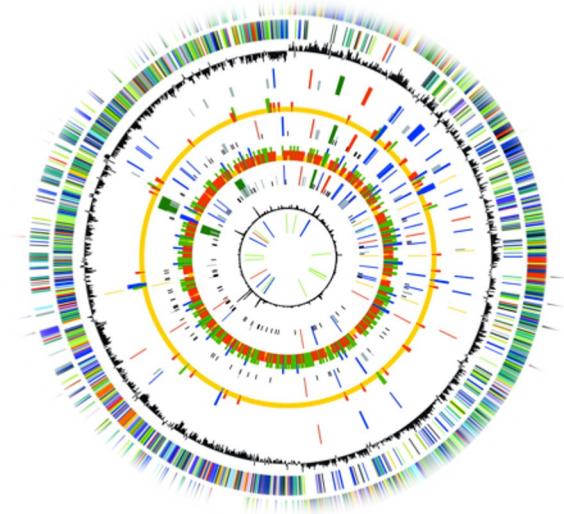


*Department of Food Science & Technology,  
NGS Workshop,  
Stellenbosch University  
South Africa*

Tuesday 23<sup>rd</sup> January, 2024

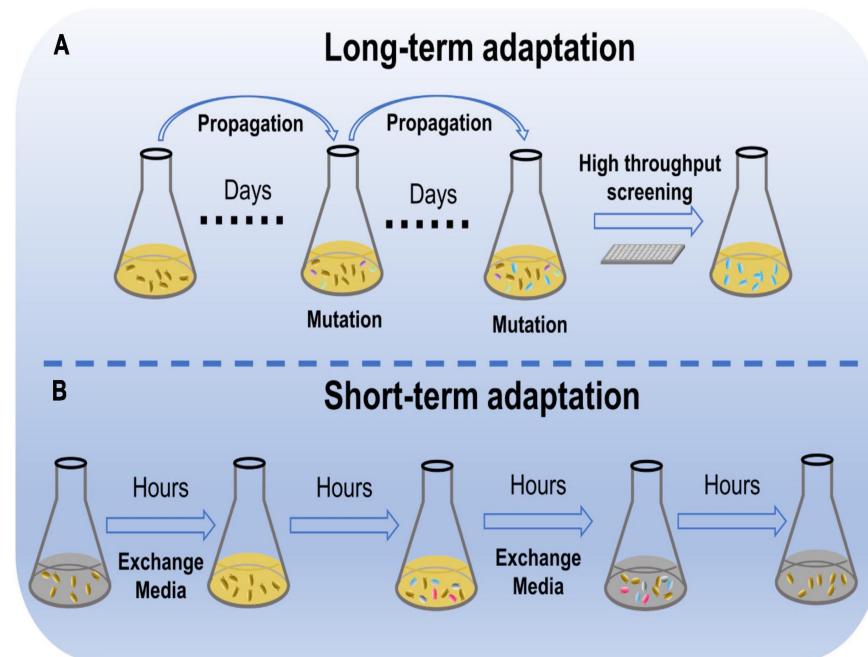


## Using genome sequencing to study food-borne bacterial adaptation-

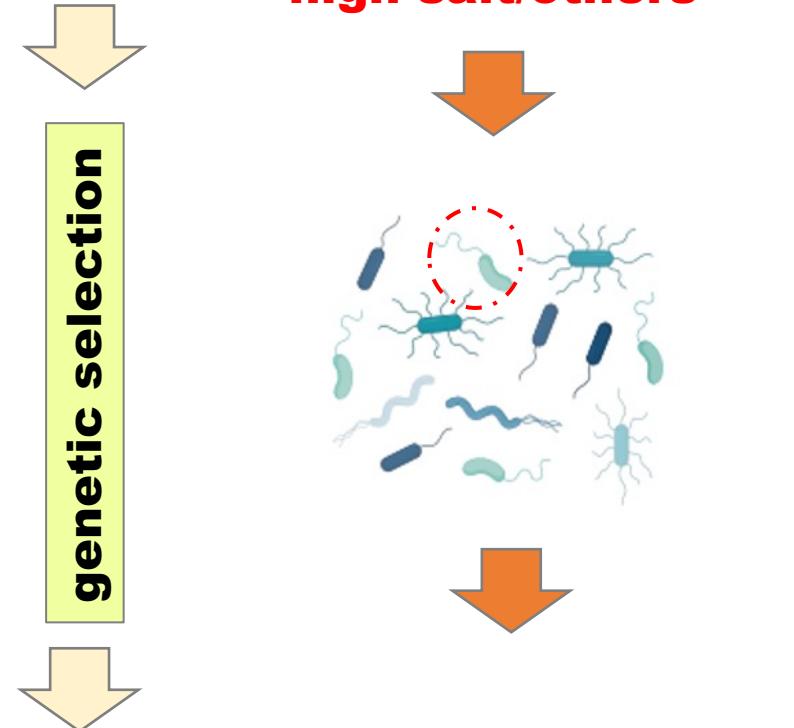
Professor Séamus Fanning,  
UCD-Centre for Food Safety,  
School of Public Health, Physiotherapy & Sports Science,  
University College Dublin, Ireland.



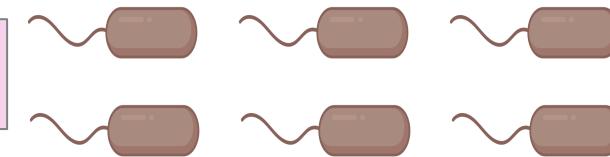
# Bacterial adaptation: *the fittest isolate wins!*



pH/low temperature/acid/sanitizer/  
high salt/others



Chromosomal mutations  
Acquire exogenous DNA



adapted bacterial isolates

Its all about **bacterial adaptation** in  
an **ecological niche** such as food-

**Genome sequencing to predict sanitizer tolerance–**  
*identifying genes functioning to enhance bacterial survival &  
applying machine learning (ML) to enhance this approach*



## Antimicrobial compounds

### Antibiotics

- Natural
- Semi-synthetic/Synthetic

$\beta$ -Lactams

Fluoroquinolones (FQ)

Phenolics

### Biocides

Antiseptics and Germicides  
Sterilizers (Sporicides)

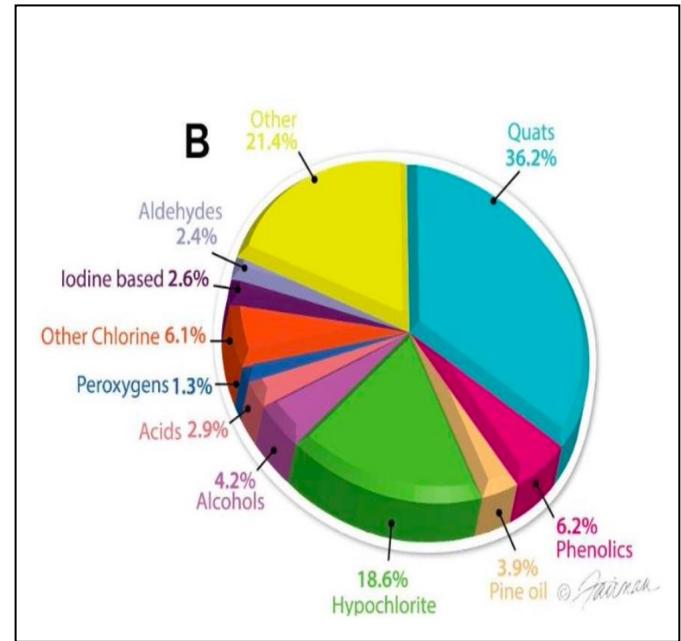
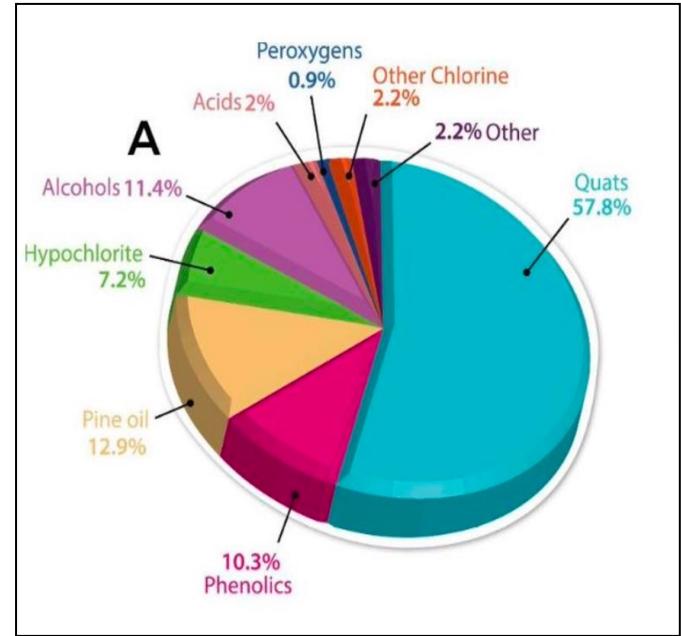
### *Disinfectants/Microbicides*

Sanitizers  
Food Preservatives  
Herbicides  
Pesticides  
Insecticides  
Fungicides

Selectively toxic

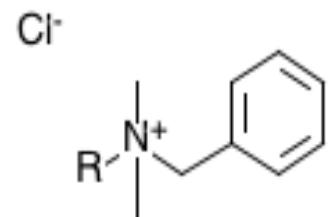


Non-selectively toxic

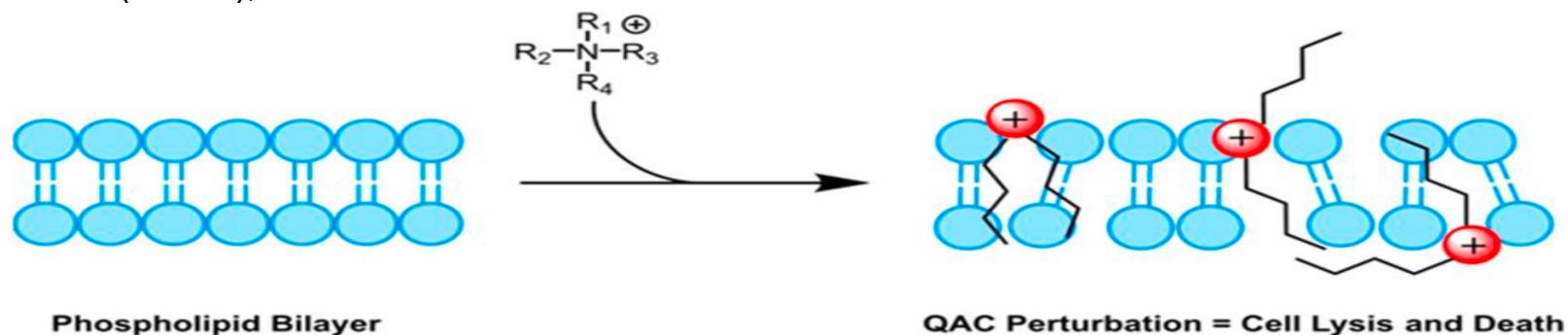


## Application of biocides in the food industry -

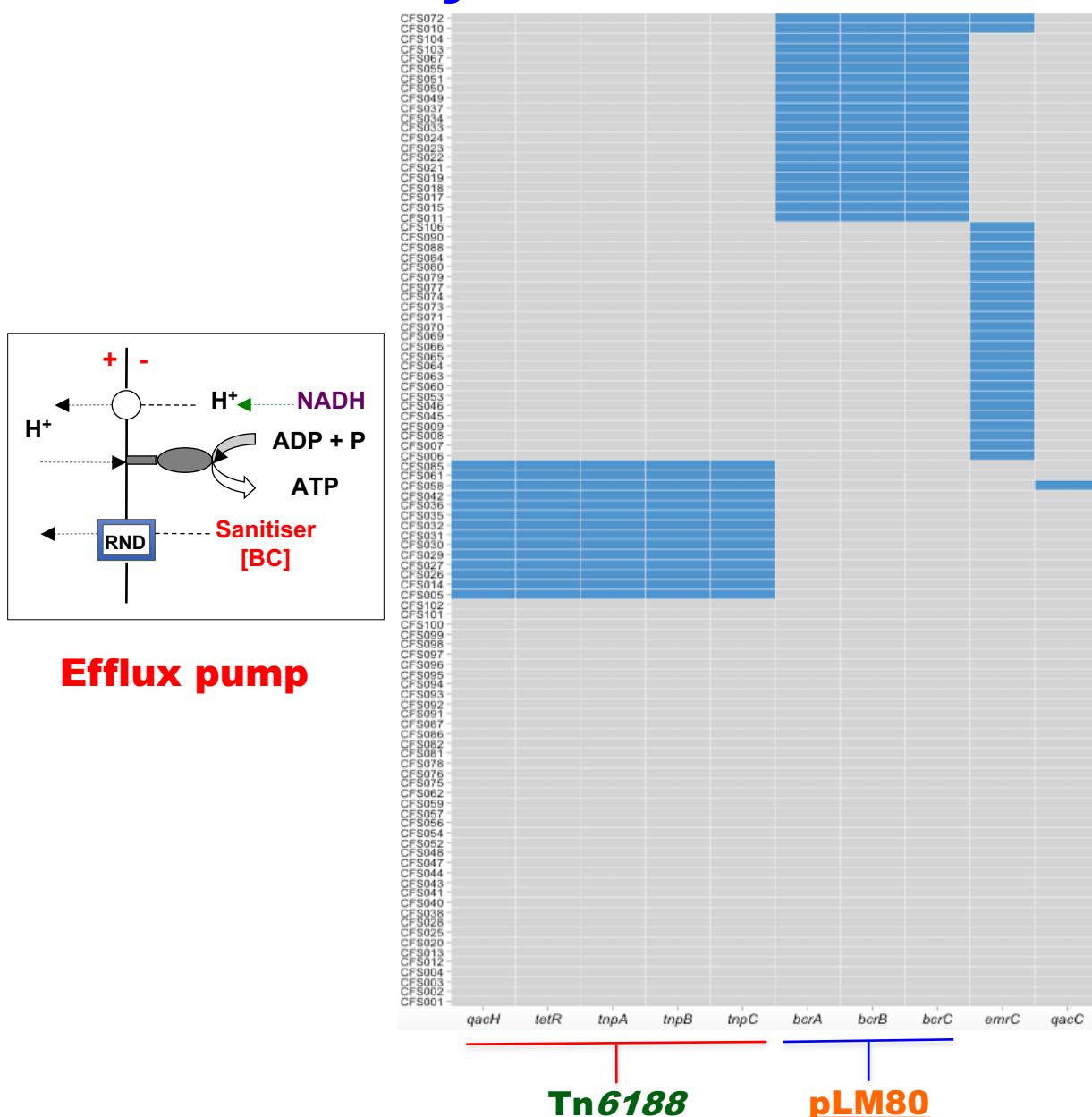
- **disinfection is an important hygienic measure** applied throughout the food industry to control microbiological contamination and reduce the risk of outbreaks;
- **quaternary ammonium compounds (QAC) have been widely used** for sanitizing production environments and benzalkonium chloride (BC), an important active ingredient is well-studied, exhibiting good anti-bacterial activity
- biocidal products contain one or more active substance(s), but may also contain other non-biocidal co-formulants which may aid in effectiveness or delivery of desired qualities (e.g. pH, viscosity, odour, colour, etc)
- all biocides must be registered as required by legislation
  - Biocidal Products Directive (BPD), EU
  - Federal Pesticides Law (FIFRA), USA



