tularensis*, *Burkholderia pseuodomallei* and *Bacillus anthracis*



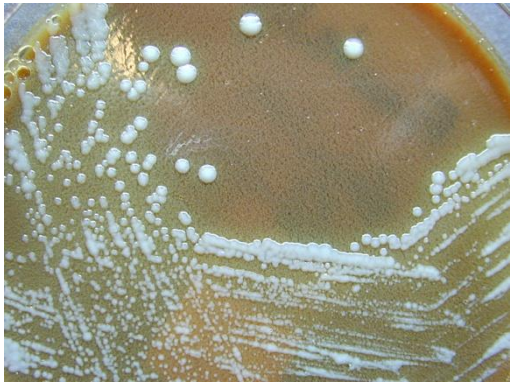
Target 2 ***Burkholderia pseudomallei (Bp)***

Bp **is the etiologic agent of meloidosis, particularly relevant in Thailand and northern Australia.**



Target 3 ***Francisella tularensis (Ft)***

Ft **is the causative agent of tularemia, and can infect humans and animals. It is endemic in North America**

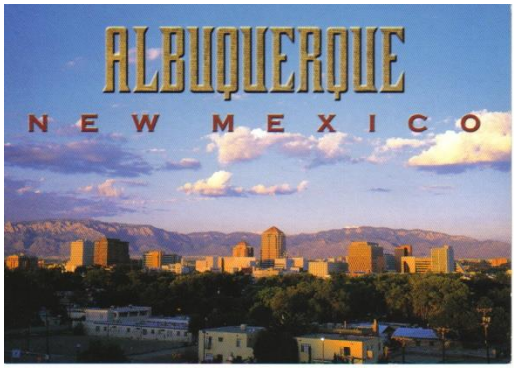


These two bacteria have been recognized as probable biological weapon by US Army

We selected Tibetan strains to perform new antimicrobial screenings

We performed 16S analysis on the most promising strains

***Pseudomonas sp.* MD1**
***Pseudomonas sp.* MD2**
***Pseudomonas sp.* MD3**
***Pseudomonas sp.* MD4**
***Pseudomonas sp.* KRL1**
***Pseudomonas sp.* KRL3**
***Exiguobacterium sp.* KRL4**



THE UNIVERSITY of NEW MEXICO

1. Collection of environmental samples → 2. Isolation of bacteria → 3. Cell-based bioassays → 4. Preparation of crude extracts → Extract based bioassay → Identification of active compounds

In collaboration with Prof. Chad E. Melancon III, Department of Chemistry, University of New Mexico

7 psychrophilic strains

↓

Bacteria growth in 2 different media at 2 different temperatures

↓

3 different extraction procedures

↓

Library of 84 extracts for antimicrobial screening

1. Collection of environmental samples → 2. Isolation of bacteria → 3. Cell-based bioassays → 4. Preparation of crude extracts → 5. Extract-based bioassays → Identification of active compounds

