Virtual Training 15: Focus on the Agents of Diphtheria

Diphtheria agents: Taxonomy and strain diversity

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Intended Learning Objectives

Specific objectives of this session:

1. Diphtheria agents and their taxonomic diversity

2. Population diversity of *C. diphtheriae*: strain diversity and phylogeny

3. *tox* gene distribution, diphtheria toxin production and biovars

4. Antibiotic resistance prevalence and mechanisms in *C. diphtheriae*

slido

Do you work regularly on diphtheria or its agents?

ⓘ Start presenting to display the poll results on this slide.

Diphtheria: clinical forms

Case fatality rate: 5 – 20%

Clinical presentations:

❖ **Respiratory (classical)**:

    Pharyngeal infection (angina)

    Pseudomembranes

    Lymphadenitis (bull's neck)

    Laryngeal obstruction (croup)

❖ **Cutaneous infection**

    Ulcer (non-healing, membranes)

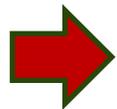❖ **Invasive infection (*tox*-negative)**

Systemic manifestations
(diphtheria toxin)

- Cardiac
- Neurological
- Renal

Classical diphtheria

- Respiratory infection

- *Corynebacterium diphtheriae*

- Diphtheria toxin-producing (= toxigenic) strains

- Human-to-human transmission

- Outbreaks

- High case fatality rate (5 – 20%), mostly in children < 5 years old

- *Excludes non-toxigenic strains, and other species*

- *'Diphtheria in the strict sense'*

Diphtheria: once a major killer of children
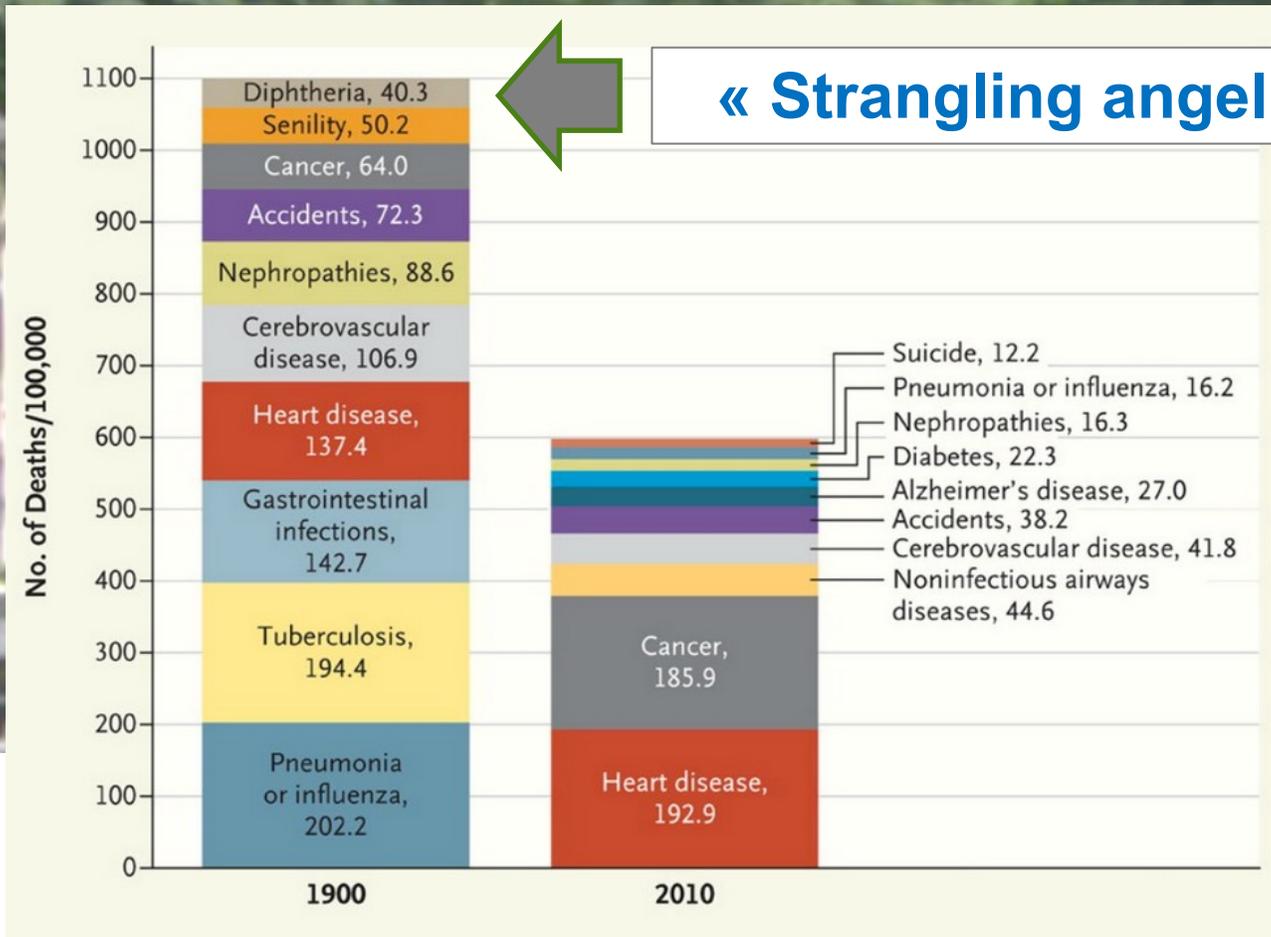
« Strangling angel of children »

Randy Olson @randal_olson · Feb 7
Top 10 causes of death: 1900 vs. 2010. #dataviz

nejm.org/action/showIma… pic.twitter.com/NpLxgtPKeW

https://study.com/academy/lesson/diphtheria-antitoxin-vaccine.html

Diphtheria control and case management

Diphtheria vaccine (including against *C. ulcerans*)

  Inactivated diphtheria toxin (toxoid)

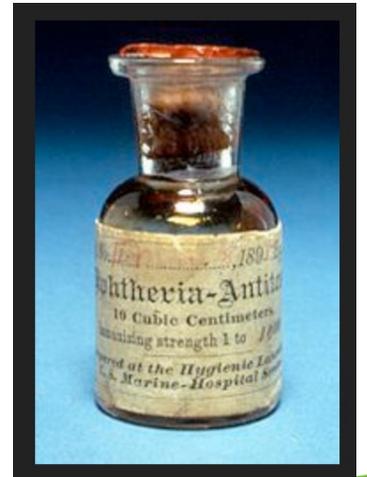 (Purified) supernatant; formalin+heat treatment

Antimicrobial therapy

  Amoxicillin (France); Penicillin G in LMICs

  Macrolides (Erythromycine, Azithromycine, …)

  Others (clindamycine (Cd), vancomycine)

Diphtheria AntiToxin (DAT)

  Only if systemic symptoms (or resp. pseudomembranes)