Benzalkonium chloride

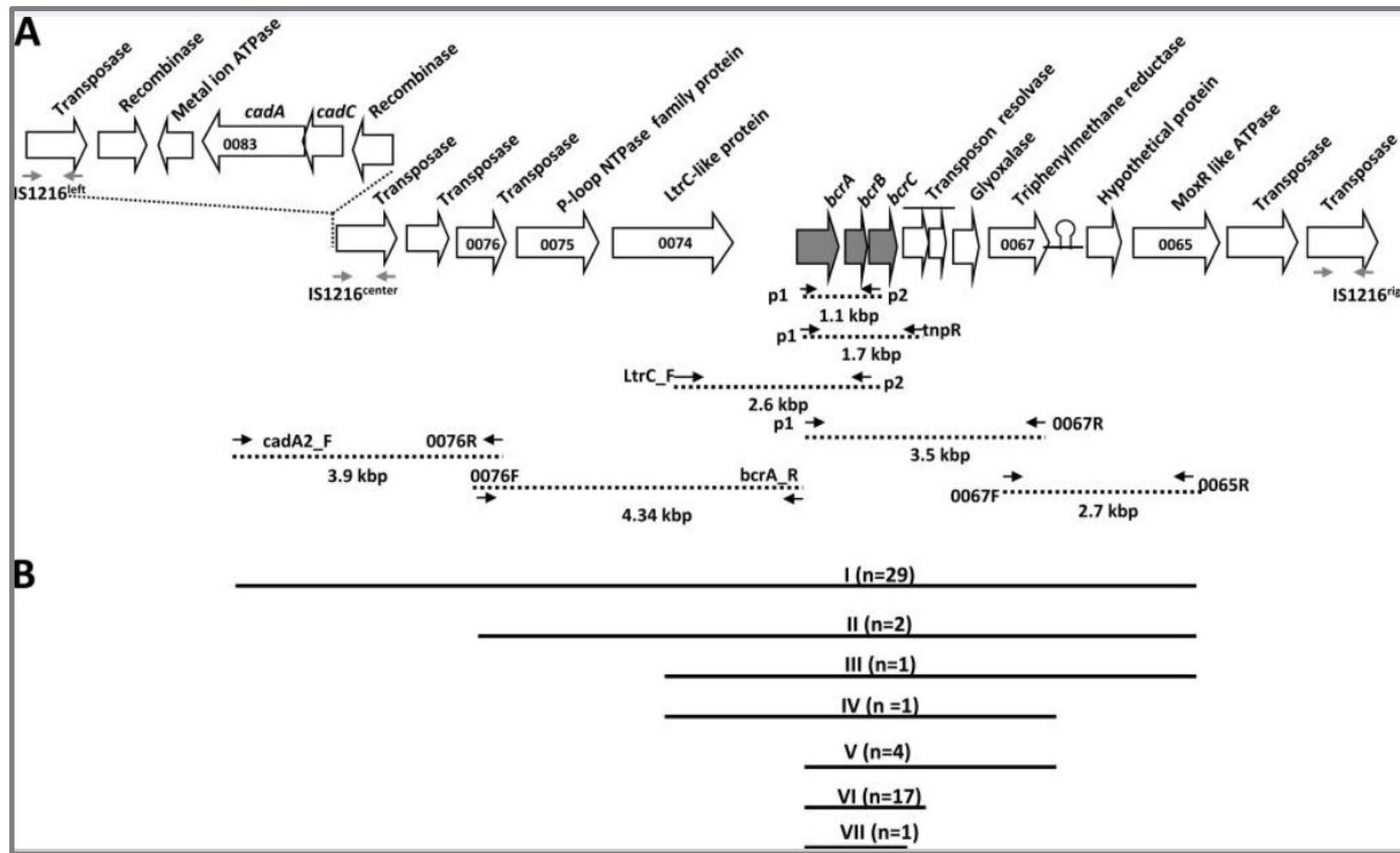


# Tolerance to benzalkonium chloride- a biocide of relevance to the food industry



- Bacterial isolates were tested for their susceptibility to **Benzalkonium Chloride** (BC) by broth microdilution and **34.65% of the isolates were found to exhibit BC tolerance**
  - **21.78% were positive for the *bcrABC* cassette mapped to plasmid pLM80**
  - **12.87% were positive for the complex transposon, Tn6188**
- **all SL121 isolates carried Tn6188**
- Similarly, persistent **SL121 isolates harboured a newly described Stress Survival Islet (SSI-2)**
  - SSI-2 carries homologues of genes found in *L. innocua*
  - these included *lin0464* and *lin0465*
  - **mutants of lin0465 were attenuated for survival when placed under oxidative and alkaline stress conditions**

# Plasmid pLM80 encoding a QAC resistance mechanism -



# Strategy for machine learning (ML) applied to a GWAS study-

## □ Feature Preparation-

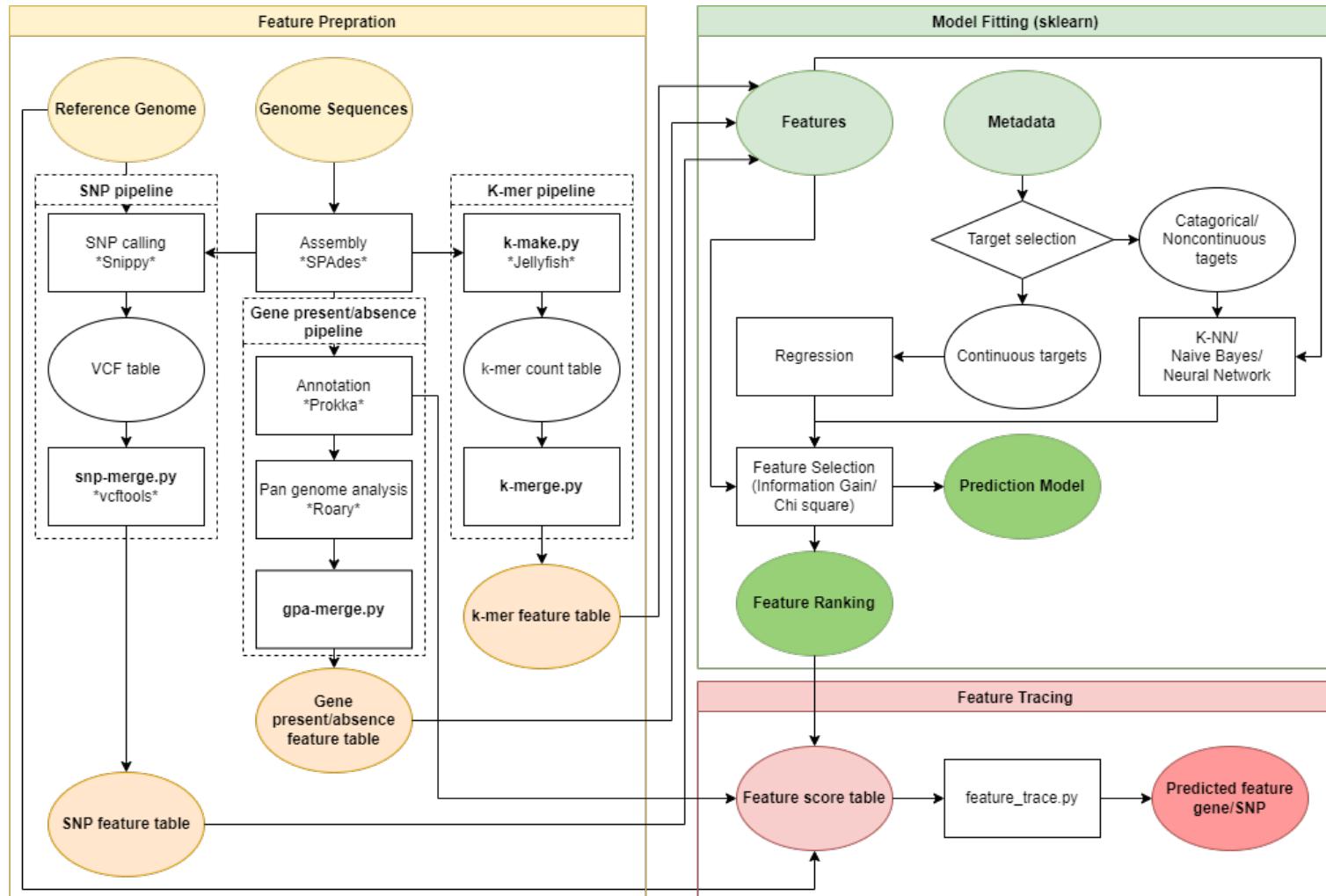
- generate an original *K*-mer/SNP/gene feature table from genome sequences
- merge features with identical patterns

## □ Model Fitting-

- for continuous numerical targets, use regression algorithms
- for categorical targets, us K-NN/Naïve Bayes/Neural Network algorithms
- feature selection to rank the best-related genome features

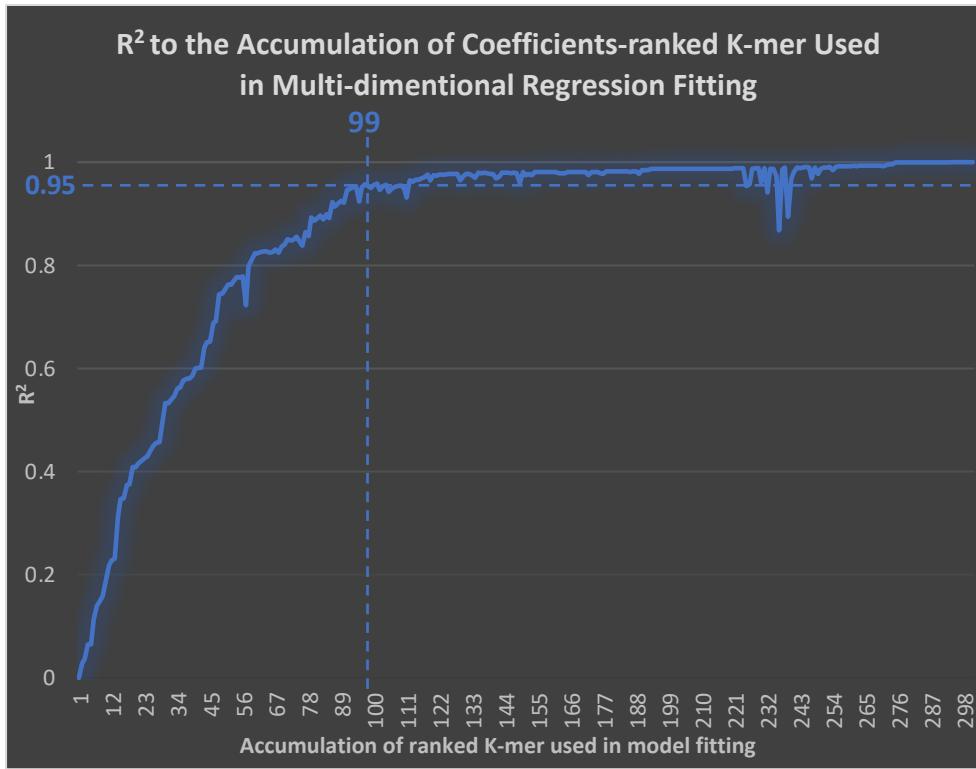
## □ Feature Tracing-

- trace the best-related *K*-mers/SNPs/genes to the original pan-genome
- **find the potential functional genes or SNPs to the targeted characteristic**



## Results of ML applied GWAS study (benzalkonium chloride)-

- 18,227 merged *K-mer* features in total
- top 99 *K-mer* features contribute to >0.95 accuracy in model prediction
- top 99 features traced to 30 genes



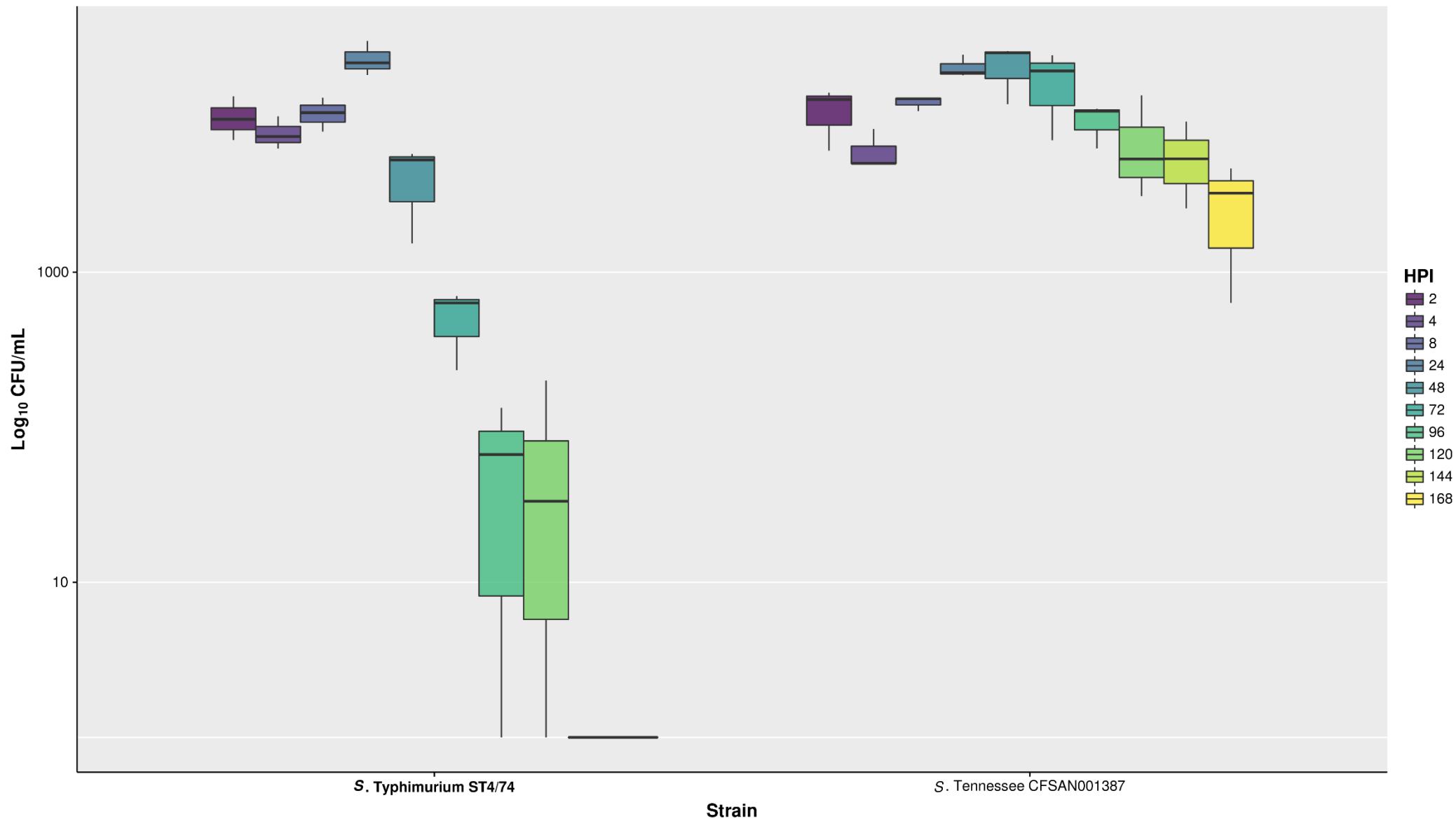
Gene Name	Description	k-mer count	Reference species
<i>eno</i>	phosphopyruvate hydratase	8	<i>L. monocytogenes</i>
<i>rhsA</i>	Rhs family protein	7	<i>L. monocytogenes</i>
-	DUF190 domain-containing protein (plasmid)	6	<i>L. monocytogenes</i>
-	phage tail protein	6	<i>L. monocytogenes</i>
-	YidB family protein	5	<i>E. coli</i>
<i>bolA</i>	transcriptional regulator BolA	5	<i>C. malonaticus</i>
-	BppU family phage baseplate upper protein	4	<i>L. monocytogenes</i>
<i>mscK</i>	mechanosensitive channel MscK	4	<i>E. coli</i>
<i>emrY</i>	multidrug efflux MFS transporter periplasmic adaptor subunit EmrK	4	<i>E. coli</i>
-	DUF2479 domain-containing protein	3	<i>L. monocytogenes</i>
-	plasmid recombination protein (partial)	3	<i>L. monocytogenes</i>
-	plasmid recombination protein (partial)	2	<i>L. monocytogenes</i>
-	plasmid recombination protein (partial)	2	<i>L. monocytogenes</i>
-	hypothetical protein	2	<i>L. monocytogenes</i>
<i>truB</i>	tRNA pseudouridine(55) synthase TruB	2	<i>L. monocytogenes</i>
-	6-phospho-beta-galactosidase	2	<i>L. monocytogenes</i>
-	cell surface protein	1	<i>L. innocua</i>
-	hypothetical protein	1	<i>L. innocua</i>
-	hypothetical protein	1	<i>L. innocua</i>
-	peptidoglycan-binding protein	1	<i>L. monocytogenes</i>
-	PTS sugar transporter subunit IIA	1	<i>L. monocytogenes</i>
-	DNA topology modulation protein	1	<i>L. monocytogenes</i>
-	LPXTG cell wall anchor domain-containing protein	1	<i>L. monocytogenes</i>
-	MucBP domain-containing protein	1	<i>L. monocytogenes</i>
-	glycoside hydrolase family 1 protein	1	<i>L. monocytogenes</i>
-	gfo/lldh/MocA family oxidoreductase	1	<i>L. monocytogenes</i>
-	cation diffusion facilitator family transporter	1	<i>L. monocytogenes</i>
-	aryl-phospho-beta-D-glucosidase	1	<i>L. monocytogenes</i>
<i>yham</i>	3'-5' exoribonuclease yham	1	<i>L. monocytogenes</i>