Screening for

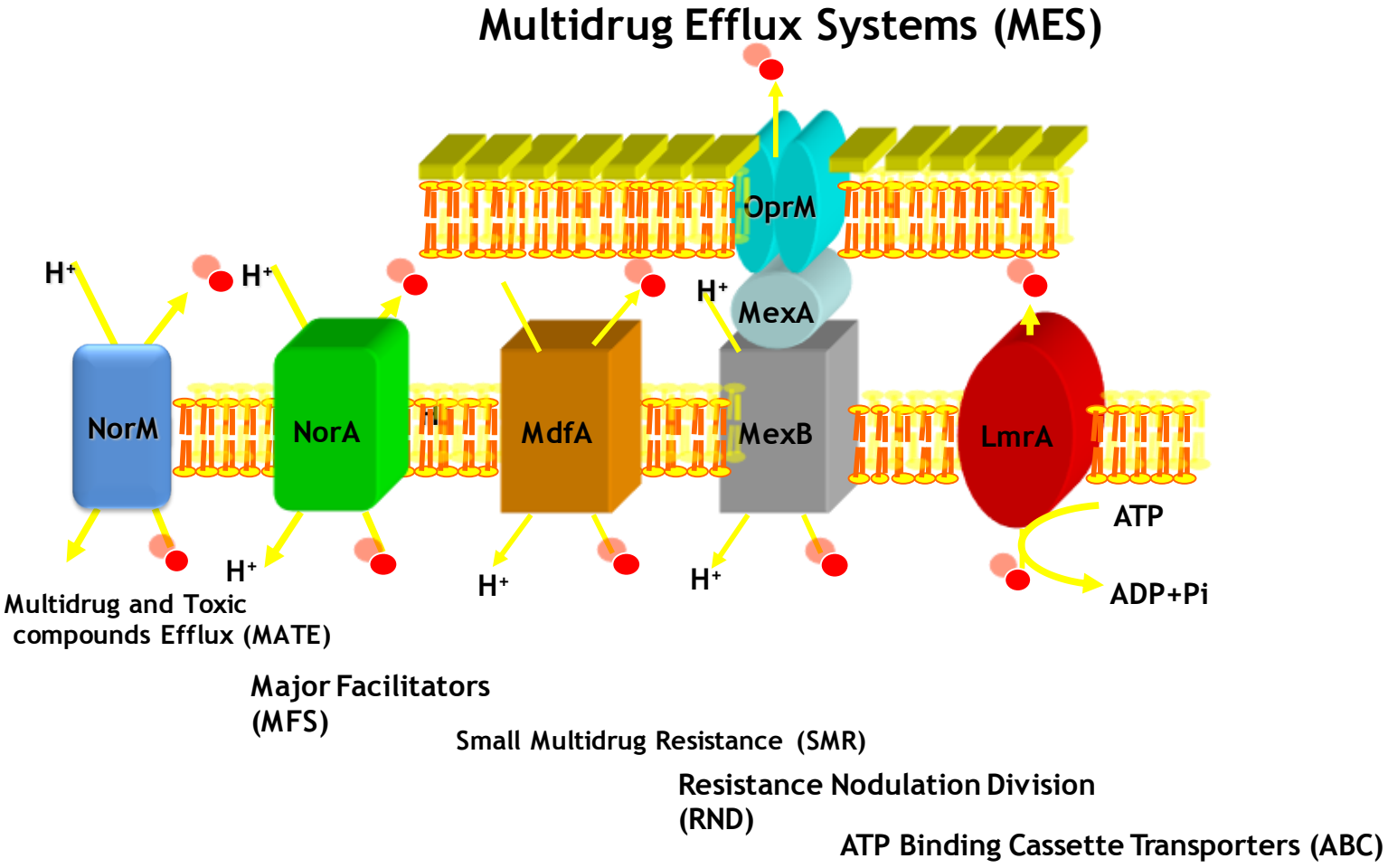
1-Antibiotic extracts

B. Pseudomallei
B. cenocepacia
F. tularensis

2-Efflux pumps inhibitors (EPIs)