  Low availability

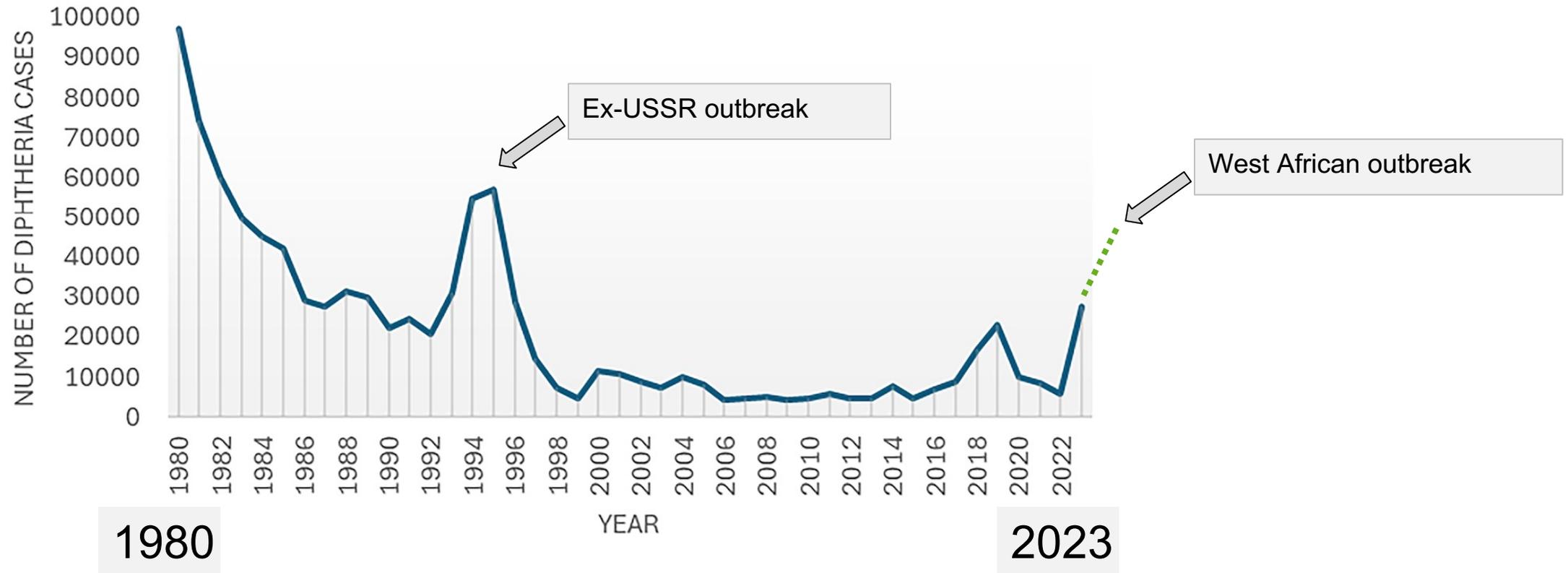 Adverse reactions (allergy, serum sickness, …)

Contact tracing to stop transmission

  Vaccination catch-up, antibio-prophylaxis, clinical surveillance

Diphtheria, Global, 2024

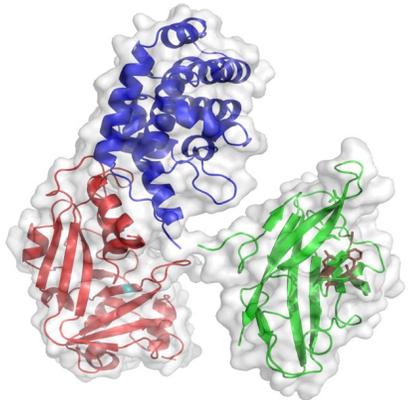Global annual cases of diphtheria, reported to the WHO, from 1980 to 2023

1980

2023

Diphtheria antitoxin treatment: from pioneer to neglected

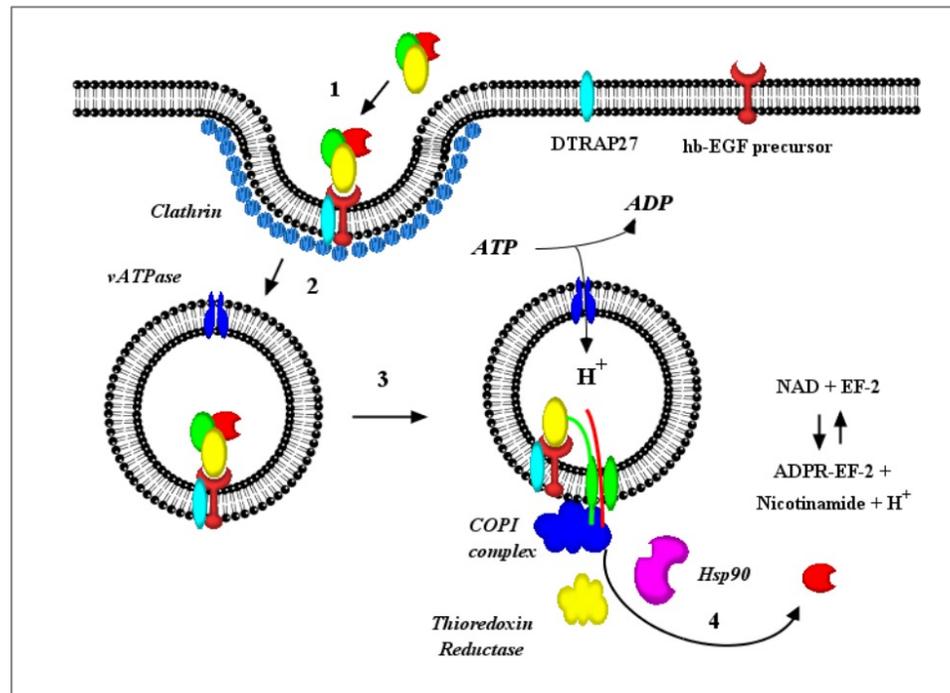Lucia Grandière Pérez[1,2], Sylvain Brisse[3,4]/+

Main virulence factor: diphtheria toxin *(DT)*

C-domain T-domain R-domain

A-fragment ← B-fragment →

Wenzel *et al.,* 2020

- **DT: EF-2 ADP-ribosyl transferase**
- **Inhibition of protein synthesis**
- **1 molecule can kill a cell**