**Transposon-mediated mutagenesis: gene discovery to aid risk assessment –**  
***genes functioning in infection in Salmonella &***  
***adaptation of Listeria to Lyoner sausage matrices***

## US-FDA top 10 foodborne *Salmonella* species -

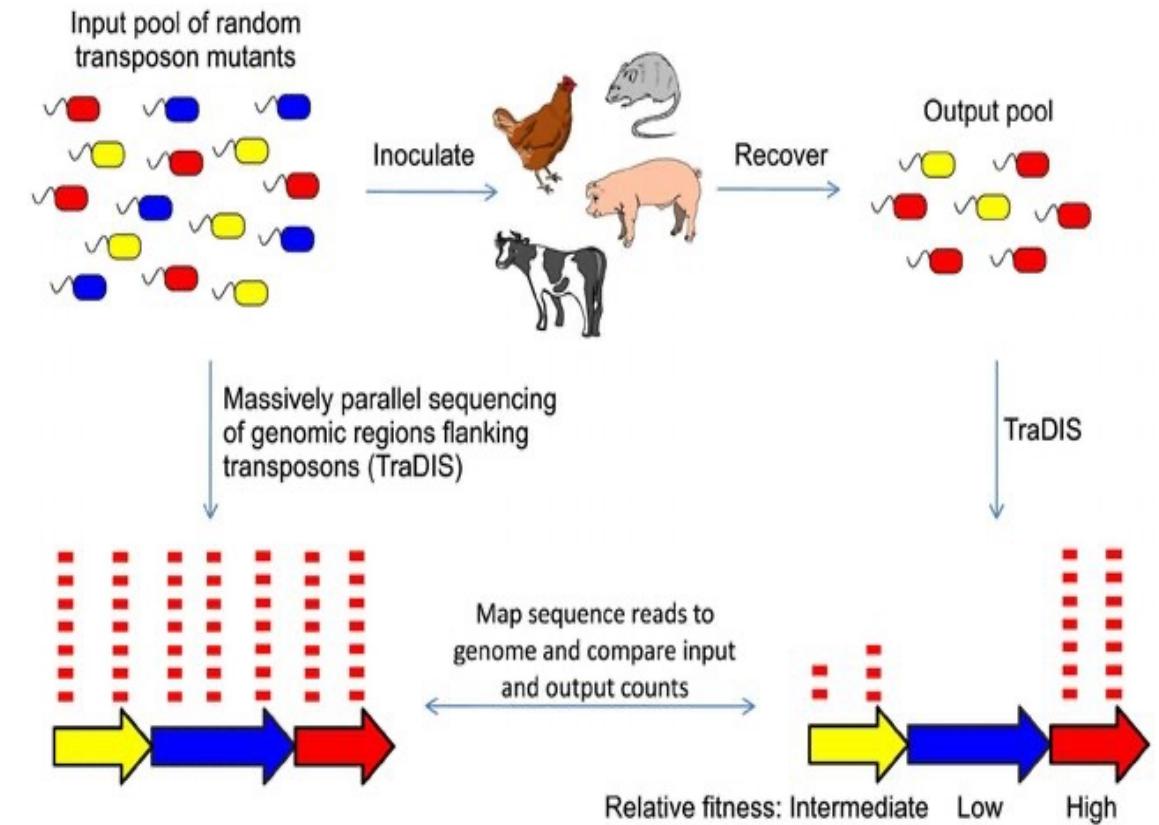
Serovar/Strain	Outbreak	Isolation date	Origin
S. Anatum CFSAN003959	Papaya	2012	Mexico
S. Bareilly CFSAN001111	Tuna	2012	India
S. Cubana CFSAN002050	Alfalfa sprout	2012	USA - Arizona
S. Heidelberg CFSAN002063	Chicken	2012	USA - Washington
S. Javiana CFSAN000905	Green onion	2009	Mexico
S. Montevideo CFSAN000264	Black pepper	2012	USA - Rhode Island
S. Newport CFSAN003345	Eastern shore sampling	2011	USA - Maryland
S. Saintpaul CFSAN004090	Jalapeño/Serranno Pepper	2008	Mexico
<b>S. Tennessee CFSAN001387</b>	<b>Peanut butter</b>	<b>2007</b>	<b>USA - Georgia</b>
S. Weltevreden CFSAN001415	Prison tuna	2010	USA - Virginia
<b>S. Typhimurium 14028S</b>			<b>Reference Strains</b>
<b>S. Typhimurium ST4/74</b>			

## *S. Tennessee CFSAN001387* persists in human (THP-1) macrophages -

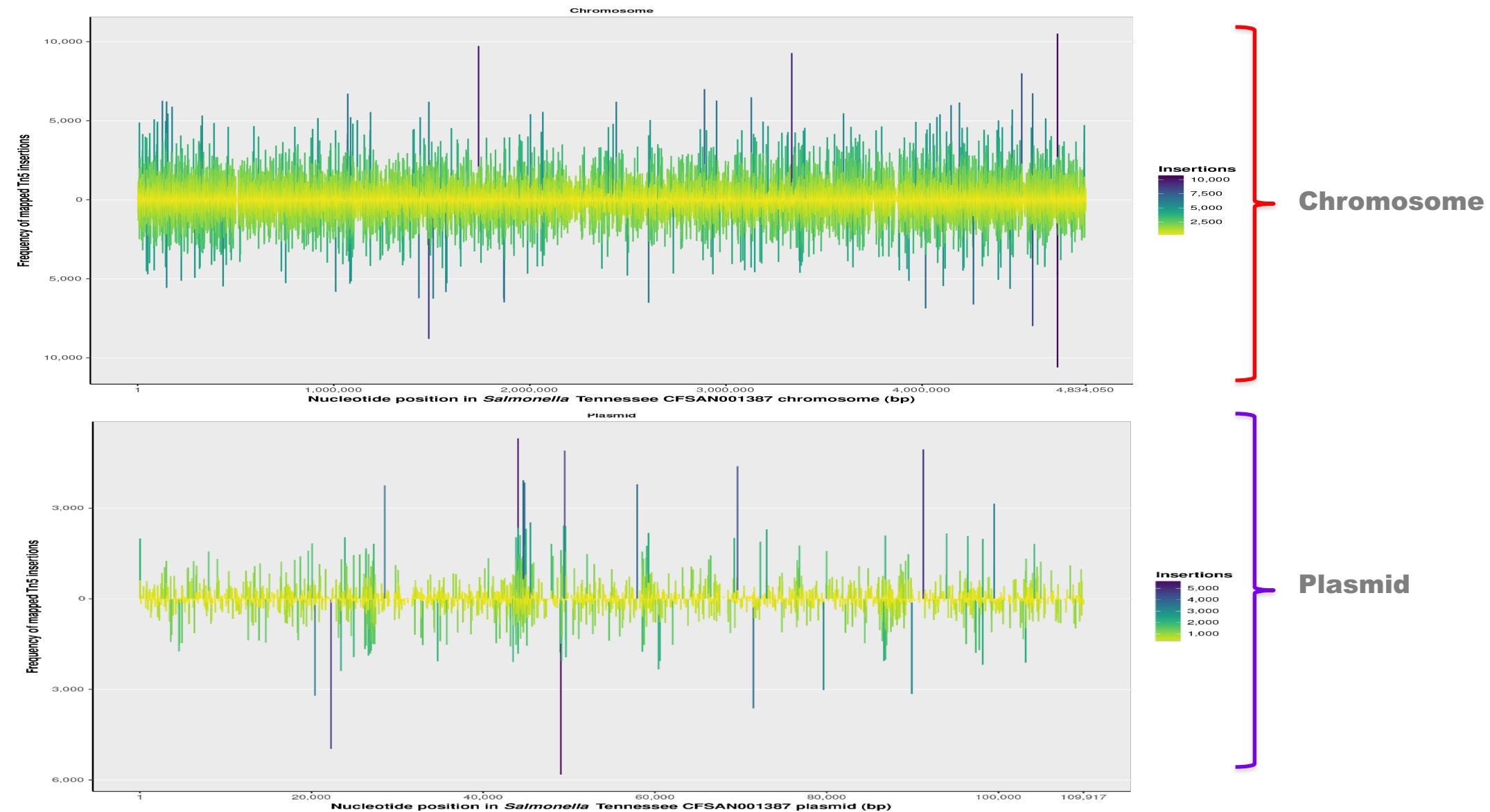


## TraDIS (Transposon Direction Insertion Sequencing)

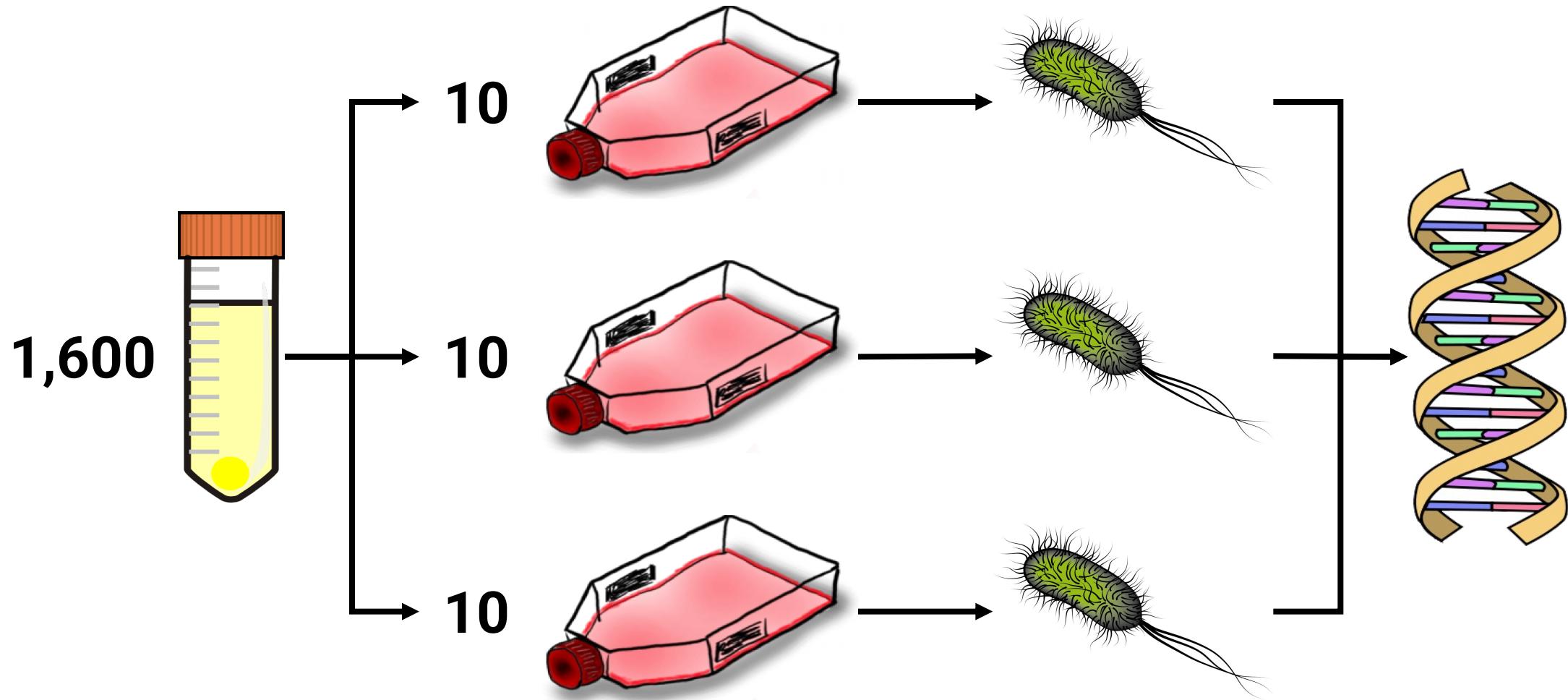
- Functional analysis of every gene in the genome simultaneously
- Random transposon-induced mutagenesis
- Enrichment of transposon-chromosome junctions
- High-throughput dark-cycle sequencing
- If a transposon inserts into an essential gene,  
***the gene is silenced and that mutant cell will not grow***
- Gene essentiality determined by negative inference