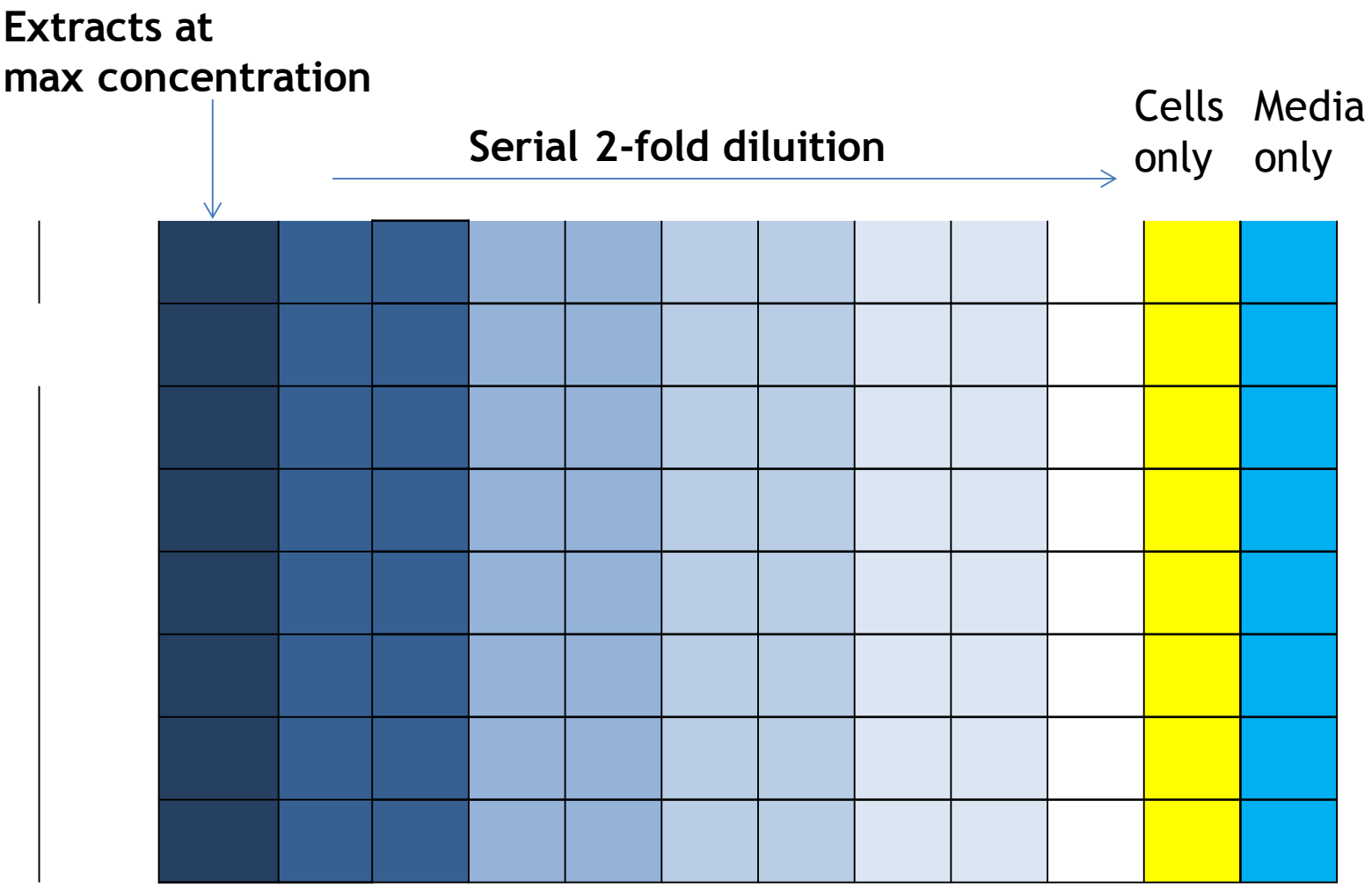
B. pseudomallei
B. cenocepacia

EPI are inhibitors of the Multidrug efflux systems



The MES is the principal mechanism of antibiotic resistance of MDR pathogens

1- Antibiotic activity screening: **LIQUID MIC ASSAY**



1- Antibiotic activity screening **LIQUID MIC ASSAY**

40.000 CFU added to each well and then plate incubated at 37°C for 48 h

Cells growth measured with a plate reader monitoring the absorbance at 600 nm

We defined an antibiotic extract as an extract able to completely inhibit the growth of the target strains after 48h at 37°C

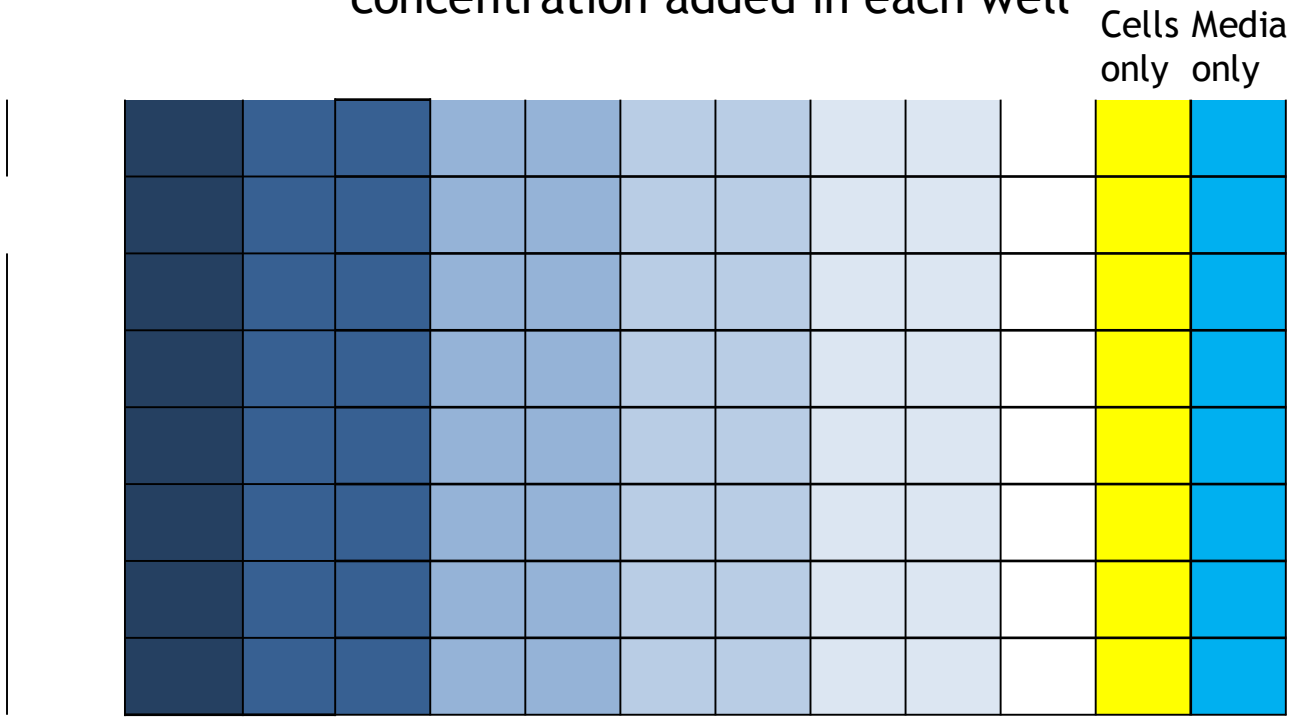
2- EPIs SCREENING **POTENTIATOR ASSAY**

Enhancement of the effect of an antibiotic

Extracts at max concentration

Serial 2-fold dilution

Antibiotic at sub-inhibitory concentration added in each well



1- EPIs screening **POTENTIATE ASSAY**

40.000 CFU added to each well and then plate incubated at 37°C for 48 h

Cells growth measured with a plate reader monitoring the absorbance at 600 nm

We define an EPI extract as an extract able to completely inhibit the growth of the target strains after 48h at 37°C in the presence of a sub-inhibitory antibiotic concentration