Murphy, Toxins, 2011

- Very potent toxin

- Targets elongation factor 2: interrupts protein synthesis

- Responsible for systemic signs and death (cardiac arrest)
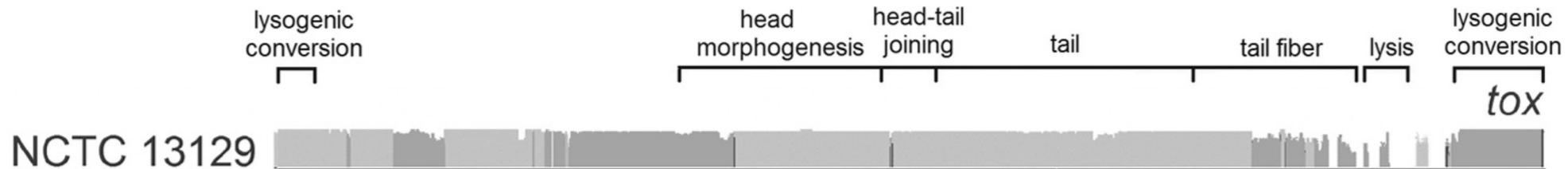
Diphtheria toxin (DT) is encoded on a prophage (*tox* gene)

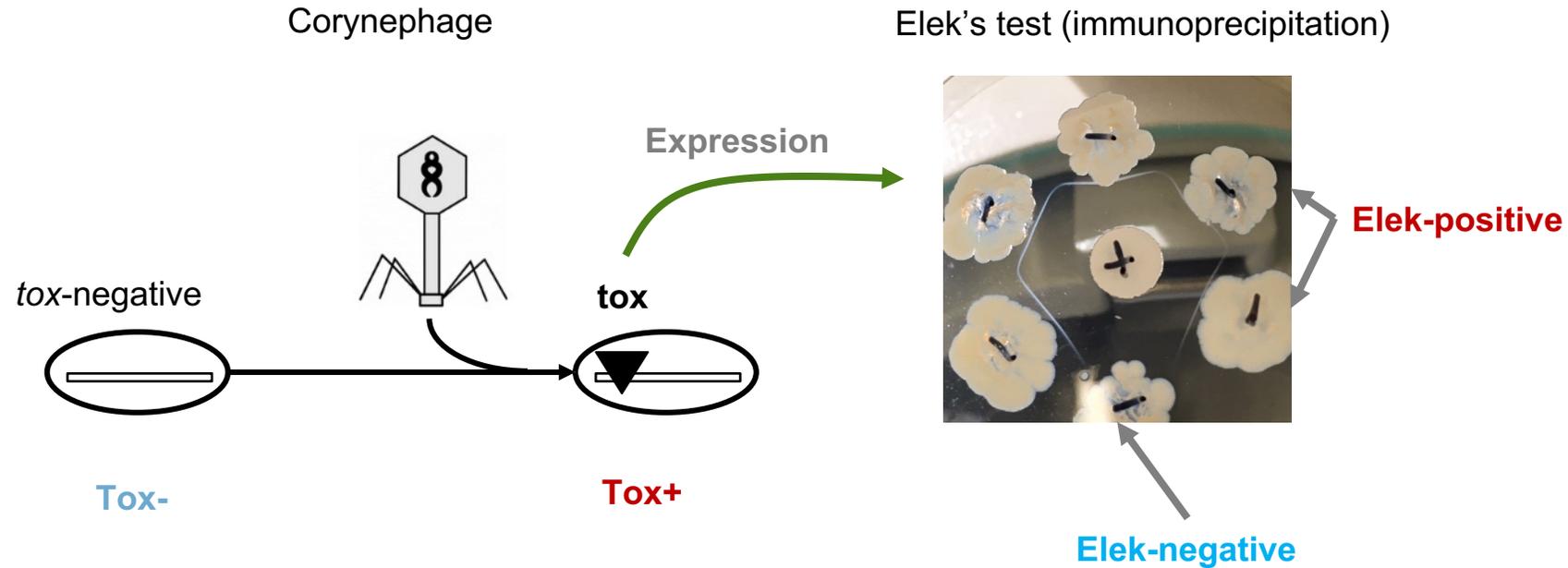« avirulent cultures of *C. diphtheriae* can become virulent in the presence of a specific bacteriophage »
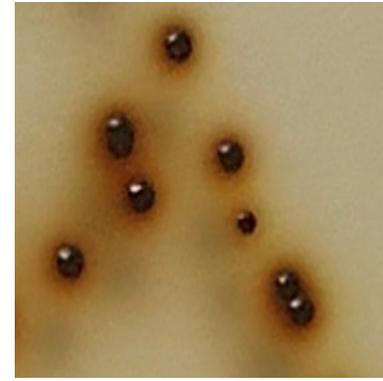
Victor Freeman, J. Bacteriol, 1951

tox gene = 5' end of prophage genome

Laird & Groman, 1976; Trost *et al*., 2012

Diphtheria agents: toxigenic strains

Corynephage

Elek's test (immunoprecipitation)