# Density of insertion sites mapped to *Salmonella Tennessee* genome-



# THP-1 macrophage screen of transposon Tn5 mutant pool -



# Temporally track emergence of *essentiality* during infection -

Viable mutants recovered at 2, 8, 24, 72 and 120 HPI

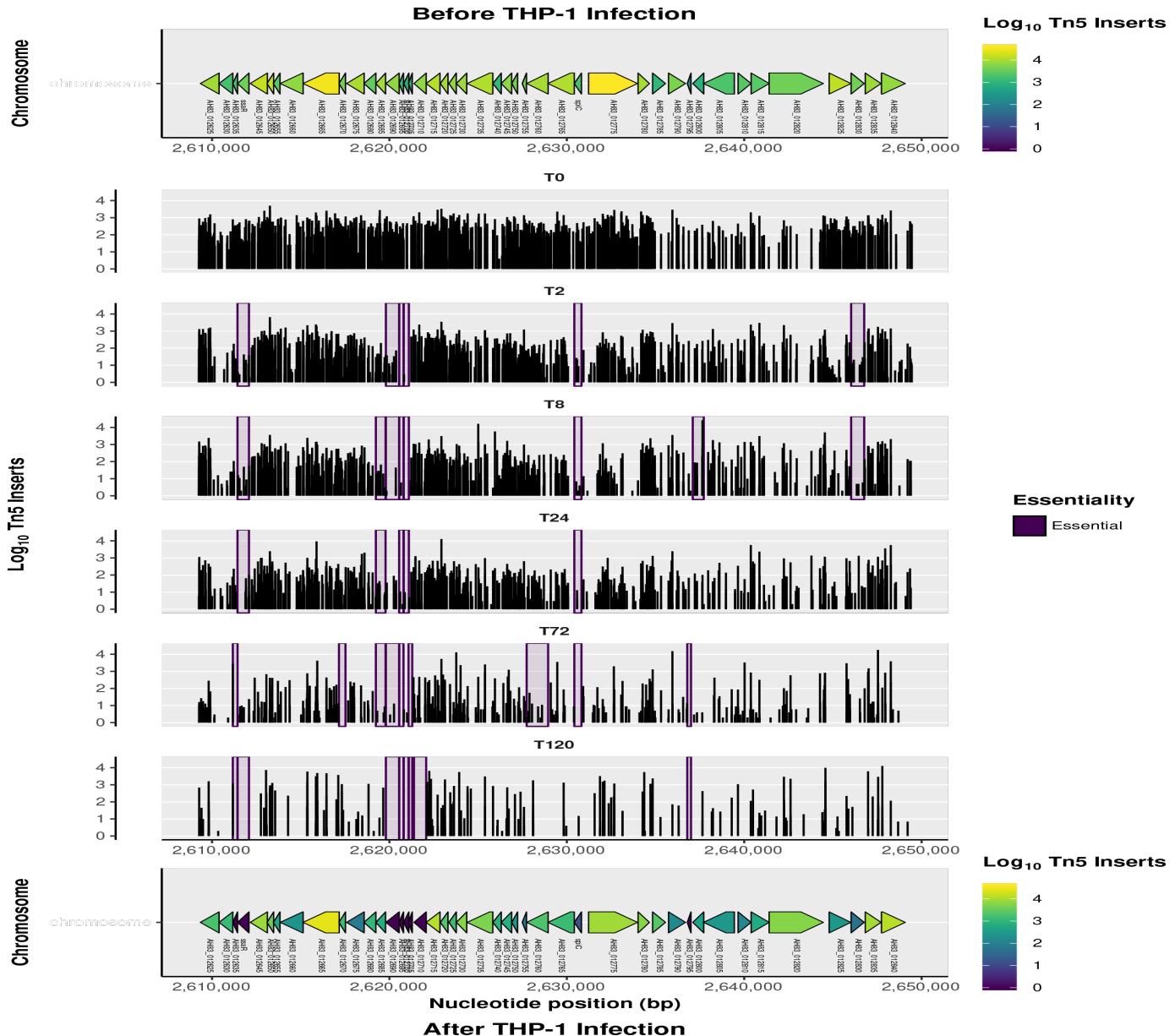
Downstream infection samples compared back to original input pool of mutants

290 genes (6.1%) determined to be essential in *S. Tennessee CFSAN001387*

No viable mutants recovered from input pool

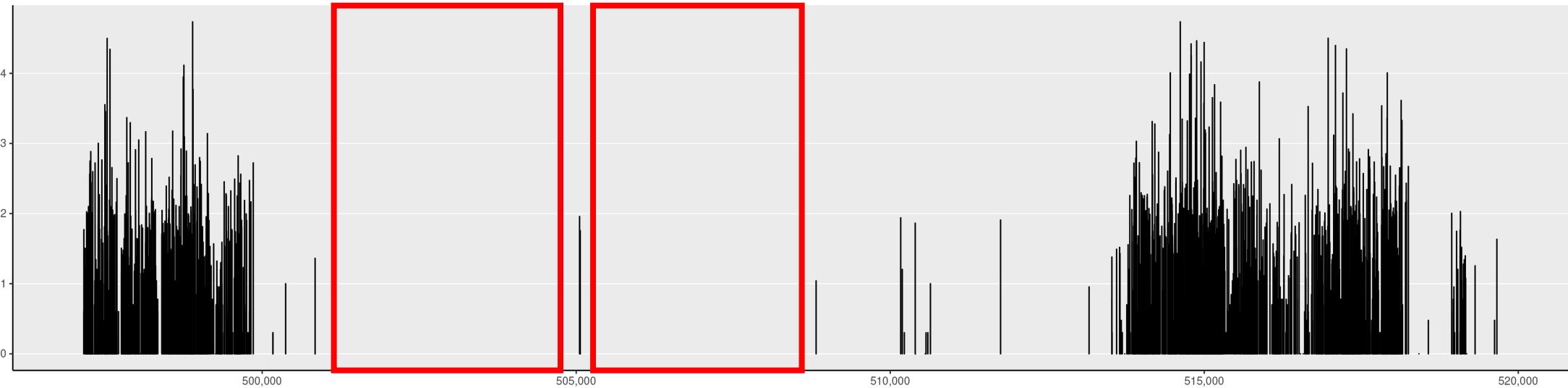
Similar numbers observed in other *Salmonella* serovars

160 (2 HPI), 174 (8 HPI), 180 (24 HPI), 377 (>72 HPI)