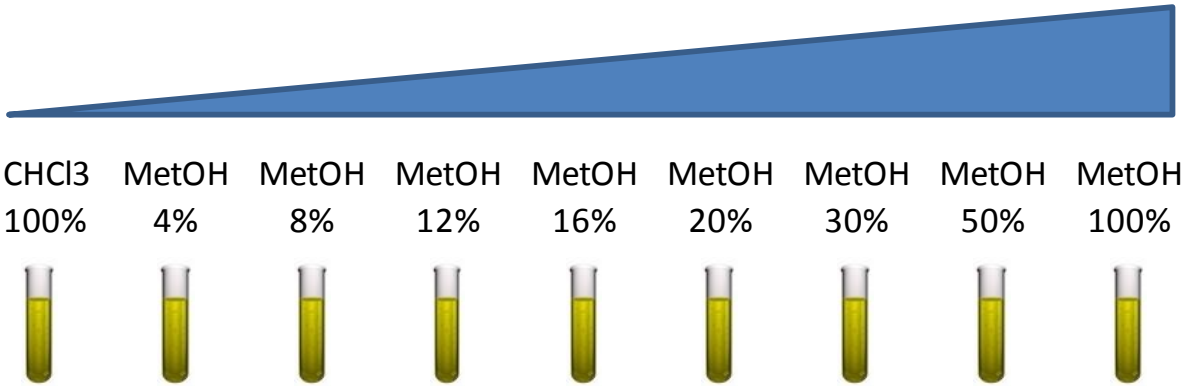
Antibiotic activity - *Ft*

	#1	#2	#3	#4	#5	#6	#7	#8	#9	#10	#11	#12	#13	#14	#15	#16	#17	#18	#19	#20	#21	DMSO
EXT 200 ug/mL	0,31	0,31	0,28	0,22	0,23	0,23	0,17	0,24	0,24	0,2	0,23	0,22	0,2	0,23	0,22	0,14	0,23	0,24	-0	0,26	0,25	0,447
EXT 100 ug/mL	0,31	0,26	0,2	0,17	0,18	0,17	0,12	0,18	0,17	0,16	0,18	0,17	0,16	0,18	0,17	0,15	0,17	0,16	0,001	0,19	0,19	0,288
EXT 50 ug/mL	0,35	0,21	0,21	0,16	0,16	0,15	0,13	0,14	0,14	0,12	0,15	0,14	0,12	0,14	0,14	0,11	0,13	0,13	0,038	0,14	0,17	0,282
EXT 25 ug/mL	0,33	0,2	0,14	0,11	0,11	0,1	0,08	0,1	0,1	0,1	0,14	0,1	0,1	0,11	0,11	0,11	0,12	0,13	0,094	0,15	0,21	0,316
EXT 12,5ug/mL	0,42	0,26	0,24	0,21	0,2	0,19	0,16	0,17	0,16	0,14	0,13	0,14	0,11	0,13	0,12	0,11	0,11	0,13	0,103	0,14	0,17	0,287
EXT 6,25 ug/mL	0,33	0,22	0,15	0,13	0,11	0,11	0,1	0,11	0,11	0,11	0,1	0,1	0,1	0,11	0,11	0,11	0,12	0,12	0,121	0,13	0,17	0,292
EXT 3,125 ug/mL	0,36	0,24	0,19	0,14	0,14	0,13	0,12	0,13	0,13	0,13	0,12	0,12	0,11	0,13	0,11	0,13	0,13	0,13	0,13	0,14	0,19	0,309
Cells only	0,39	0,31	0,22	0,21	0,18	0,18	0,17	0,17	0,16	0,16	0,16	0,16	0,16	0,16	0,16	0,15	0,18	0,17	0,192	0,22	0,25	0,34
Media only	0	0,01	0,01	0	0	0,01	0,01	0	0,01	0	0,01	0,01	0	0,01	0,01	0,01	0,01	0,01	0,006	0	0,01	0,006

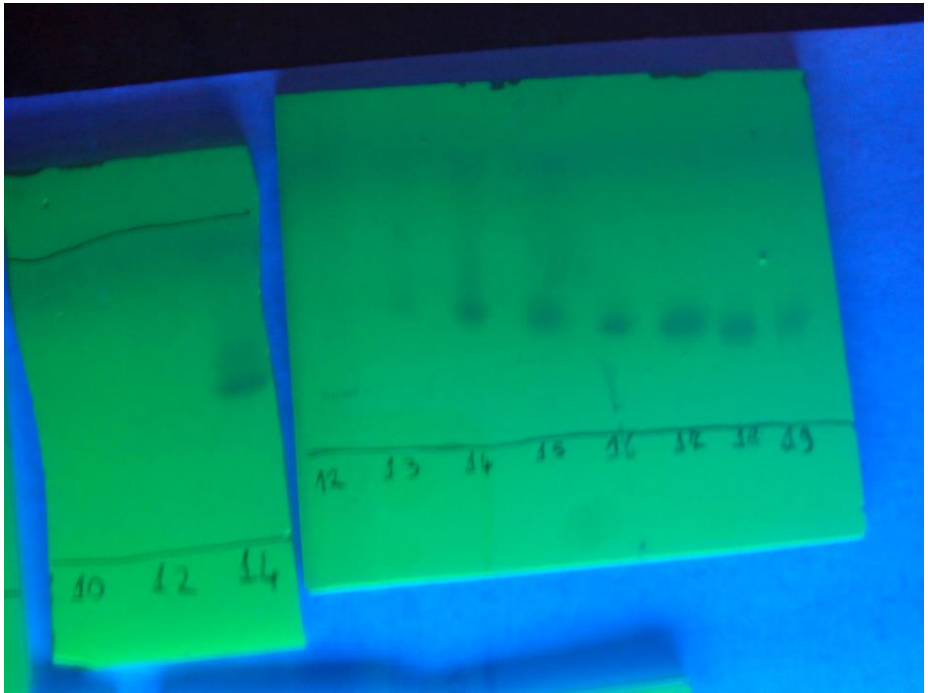
Extract #19 is the ethyl acetate extraction of the Isolate MD3