Expression

tox-negative

tox

Tox-

Tox+
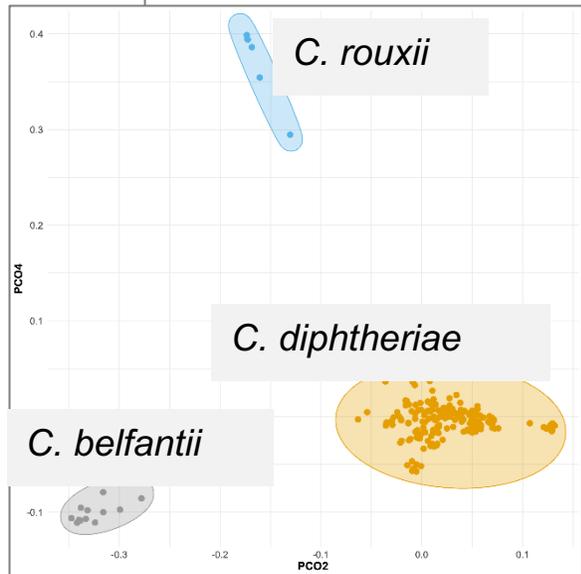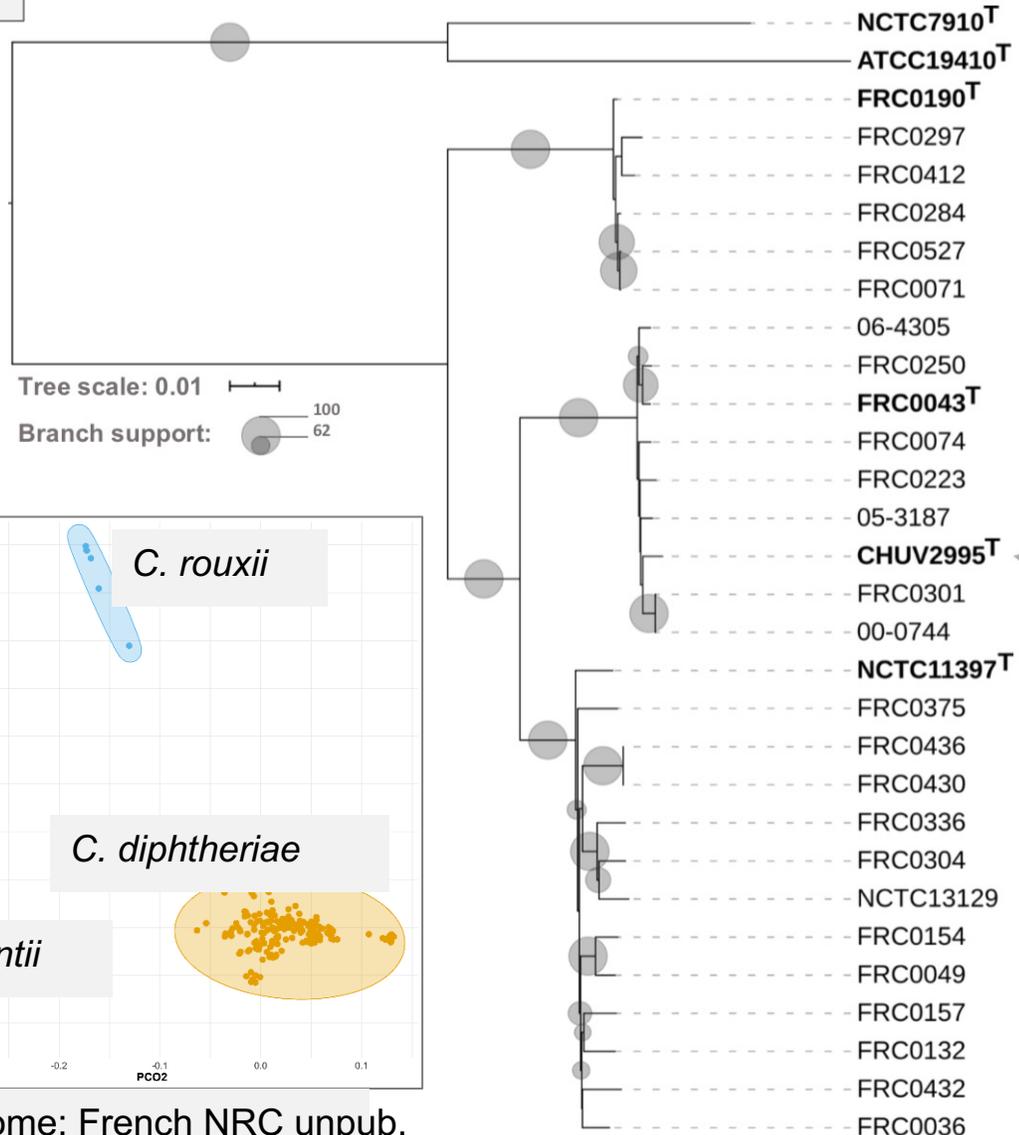
Elek-positive

Elek-negative

3 conditions are needed for strains to be toxigenic:
- Lysogenized
- *tox* gene expression
- Full *tox* gene (not disrupted)

Corynebacterium diphtheriae Species Complex (CdSC)

tox-negative
tox-positive

C. ulcerans — 1995

C. pseudotuberculosis

FRC0190^T
FRC0297
FRC0412
FRC0284
FRC0527
FRC0071

C. rouxii — 1911

2020

NCTC7910^T
ATCC19410^T

06-4305
FRC0250
FRC0043^T
FRC0074
FRC0223
05-3187
CHUV2995^T
FRC0301
00-0744

C. belfantii — 2018
C. diphtheriae subsp. lausannense

Biovar Belfanti

NCTC11397^T
FRC0375
FRC0436
FRC0430
FRC0336
FRC0304
NCTC13129
FRC0154
FRC0049
FRC0157
FRC0132
FRC0432
FRC0036
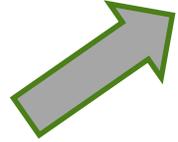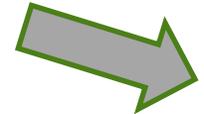
C. diphtheriae — 1886

Biovars Gravis & Mitis, and rarely Belfanti

Tree scale: 0.01
Branch support: 100 / 62

C. rouxii
C. diphtheriae
C. belfantii

Pangenome; French NRC unpub.

Corynebacterium diphtheriae Species Complex (CdSC)*

tox-positive & negative

Other species: *tox* negative

Corynebacterium pseudotuberculosis
ATCC19410^T

Corynebacterium ramonii
FRC0011^T
(lineage 2)

Corynebacterium ulcerans
NCTC7910^T
(lineage 1)