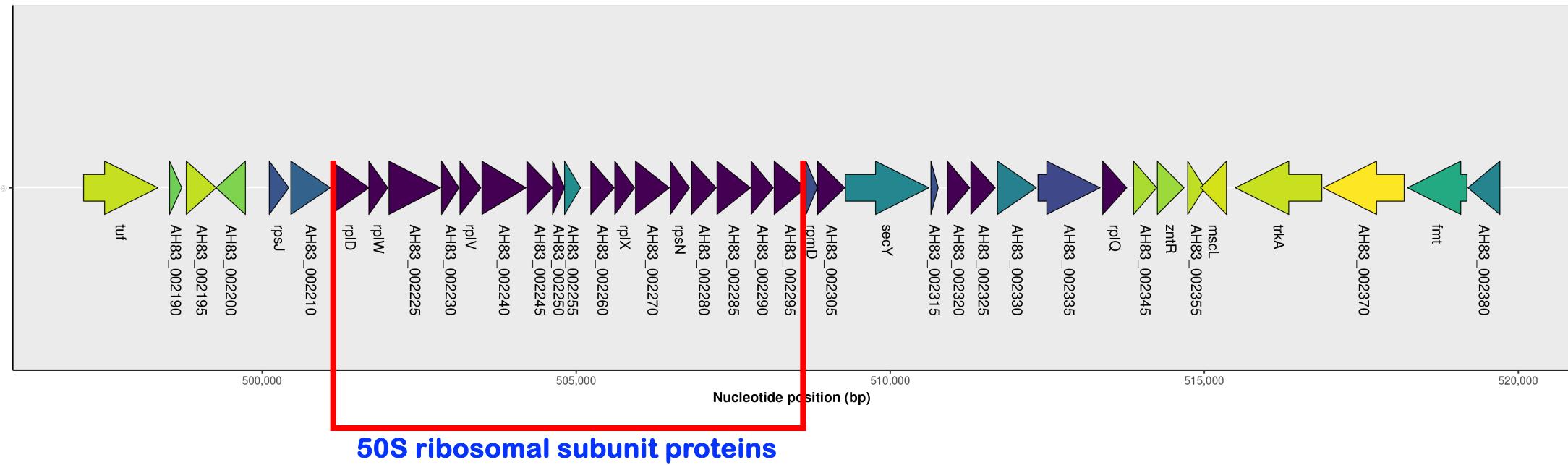


## Landmark genes essential for survival confirmed in input pool -

$\log_{10}$  Tn5 inserts

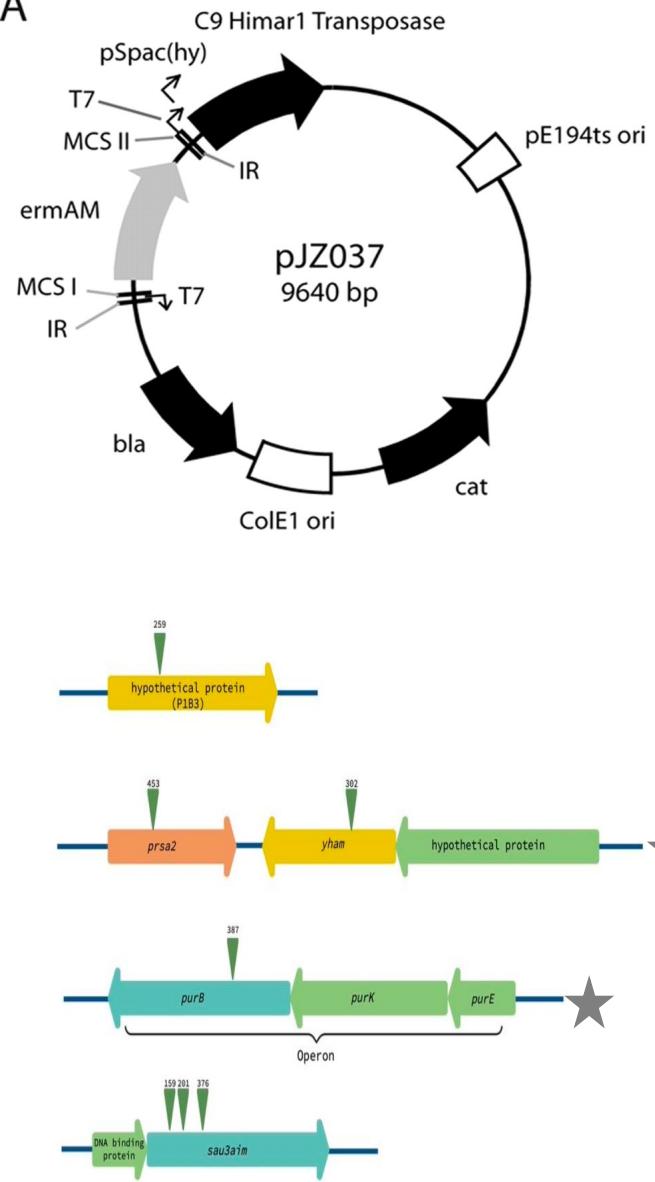


Chromosome

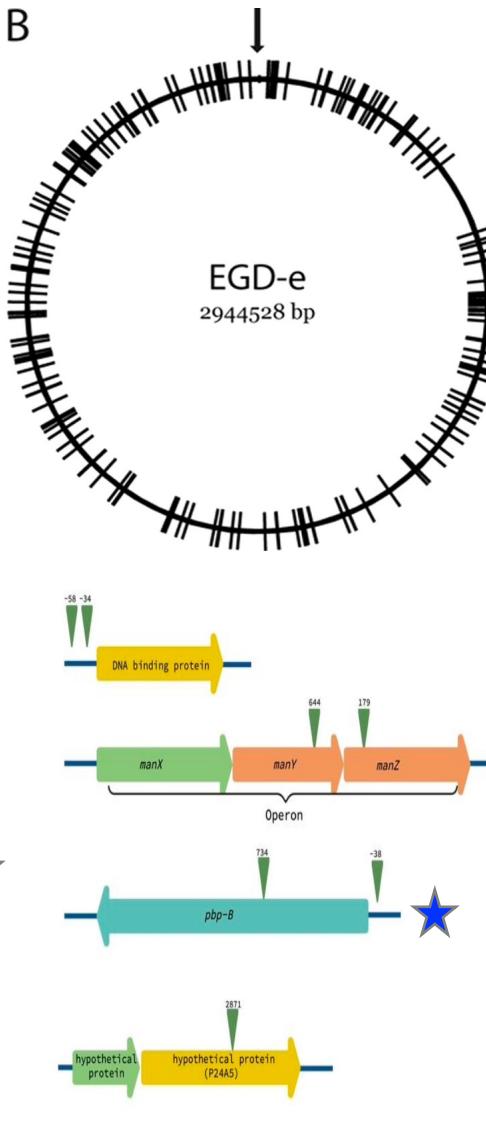


# Identification of genes essential for survival of *Listeria monocytogenes* in Lyoner sausage -

A



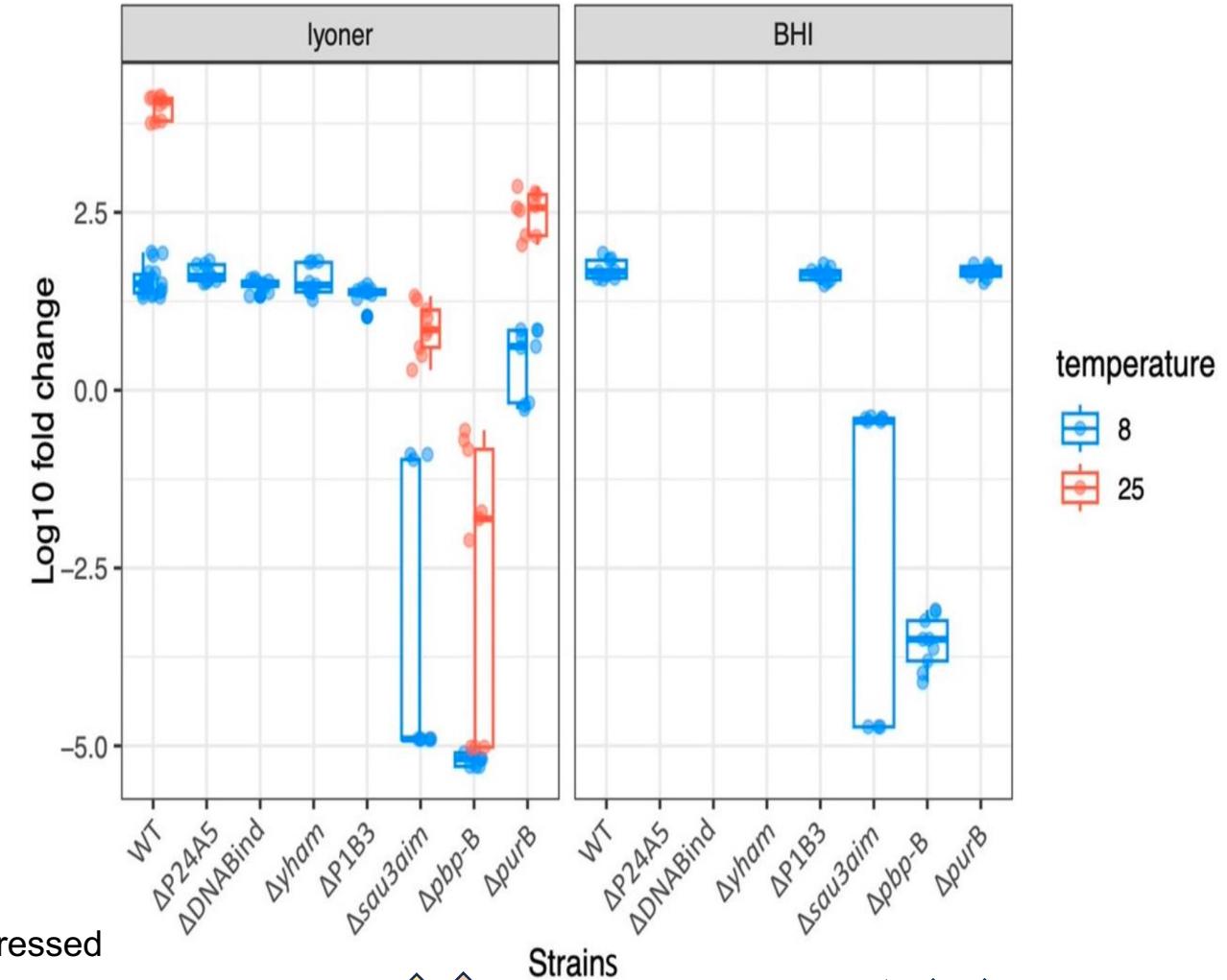
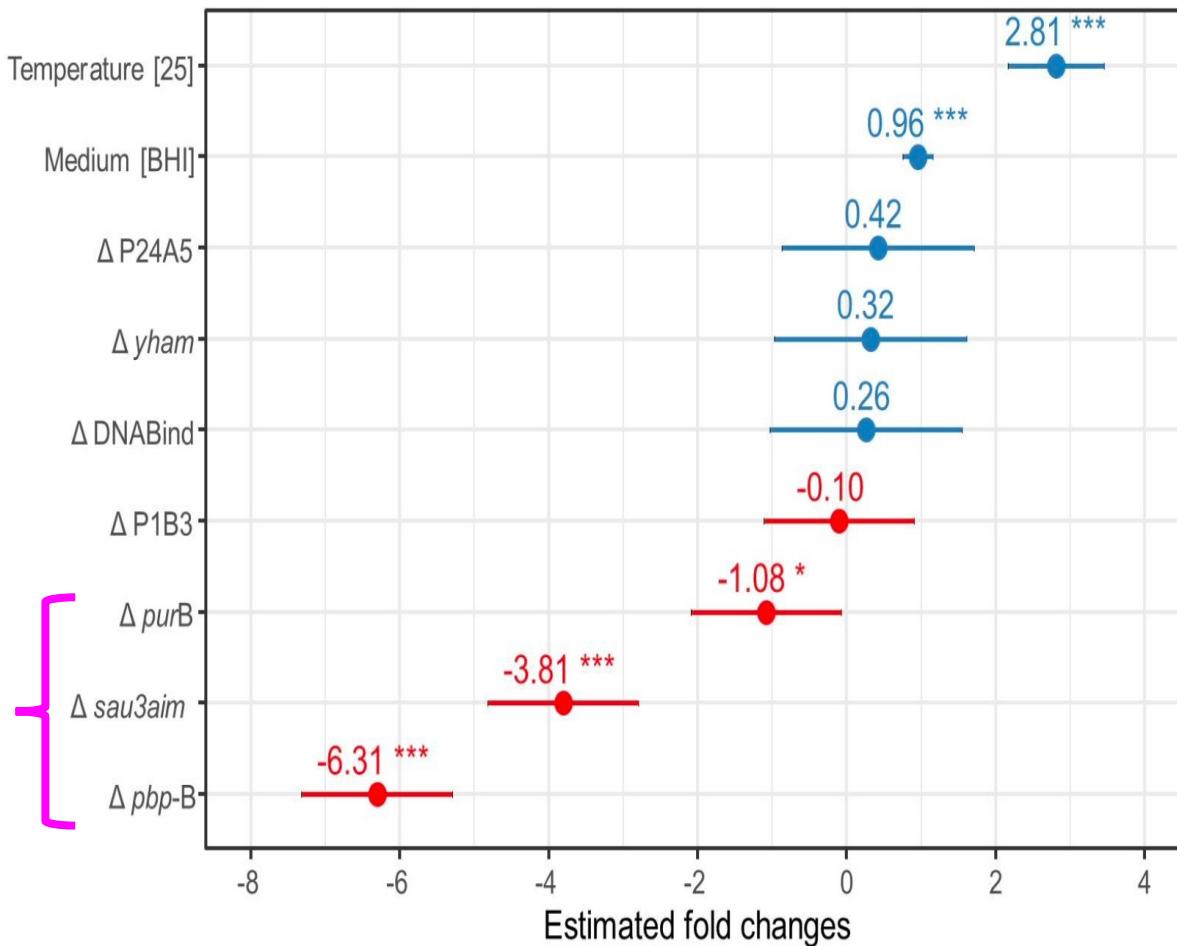
B



Mutant Name	Locus Tag	Interval	Disrupted Gene	Protein Name	Growth Difference Compared to WT (log reduction)	Function
P28C2						
P28C3	BN389_03290	346,524...347,923	sau3AIM	Cytosine Specific DNA Modification Methyltransferase	No Survival	Methylates C on GATC sequence and protect the host DNA from cleavage
P28C10						
P21G11	BN389_04850	493,081...495,132	ppb-B	Penicillin Binding Protein	No Survival	Has a role in the cell wall assembly, cell division and cell wall reshaping
P30C4						
P29H8	BN389_01150 BN389_01160	115,272...116,078 116,100...117,011	manY-manZ	Phosphoenolpyruvate-dependent sugar phosphotransferase system	0,82±0,2	A major carbohydrate active transport system, involves in mannose transport
P1B3	BN389_7980	796,753...797,505	Hypothetical Protein 1	-	0,76±0,07	Similar to transcriptional regulators of the GnTr family in <i>L. monocytogenes</i>
P24A5	BN389_01040	99,035...104,914	Hypothetical Protein 2	-	0,72±0,16	Similar to HisZ, a regulatory subunit of the ATP phosphoribosyltransferase
P18F2	BN389_27880	2,850,688...2,851,515	DNA Binding protein	-	1,29±0,40	Similar to XRE family transcriptional regulator
P16F10						
P16E1	BN389_22520	2,301,560...2,302,441	prsA2	Post translocation chaperone	2,18±0,61	Promotes the activity and stability of 2 virulence factors in <i>L. monocytogenes</i>
P27B9	BN389_18000	1,826,027...1,824,717	purB	Adenylosuccinate lyase	1,11±0,19	Takes part in the purine biosynthesis pathway, affect the colonization ability of <i>L. monocytogenes</i> in gastrointestinal tract
P16E1	BN389_22530	2,303,471...2,302,482	yham	-	2,18±0,61	3' to 5' exonuclease activity



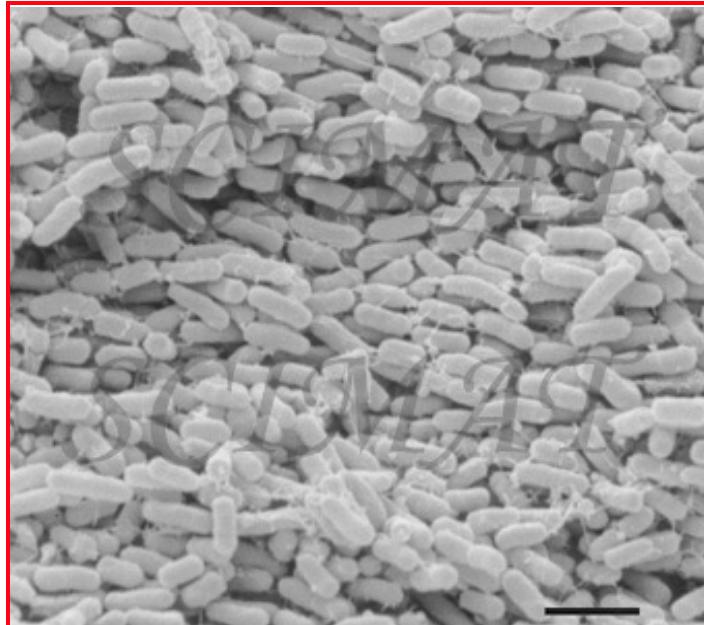
## Confirming phenotypes observed using clean in-frame deletion mutants -



- three of the mutants exhibited changes in growth parameters when stressed
- linear mixed effects models showed log<sub>10</sub> fold-changes in cell numbers, when grown in Lyoner at 8- and 25-degrees C;
- Two of these three mutants demonstrated a temperature-dependent growth inhibition in BHI compared to the wild-type and another showed a matrix effect only

**Using RNA-seq to study *Cronobacter* adaptation  
to low-moisture conditions-**

## ***Cronobacter* species are bacterial hazards of importance to neonates -**

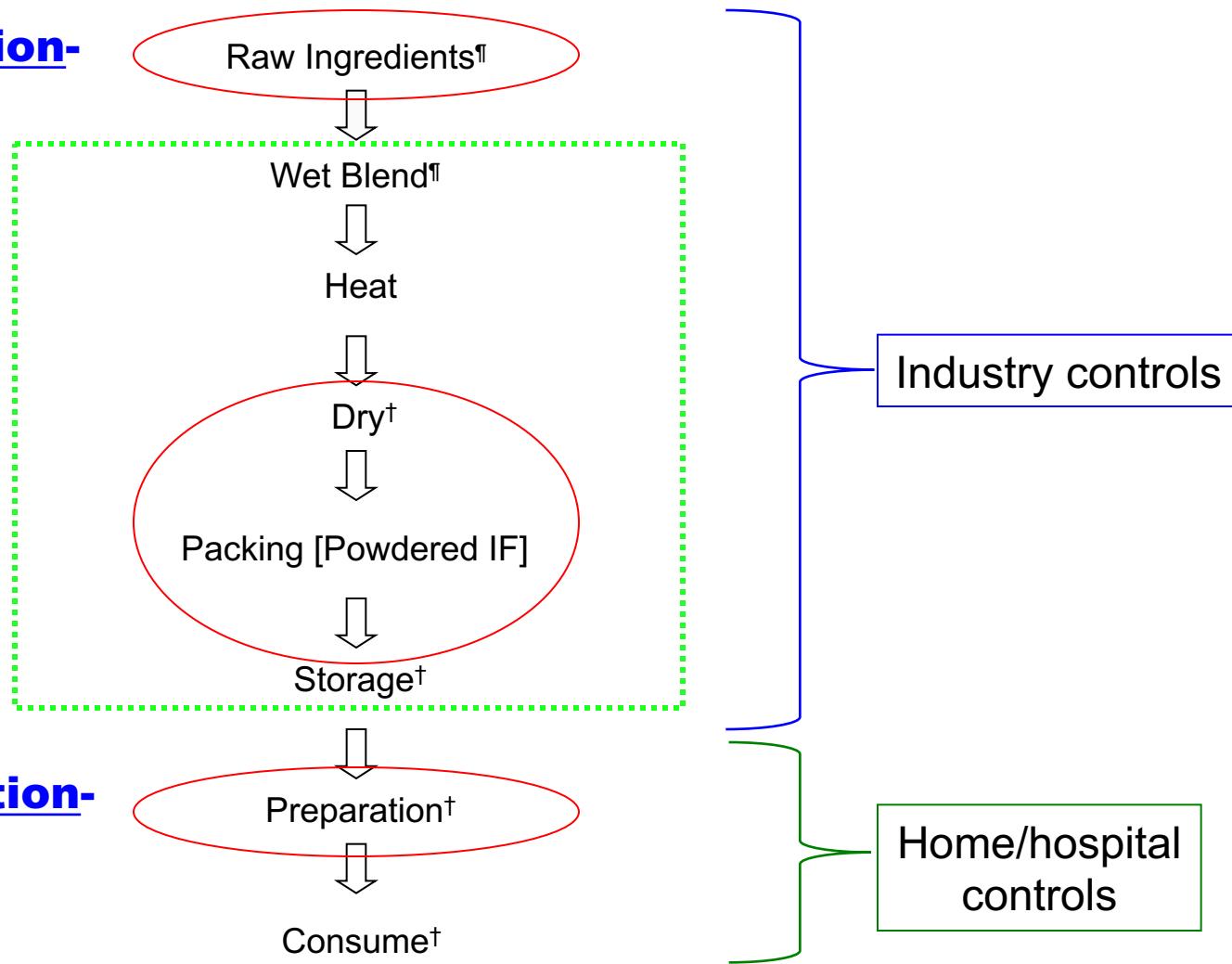


### **General characteristics**

- member of the *Enterobacteriaceae* family
- Gram-negative, motile rods
- facultatively anaerobic
- designated as a species in 1980
- taxonomy revised and a new genus recognised
- grows readily on laboratory media
- **desiccation resistant**
- rare opportunistic pathogen & causes nosocomial infections