Fractionation with Silica Gel Flash column chromatography

Chloroform-methanol system



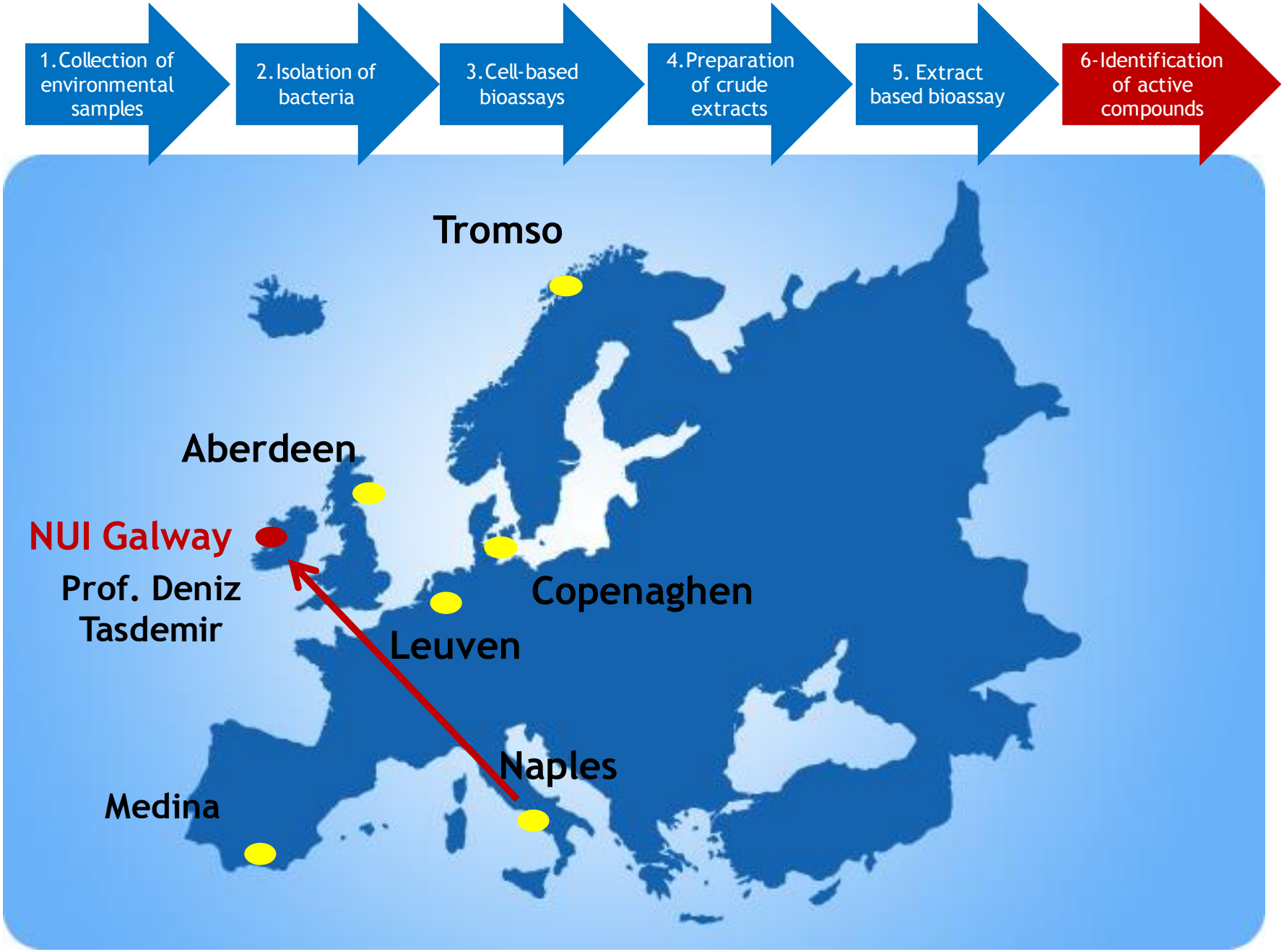
Fractions collected and analyzed performing TLC with UV-light detection



Fraction	Tubes collected	Methanol % v/v	Yield (mg)
1	Ethyl -acetate extract	0%	3
2	Methanol wash	100%	1.6
3	Flow-through + fractions 1-10	0 %-2%	1.6
4	Fractions 11-13	3%	5.5
5	Fraction 14	4%	12.1
6	Fraction 15	5%	8.0
7	Fraction 16	5%	4.0
8	Fractions 17-22	5%-7%	12.2
9	Fractions 23-30	7%-11%	4.6
10	Fractions 31-38	11%-14%	3.0
11	Fractions 39-43	14%-16%	1.2
12	Fractions 44-50	17%-19%	1.7
13	Fractions 51-58	20%-25%	2.3