Corynebacterium silvaticum
KL0182^T

0.03

Corynebacterium rouxii
FRC0190^T

Corynebacterium belfantii
FRC0043^T

Corynebacterium diphtheriae
NCTC11397^T

NTTB

* CdSC: Phylogenetic group of taxonomic species

Ecology: *C. diphtheriae*

Reservoirs   **Carriage**   **Disease**

Rarely

Skin
throat

~ Human only

Ecology: *C. ulcerans*

Reservoirs   **Carriage**   **Disease**

Rarely
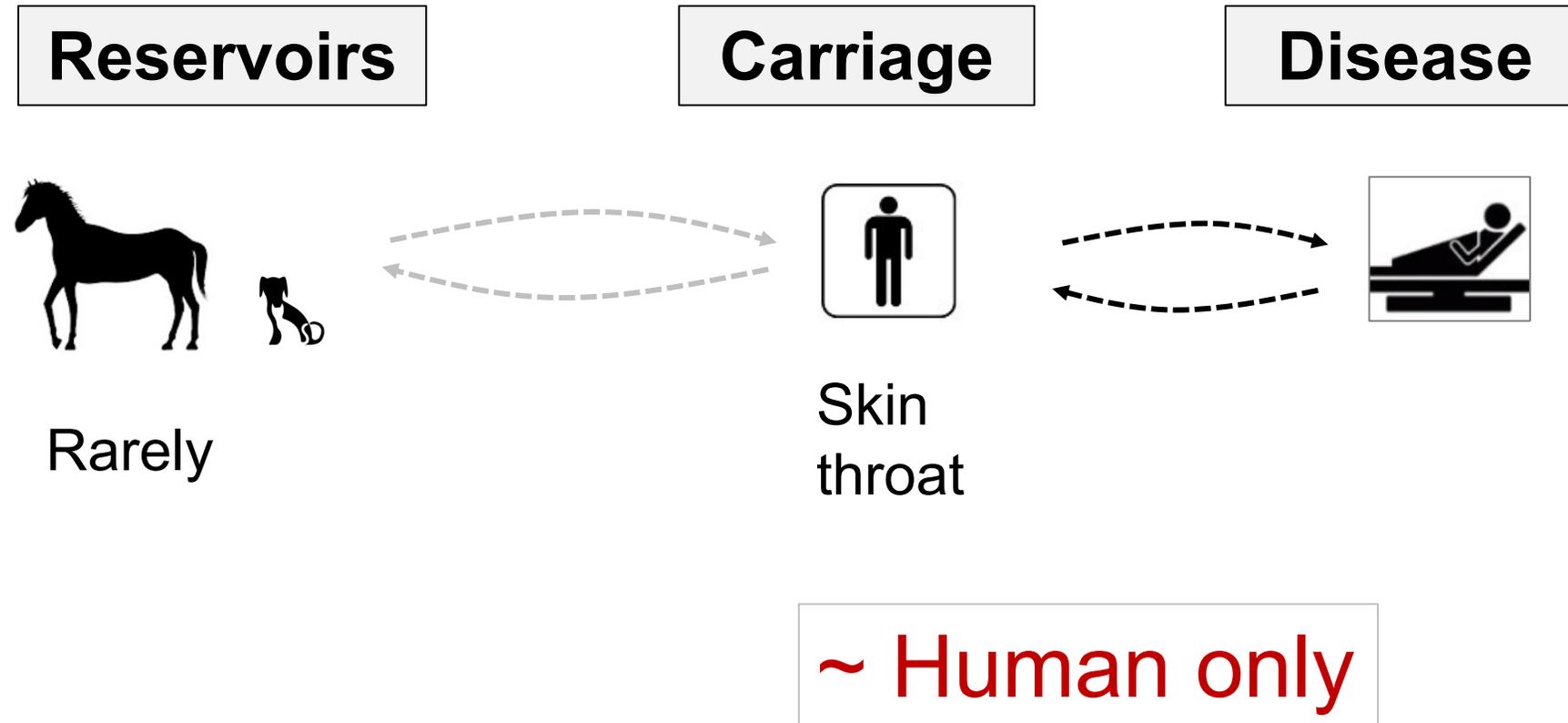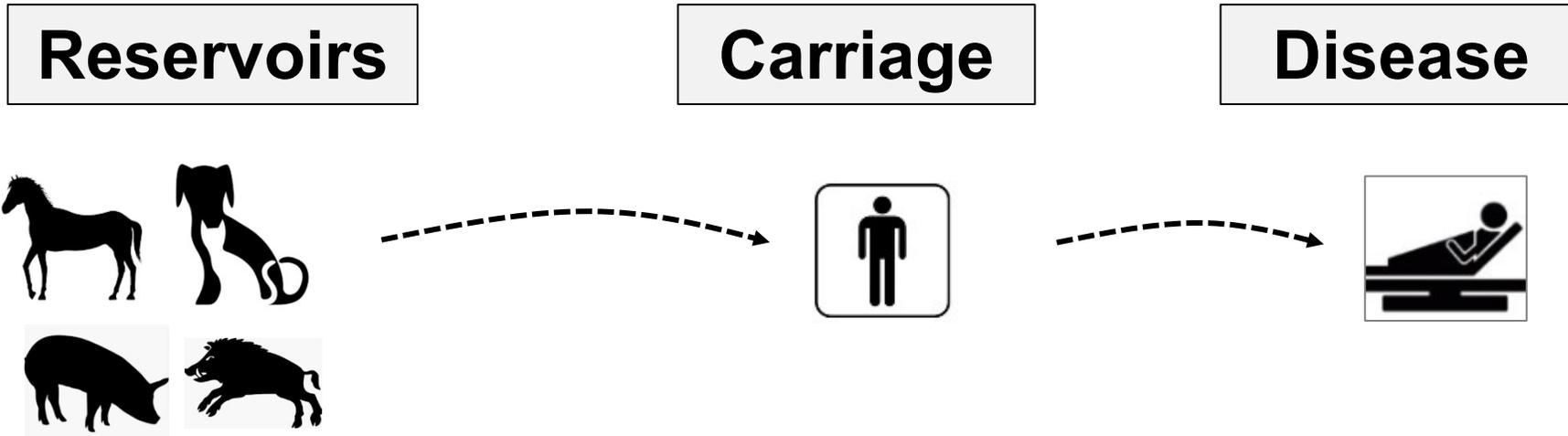
C. ulcerans:
- Ecological generalist
- <u>No</u> human-to-human transmission
- Risk factors: pets, age

Diphtheria agents and vaccination

Diphtheria toxin is highly conserved within the CdSC species complex

- DT of *Corynebacterium diphtheriae* and *C. ulcerans* ~95% identical (nt and AA)
- DT from *C. ulcerans* is also neutralized by eDAT
- Clinical severity of *C. ulcerans* infections: reduced by vaccination against diphtheria
- Diphtheria antitoxin (eDAT) recommended for toxigenic *C. ulcerans* upper airways infections
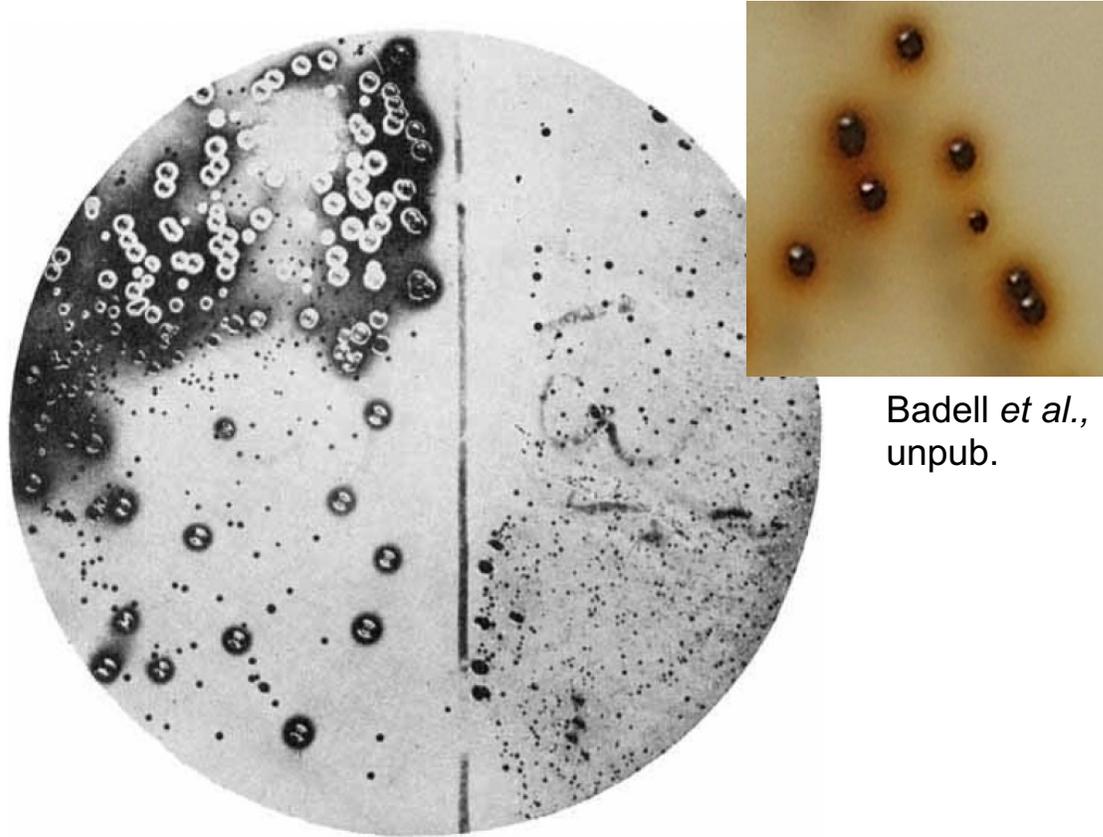
Sing et al., 2003; Wagner et al., 201; Möller et al. 2023

Diphtheria vaccine: not just the toxoid

- Diphtheria vaccines comprise hundreds of *C. diphtheriae* proteins
- Many of these are immunogenic
- Partial protection against non-toxigenic *C. diphtheriae* strains?