## Intrinsic contamination-

- (dry) heat
- low-moisture
- limited nutrients
- sanitizers
- microbiome



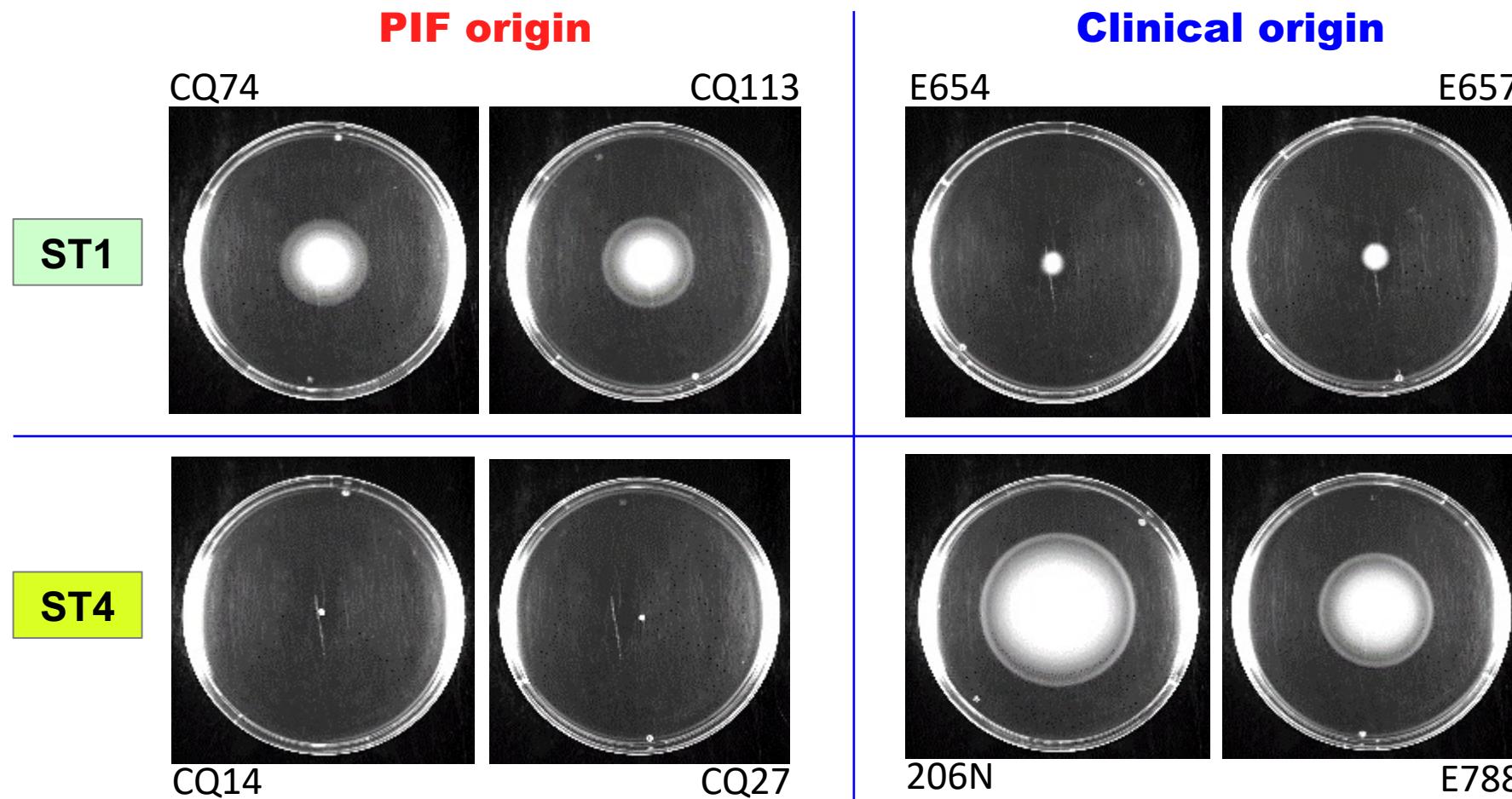
## Extrinsic contamination-

**Routes of contamination of powdered infant formula (PIF) -**

## Bacterial adaptation in low-moisture production environments-

- **Low-moisture foods are NOT sterile and outbreaks of food-borne diseases** associated with the consumption of low- $a_w$  foods and those formulated in part with low- $a_w$  ingredients have increased in frequency
- **Little is known about how bacteria behave** in low- $a_w$  food and dry food processing environments
- Conventional **hygiene protocols** may present a challenge to effective cleaning
- **Manufacturing practices** used for the production of low- $a_w$  foods must be designed to eliminate pathogens
- **Pathogens of concern include mainly *Cronobacter* species and *Salmonella* species that present food safety challenges to low- $a_w$  foods and their production environments**

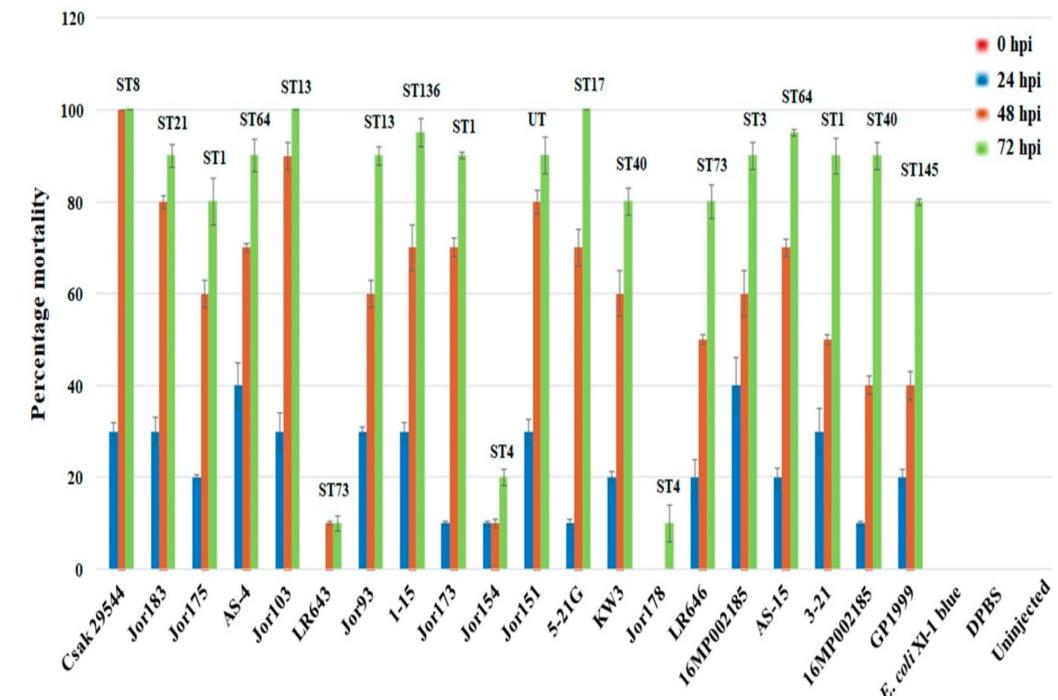
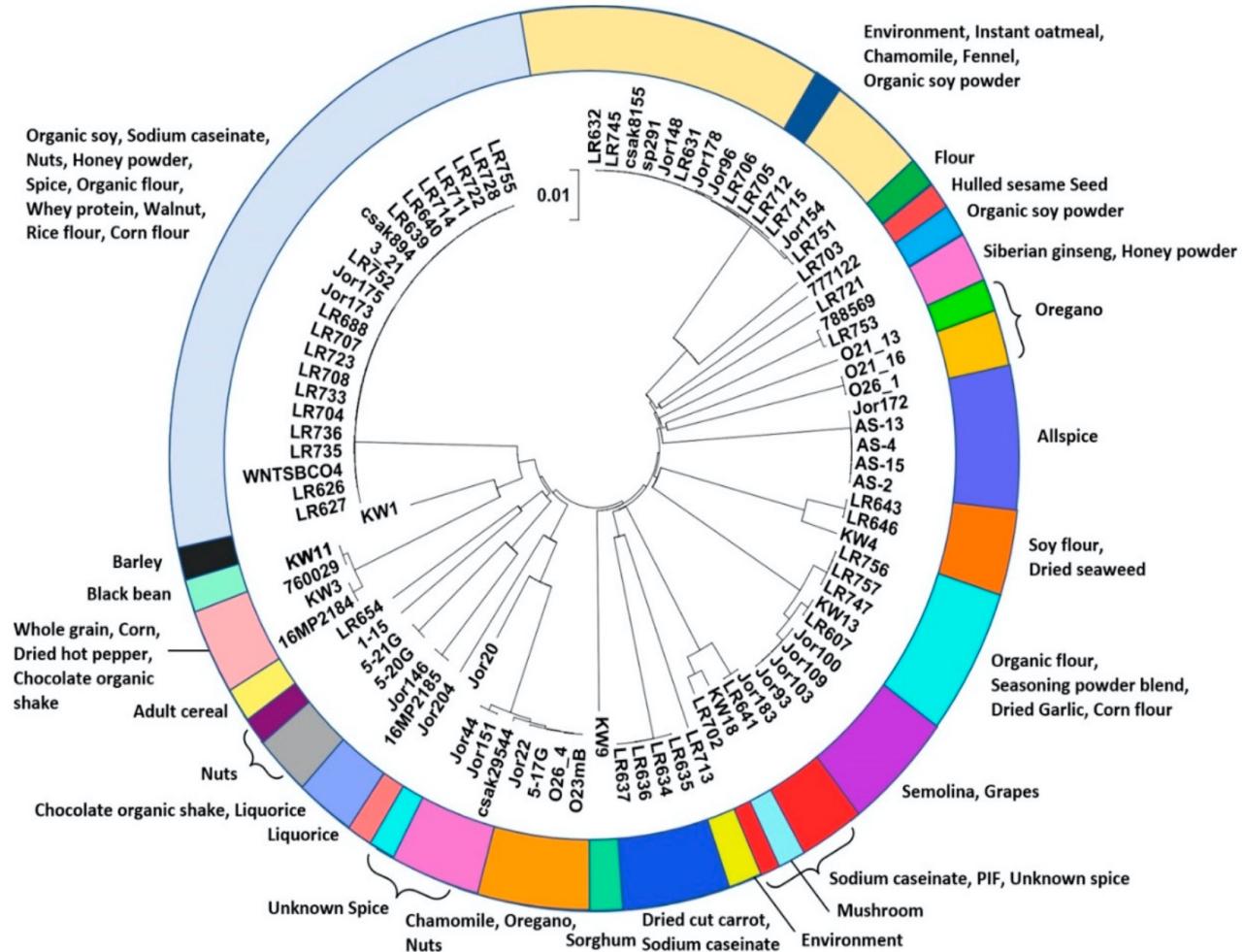
## *C. sakazakii* environmental adaptation based on swim motility -



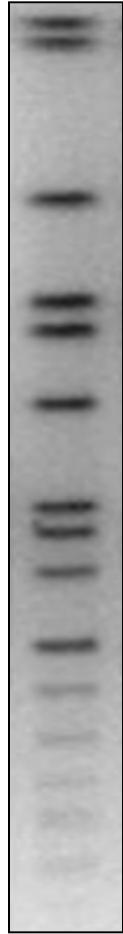
**Environment is selecting for adaptation**

### Sequence Type

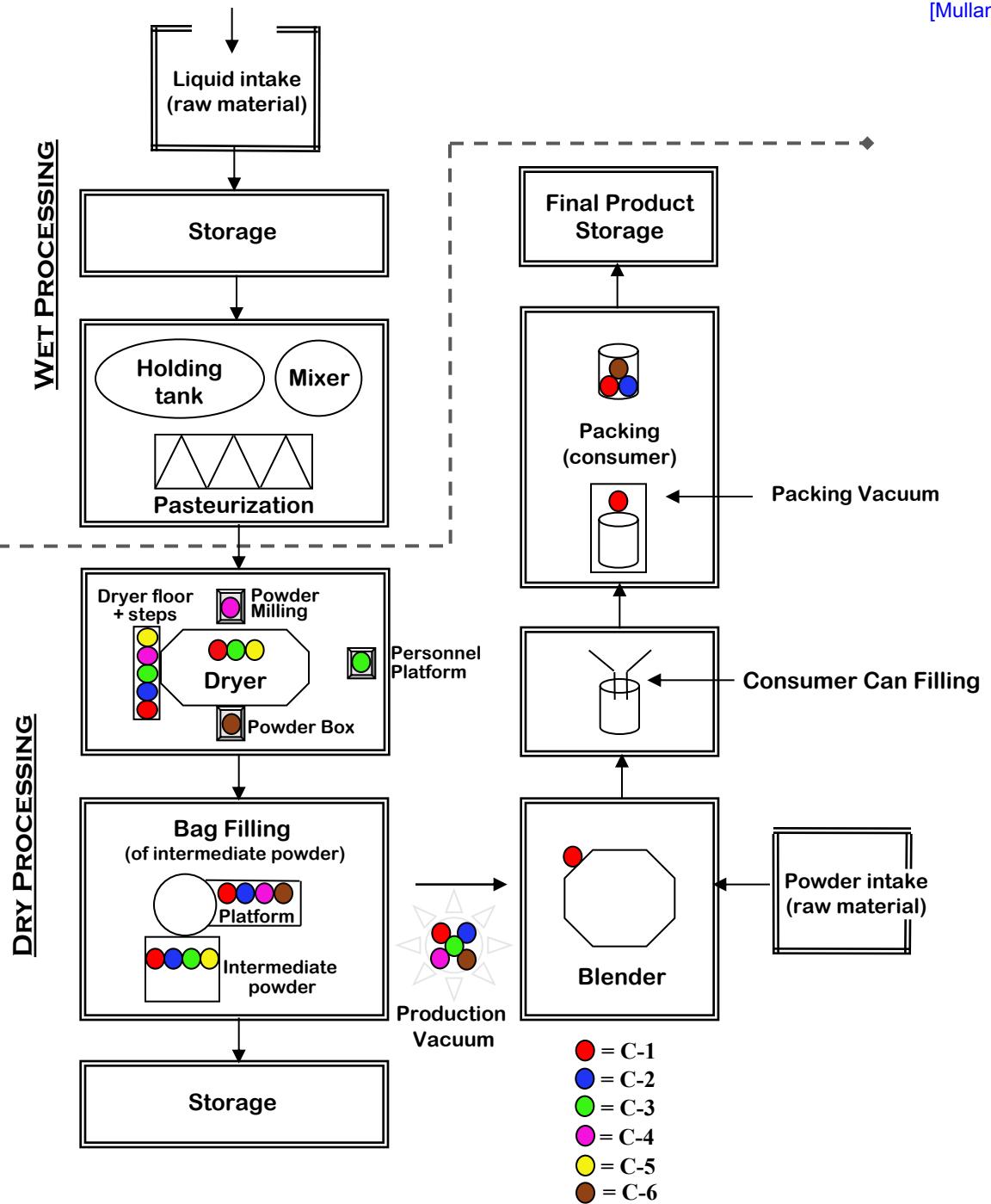
- 1, CC1
- 4, CC4
- 6 alleles
- 22
- 198, CC52
- 12
- 31, CC31
- 219, CC155
- 99, CC99
- 64, CC64
- 73, CC73
- 13, CC13
- 643
- 21, CC21
- 156, CC21
- 83, CC83
- 148, CC16
- 143, CC143
- 226, CC8
- 8, CC8
- 223
- 3, CC3
- 17, CC17
- 136
- 23, CC23
- 40, CC40
- 642
- 93