Fractions assayed for direct activity

	100 ug/mL	50 ug/mL	25 ug/mL	12,5 ug/mL	6, 25 ug/mL	3,12 ug/mL	Cells only	Media
#1	0,166	0,1745	0,1675	0,161	0,155	0,179	0,2315	0,004
#2	0,0215	0,1095	0,127	0,1095	0,1135	0,143	0,238	0,0095
#3	-0,009	0,011	0,092	0,1645	0,159	0,1575	0,241	-0,0015
#4	-0,046	-0,0225	0,026	0,152	0,2255	0,2015	0,309	0,0005
#5	-0,019	0,001	0,1395	0,2135	0,2365	0,2425	0,31375	-0,00225
#6	-0,013	-0,008	0,091	0,122	0,142	0,1645	0,3373	-0,0044
#7	-0,006	0,072	0,0975	0,101	0,1105	0,14	0,36085	-0,00655
#8	0,0685	0,11	0,1185	0,1275	0,1325	0,152	0,3844	-0,00655
#9	0,0495	0,1445	0,1815	0,215	0,222	0,2405		
#10	0,093	0,1905	0,2335	0,2795	0,255	0,3125		
#11	0,0645	0,1155	0,146	0,1565	0,1755	0,194		
#12	0,012	0,0925	0,116	0,1145	0,144	0,192		
#13	0,0725	0,129	0,1285	0,1405	0,1575	0,1955		
DMSO	0,1825	0,213	0,226	0,2195	0,2515	0,2185		



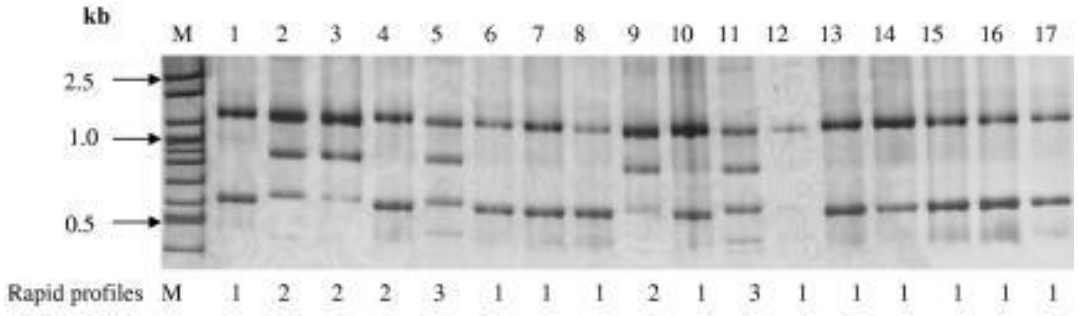
Novel bioactive compounds

2. Antarctic Isolates

In collaboration with Prof. Renato Fani from Florence University

24 Antarctic isolates

Tassonomic and genomic analysis on the 24 isolates



1. Collection of environmental samples → 2. Isolation of bacteria → 3. Cell-based bioassays → Preparation of crude extracts → Extract-based bioassays → Identification of active compounds

Cross-streaking targeting Bcc strains

Cross- streaking results

+ grown
- no grown
+/- partially grown

		Pseudomonas	*Pseudoalteromonas*		*Psychrobacter*						*Arthrobacter*
Species	Strain	BTN1	BTN2	BTN15	BTN3	BTN19	BTN20	BTN21	BTN24	BTN23	BTN4
B. ambifaria	LMG 19182	-	-	-	-	-	-	-	-	-	-
B. anthina	LMG 20980	-	-	-	-	-	-	-	-	-	-
B. arboris	LMG 24066	+	+	+	+	+	+	+	+	+	+
B. cenocepacia	LMG 16656	-	-	-	-	-	-	-	-	-	-
B. cepacia	LMG 1222	-	-	-	-	-	-	-	-	-	-
B. contaminas	LMG 23361	-	-	-	-	-	-	-	-	-	-
B. diffusa	LMG 24065	-	-	-	-	-	-	-	-	-	-
B. dolosa	LMG 18943	-	-	-	-	-	-	-	-	-	-
B. lata	LMG 22485	-	-	-	-	-	-	-	-	-	-
B. latens	LMG 24064	-	-	-	-	-	-	-	-	-	-
B. metallica	LMG 24068	-	-	+	-	-	+/-	+/-	+/-	+/-	-
B. multivorans	LMG 13010	-	-	+/-	-	-	-	-	-	-	-
B. pseudomultivorans	LMG 26883	-	-	+/-	-	-	-	-	-	-	-
B. pyrrocinia	LMG 14191	-	-	+	-	-	-	-	+/-	+/-	-
B. seminalis	LMG 24067	-	-	-	-	-	-	-	-	-	-
B. stabilis	LMG 14294	-	-	-	-	-	-	-	-	-	-
B. uborrensis	LMG 20358	-	-	+/-	-	-	-	-	-	-	-
B. vietnamensis	LMG10929	-	-	-	-	-	-	-	-	-	-