Möller et al., 2023

# Isolation of CdSC isolates on Tinsdale agar

Badell *et al.*, unpub.

Tinsdale, 1947

- Potassium tellurite inhibits throat bacteria other than corynebacteria

- L-cystine and sodium thiosulfate form the H_2S indicator system

- Black coloration of the colonies results from tellurite reductase activity

- Brown halos indicate cystinase activity

- Cystinase is CdSC specific, except *C. pseudotuberculosis*

- Diphtheroids, staphylococci, and some streptococci: small black colonies, no halo

Isolation of CdSC isolates on blood agar with fosfomycin
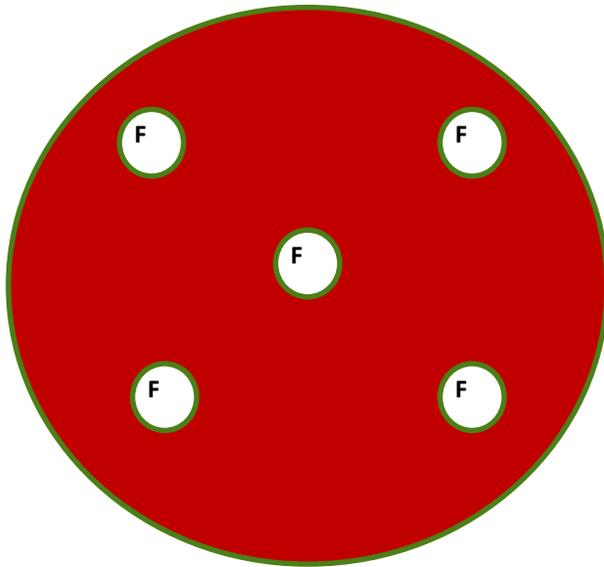
Badell *et al.,* unpub.

- Blood agar (Columbia + 5% sheep blood) with fosfomycin paper disks

- Corynebacteria are naturally resistant to fosfomycin, and will be the only ones growing around the disks

- Pick colonies close to the disks and identify

Evolutionary dynamics of the *tox* gene?

- How is the *tox* gene distributed in *C. diphtheriae*?

- How frequent are lysogenic conversion or prophage loss?

- How frequent are loss-of-function *tox* mutations, leading to NTTB status?

Diphtheria surveillance (France)

- *Mandatory notifiable disease*
- *Strains are referred to the National Reference Center for tox gene detection*

Isolates bio-collection
& attached source data
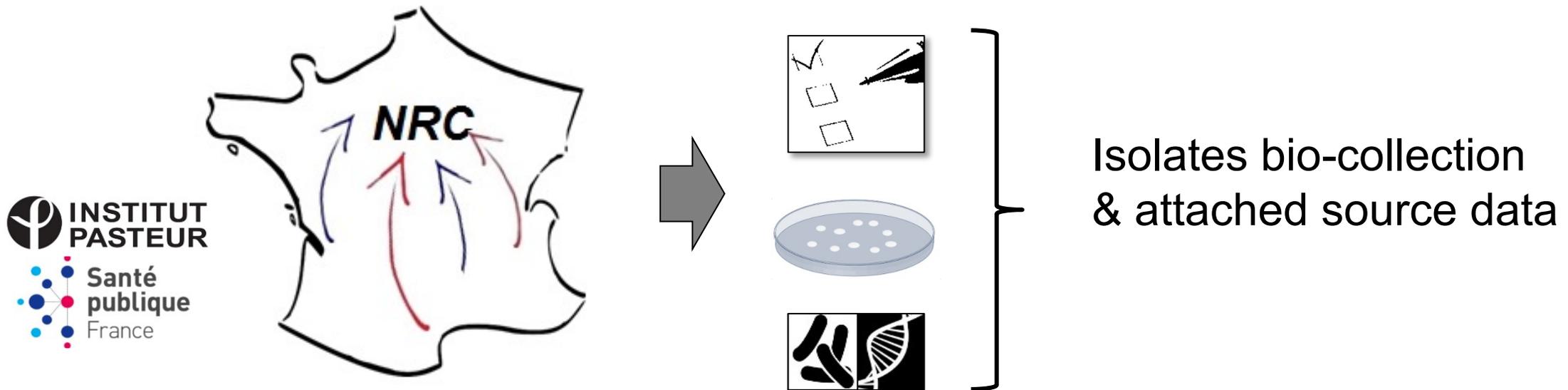
- **Rapid diagnosis:** real-time qPCR (***tox*** & species: *C. diphtheriae, C. ulcerans*, …)

- **Isolation** (Tinsdale medium)
- **Toxin production (**Elek test**) & biotyping (**biovar Gravis, Mitis, …)
- **Antimicrobial susceptibility testing**
- **Genomic sequencing**
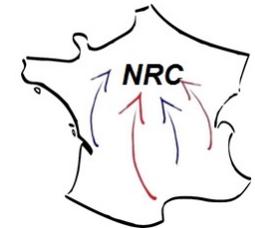
Diphtheria re-emergence, France