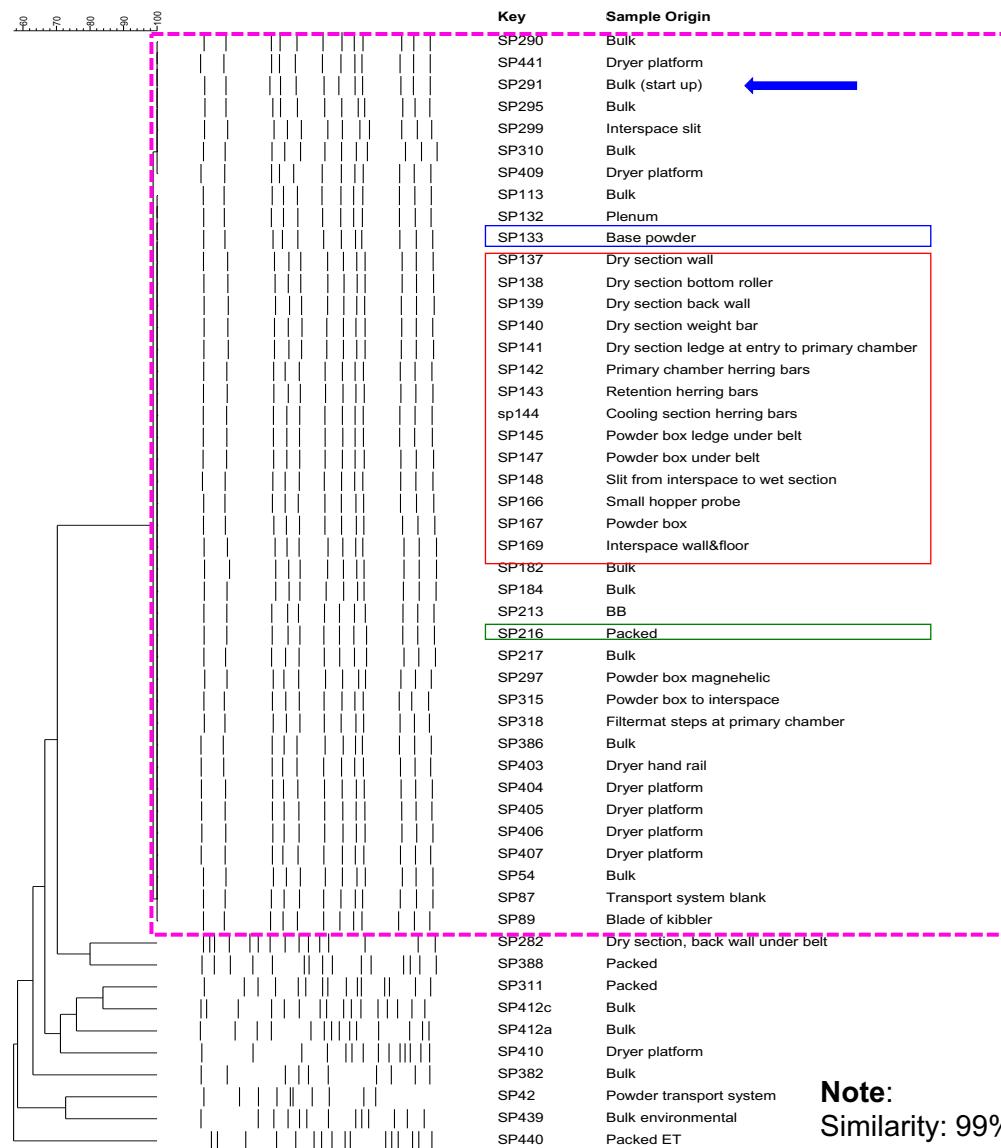


# Pulsed-field gel electrophoresis (PFGE)



●



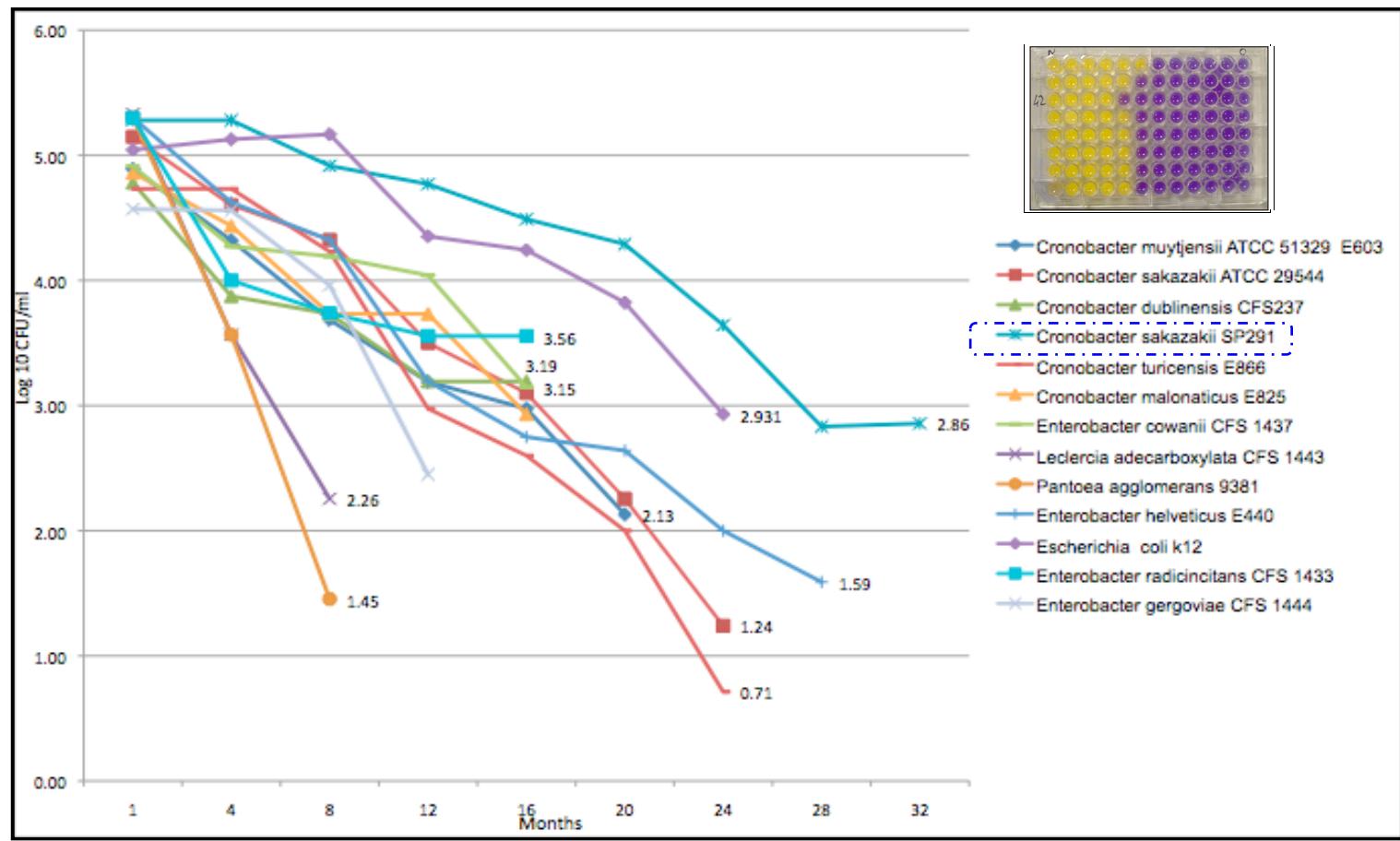


## A uniquely adapted *Cronobacter sakazakii* isolate detected in a PIF production environment using PFGE sub-typing

How does exposure of *Cronobacter*  
to dry environments  
confer an adaptation phenotype?

Note:  
 Similarity: 99%;  
 Tolerance: 1.5%;  
 Optimization: 1.5%

## Tolerance to desiccation with time -



How does *Cronobacter* persist in low-moisture environments?



## Cronobacter

CDC > Cronobacter Homepage



[Cronobacter Homepage](#)

Frequently Asked Questions

Signs and Symptoms

Transmission

People at Risk



Testing and Treatment

Prevention and Control

**Powdered Infant Formula Investigation**

Resources

# Cronobacter and Powdered Infant Formula Investigation

[Español \(Spanish\)](#)

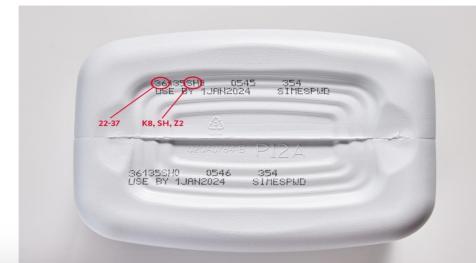
Updated March 25, 2022

On February 17, 2022, and February 28, 2022, Abbott Nutrition [recalled powdered infant formula](#) produced at its manufacturing facility in Sturgis, Michigan, because of possible *Cronobacter* contamination.

Parents and caregivers of newborns should not feed their baby recalled Similac, Alimentum, or EleCare powdered infant formulas.

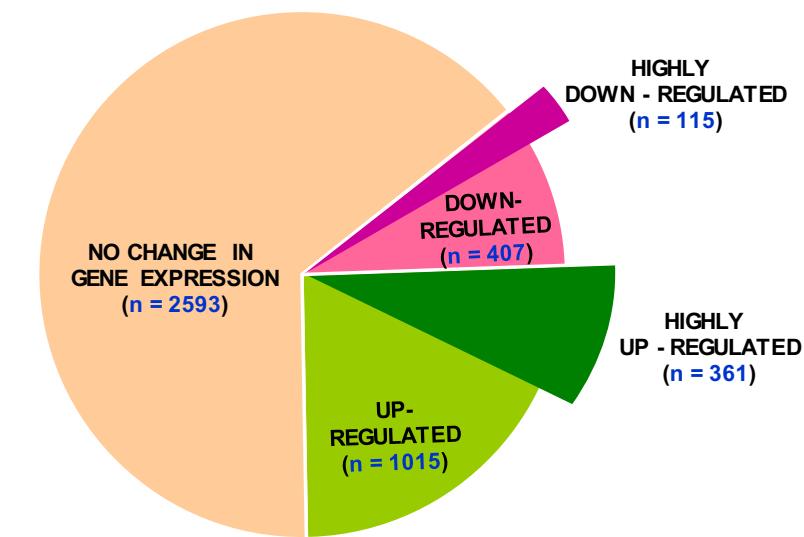
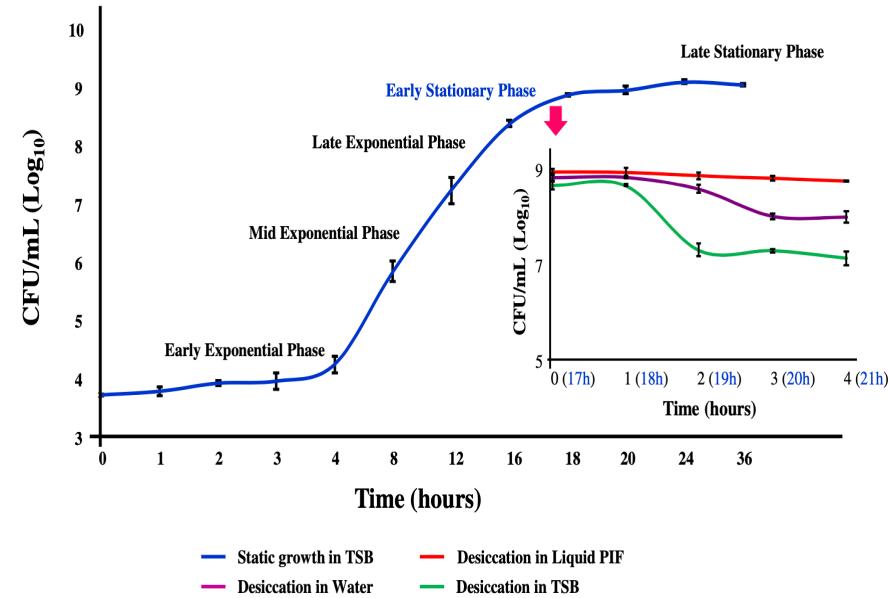
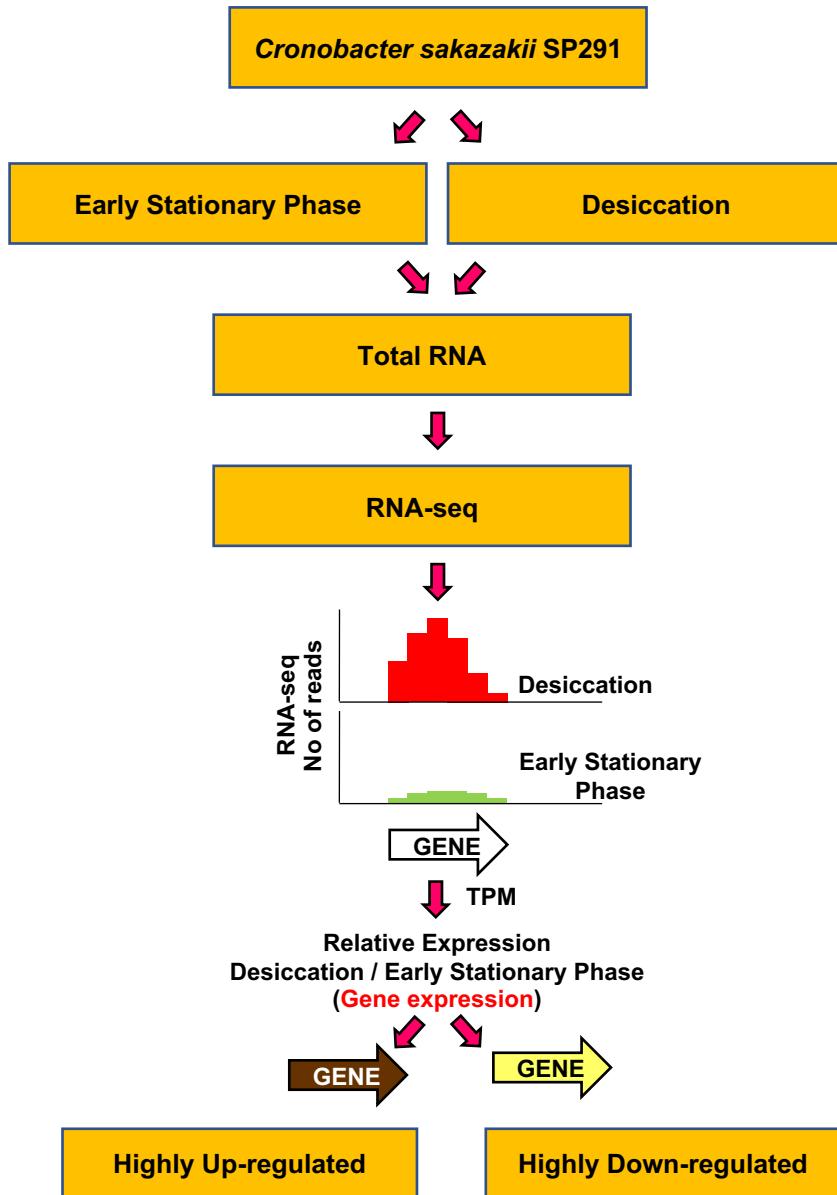
### Fast Facts

- Illnesses: 4
- Deaths: 2
- States: 3 (Minnesota, Ohio, Texas)
- Recall: Yes
- Investigation status: Active