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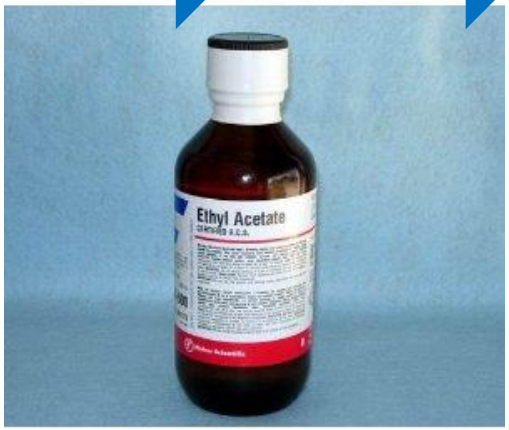


-Diaion HP20
-Amberlite XAD16
-Ethyl-acetate

1. Collection of environmental samples → 2. Isolation of bacteria → 3. Cell-based bioassays → 4. Preparation of crude extracts → 5. Extract-based bioassays → Identification of active compounds

Liquid MIC Assay

-B. cenocepacia
-F. tularensis
-P. aeruginosa

Liquid MIC Results

% of inhibition of Bcc strains with extracts at 1 mg/mL of concentration

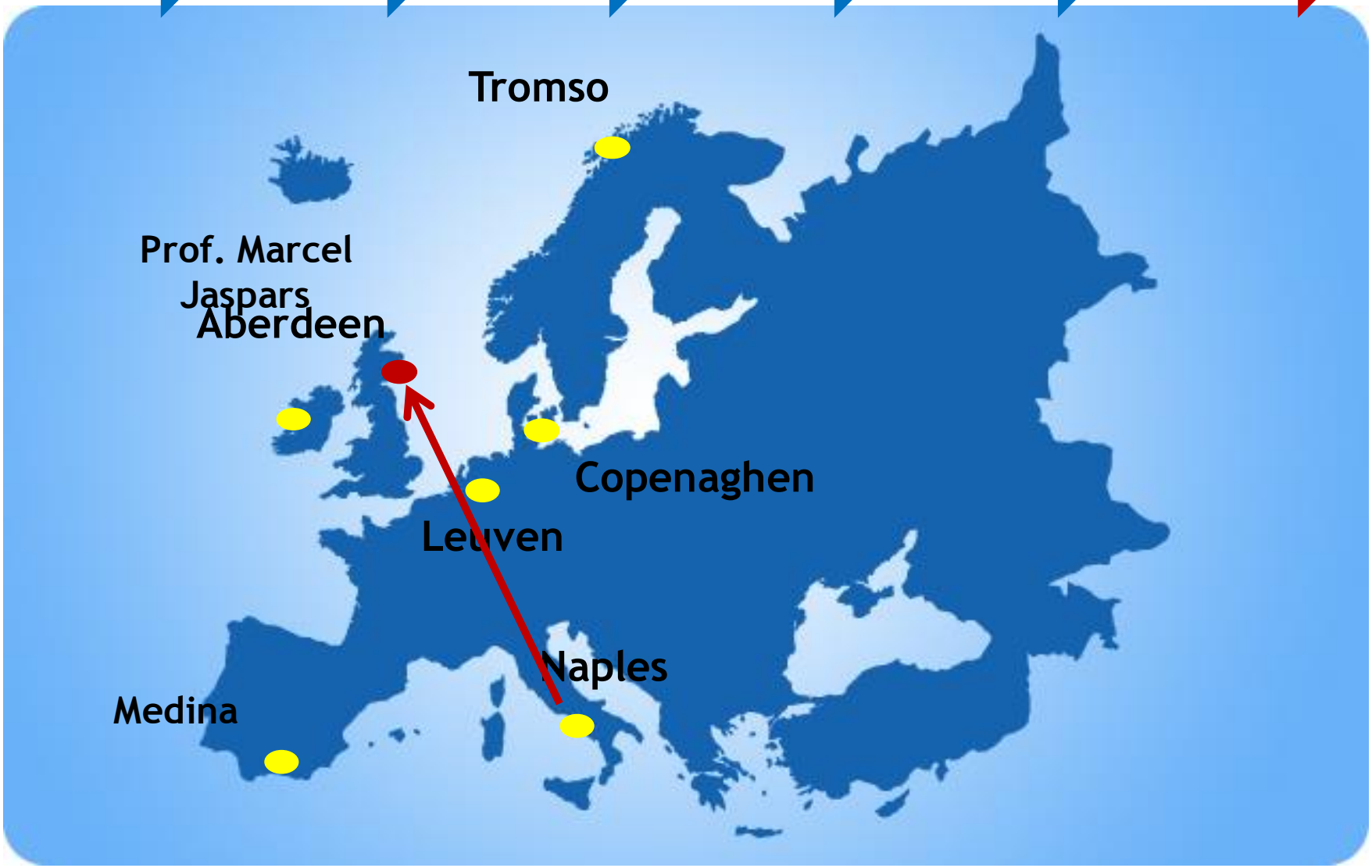
[1 mg/mL]		*Pseudomonas*	*Pseudoalteromonas*		Psychrobacter			Arthrobacter	
	%inhibition	BTN 1	BTN 2	BTN 15	BTN 3	BTN 19	BTN 21	BTN 4	BTN 5
B. diffusa	24065	90	75	77	43	45	70	63	77
B. metallica	24068	90	70	71	32	30	53	64	77
B. cenocepacia	16656	90	78	87	84	64	60	55	84
B. latens	24064	90	53	75	55	43	40	68	56
B. anthina	20980	90	38	60	30	25	46	50	38