1990-2001:
No case

- +

C. diphtheriae
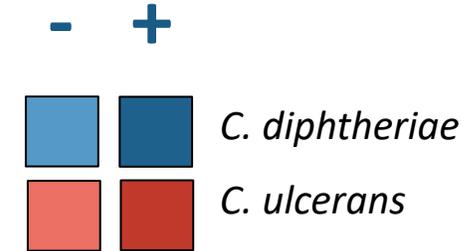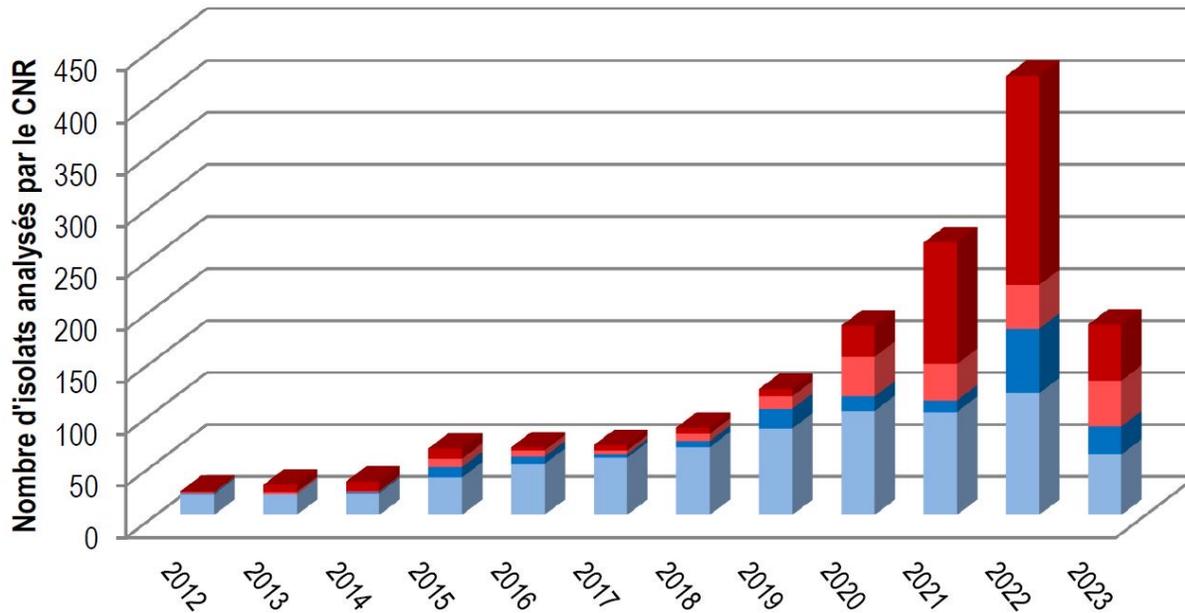
C. ulcerans

Data: SpF, CNR & HCSP, 2021
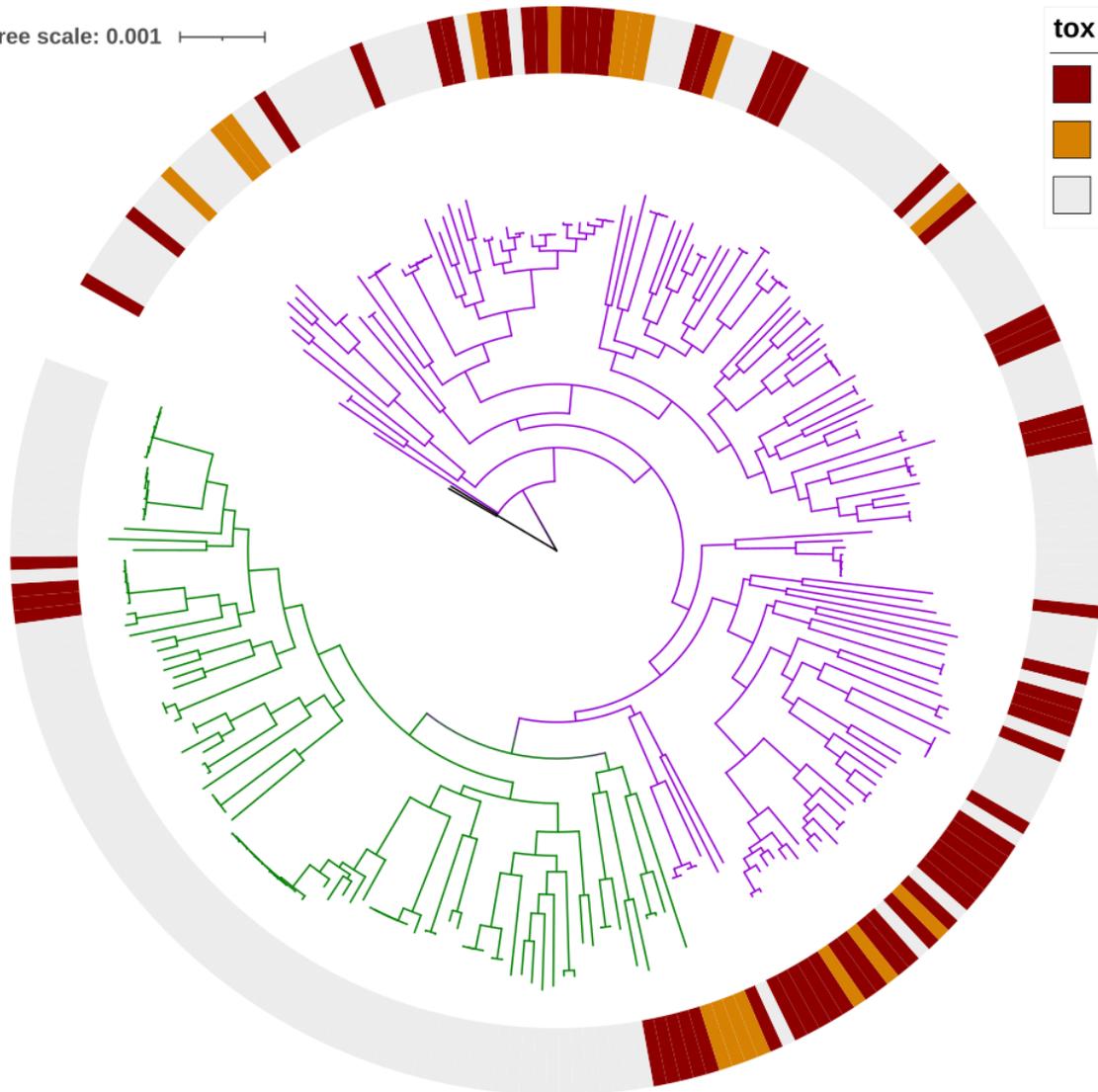
INSTITUT PASTEUR

Santé publique France

Drivers of re-emergence:

- 2003: broadened definition (*C. ulcerans* et *C. pseudotuberculosis*)

- Improved identification methods (MALDI-TOF): lab-driven detection (wounds)

- Insufficient vaccination in multiple country, combined with travel
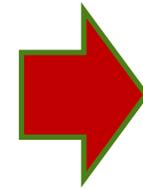
tox gene and phylogenetic structure