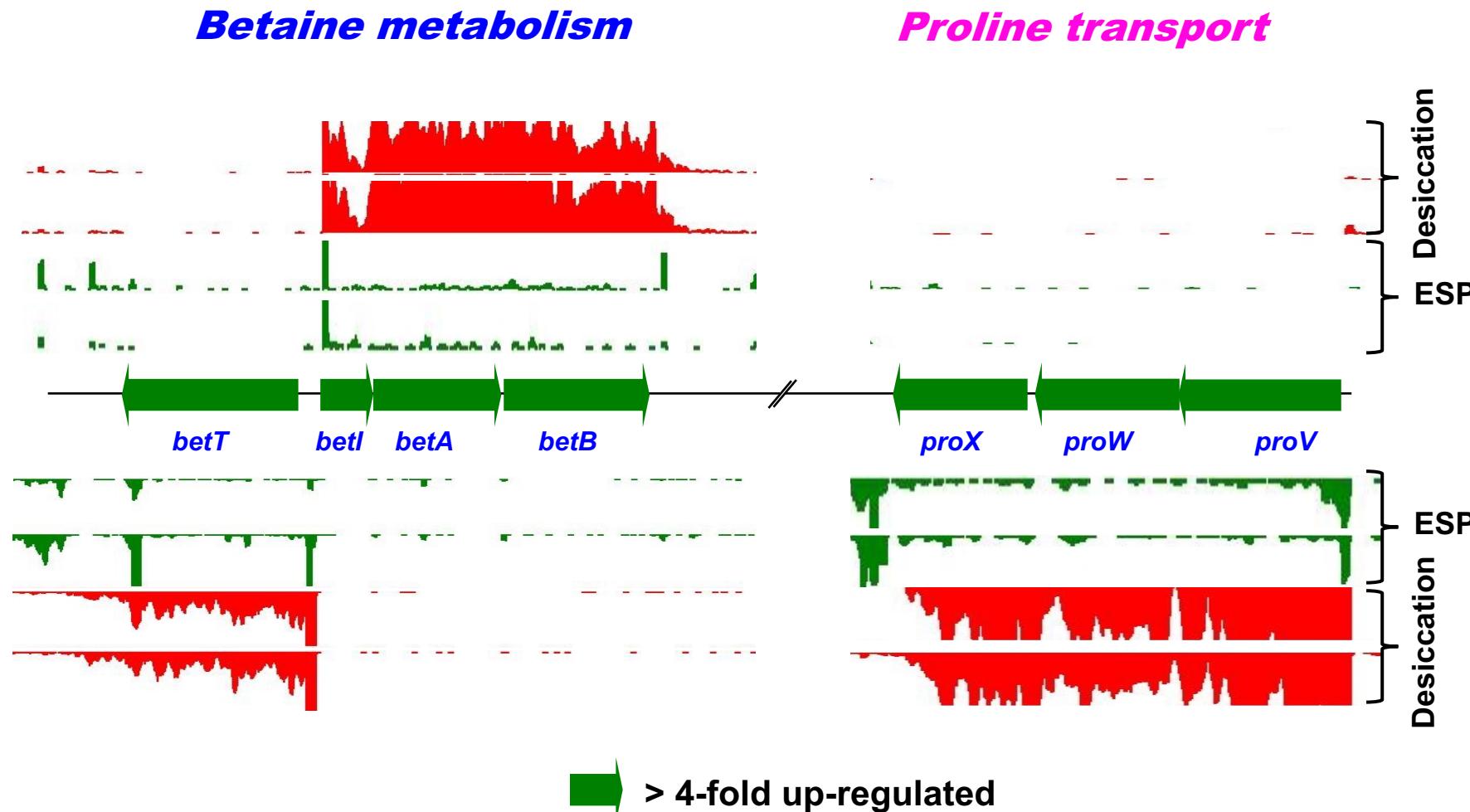


**Why do events like this happen?**

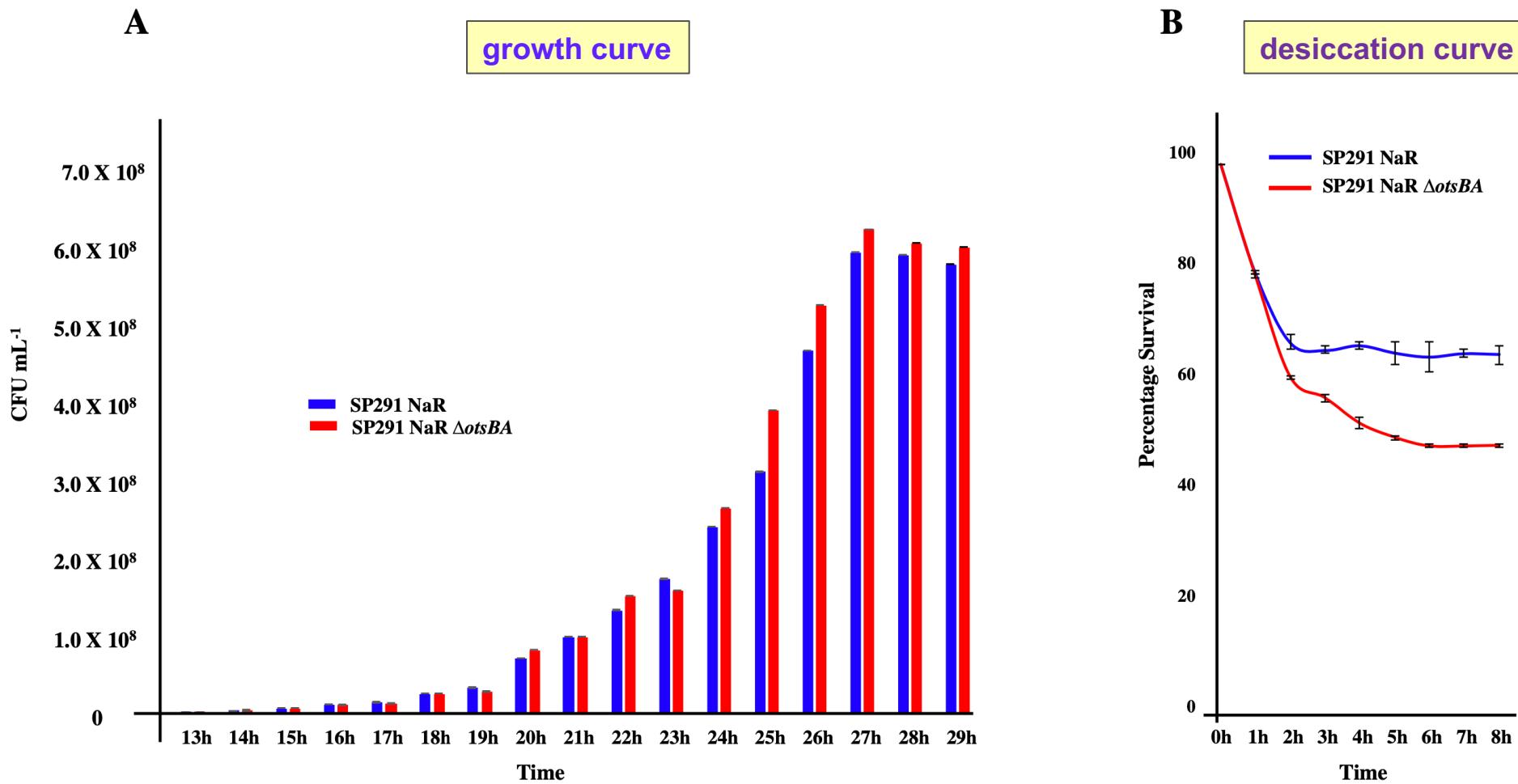
# Detecting gene expression using RNA-seq -



# Expression of stress response genes encoding osmoprotectants during desiccation -



## Trehalose metabolism is critical for survival during desiccation -



**Do all *Cronobacter sakazakii* respond to low-moisture conditions in the same way?**

## Comparison between the desiccation curve of *C. sakazakii* ATCC<sup>TM</sup>29544<sup>T</sup> (clinical) and *C. sakazakii* SP291 (environmental)-

### - Stage I (Desiccation 0-1 h)

- o no obvious change in viable cell count

### - Stage II (Desiccation 1 to 2 h)

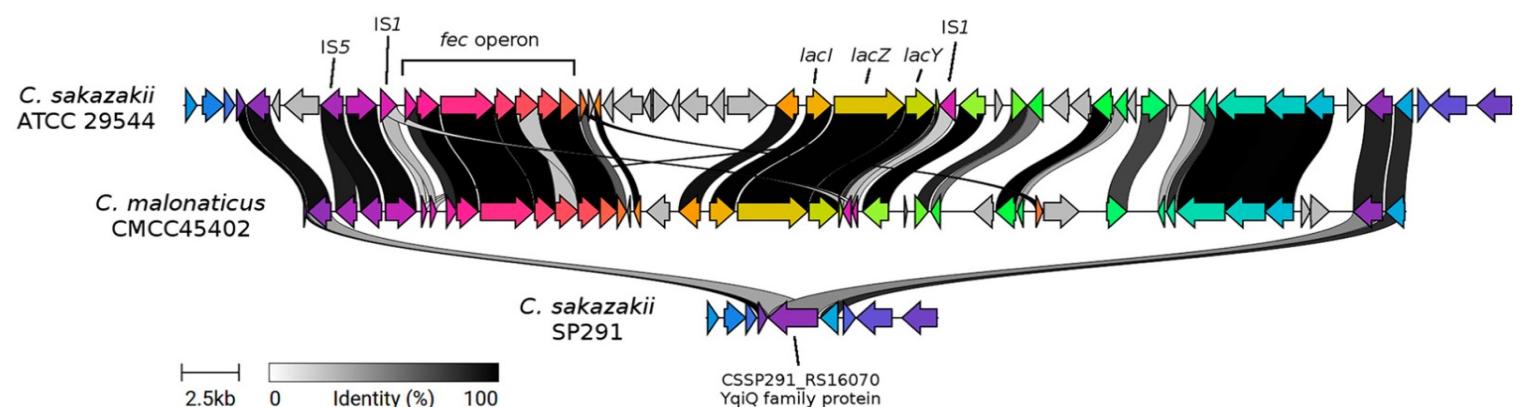
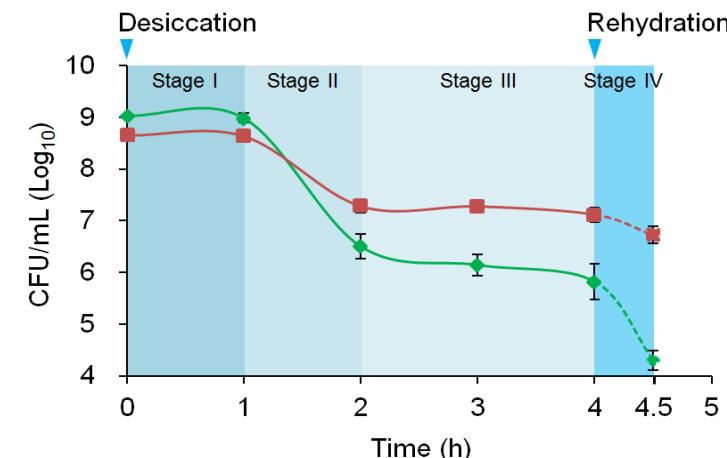
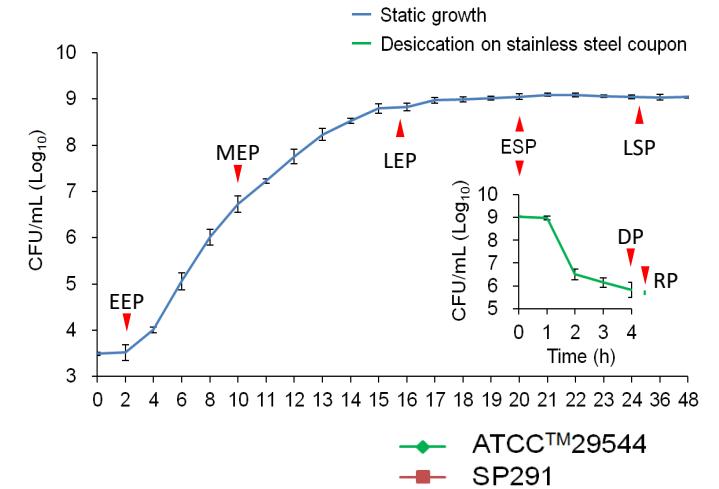
- o liquid on the coupon evaporated completely
- o a sharp reduction in the viable cell count (~ $2.5 \log_{10}$  reduction in 1 h for ATCC<sup>TM</sup>29544)

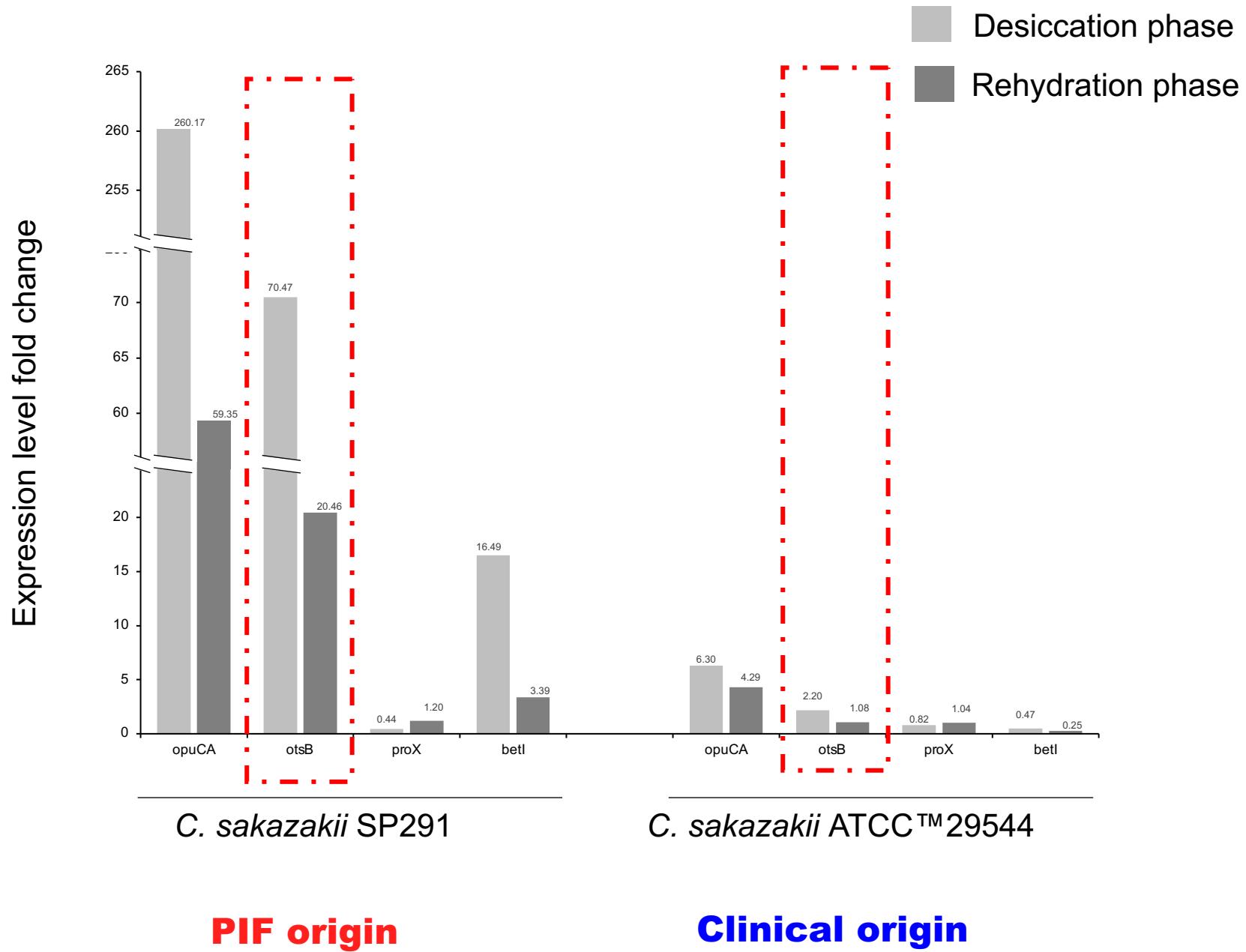
### - Stage III (Desiccation 2 to 4 h)

- o bacteria were continuously desiccated on the coupon
- o decrease in viable cell counts at a much slower rate (~ $1 \log_{10}$  reduction in 2 h for ATCC<sup>TM</sup>29544)

### - Stage IV (Rehydration 0-30 min)

- o viable cell count change for *C. sakazakii* ATCC<sup>TM</sup>29544 was larger than that for SP291 during each stage





## **Conclusions -**

- ***Genome sequencing can be used to study bacterial adaptation to relevant stresses***
- ***Biomarkers of importance to these adaptations can be identified and used for (future) diagnostic purposes***
- ***Understanding the nature of these adaptations offer the prospect of developing mitigating measures to improve food safety***



**Thank you**