strong inhibition

weak inhibition

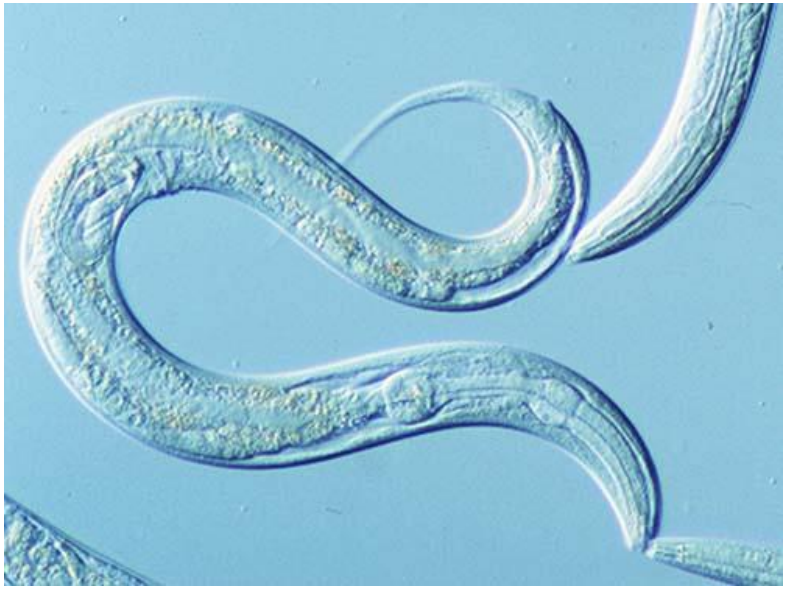
Fractionation of BNT1, BTN15, BTN5 extracts with SPE

1. Collection of environmental samples
2. Isolation of bacteria
3. Cell-based bioassays
4. Preparation of crude extracts
5. Extract based bioassay
6- Identification of active compounds



Future Perspective

-*In vivo* proof of efficacy of the bioactive compounds using the nematode C. *elegans* as model system



In vivo **proof of efficacy is a valuable procedure to establish the effectiveness of a drug in real infection system**

Preliminary studies of the pathogen-nematode interaction

Poster #44

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•FFC 2011-2013: New drugs for Burkholderia cepacia from Antarctic bacteria

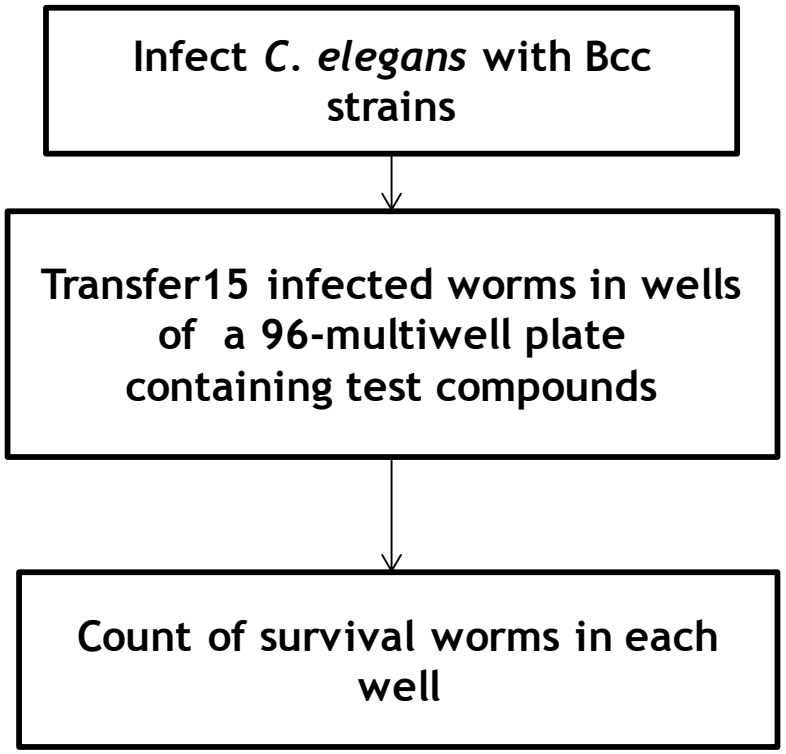
•PNRA 2014-2016: New drugs for cystic fibrosis opportunist pathogens

"PROGETTO CREME CAMPANIA RESEARCH IN EXPERIMENTAL MEDICINE" POR CAMPANIA FSE 2007/2013"

Collaborations

Thank you for your kind attention

In vivo validation of novel antimicrobic compounds



24-36 h of incubation

Incubation
Up to 5 days

Selection of compounds that prolong worms survival