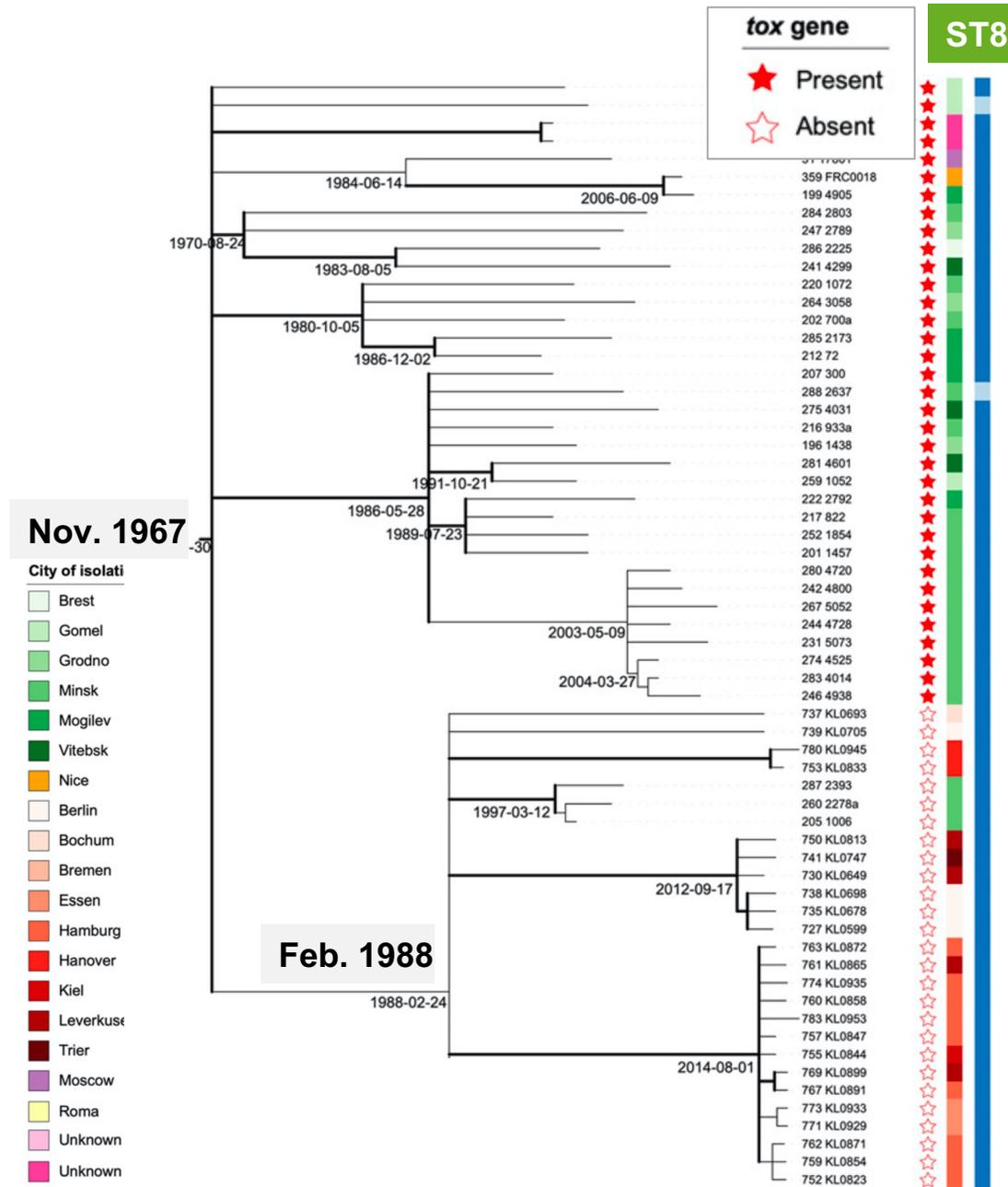Tree scale: 0.001

tox gene and Elek's test
- ■ tox + and Elek +
- ■ NTTB
- □ tox - and Elek +

NTTB:
non-toxigenic, *tox*-bearing

- ***tox*** in multiple sublineages: gain/loss events at deep evolutionary timescales

- Multiple loss of function events (NTTB)

- Association with Mitis branch

- Gravis: more severe diphtheria!?

Hennart *et al.*, Genome Med 2020

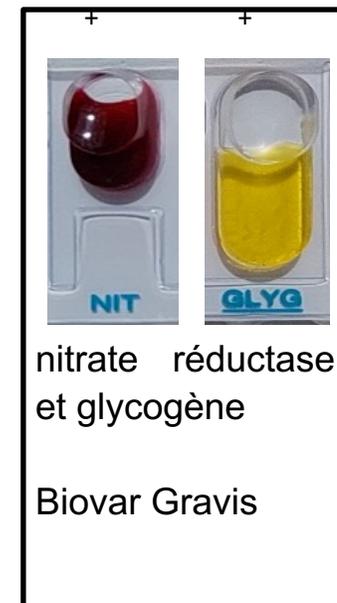# *How stable is the tox* gene/prophage in Cd genomes?

- *tox* positive or *tox*-negative clades are stable over decades

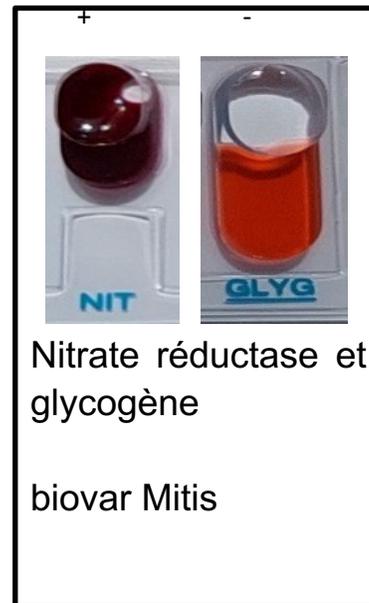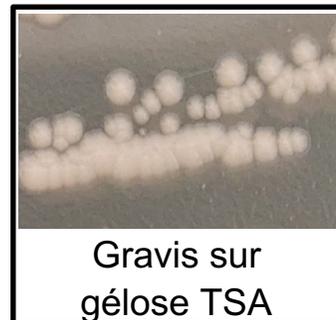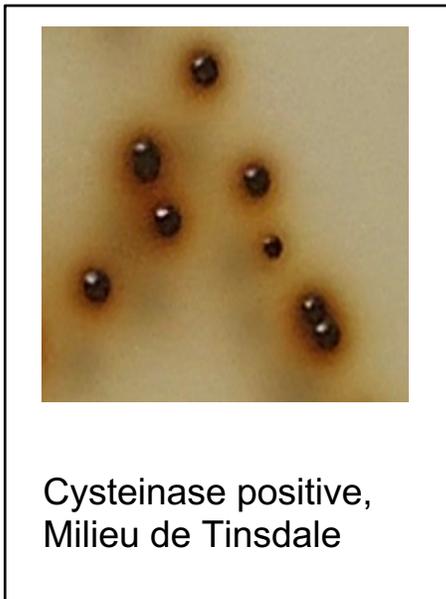➡ Phage conversion or loss of *tox* gene are rare events (at epidemiological timescales)

Guglielmini *et al.*, 2018

Biovars of *C. diphtheriae*

Burkovski, 2014

	Tests phénotypiques conventionnels											
	Hémolyse	ADNase	Cystinase (H₂S)[b]	Pyrazina-midase	Réduction des nitrates	Hydrolyse de l'urée	Glucose	Maltose	Sucrose	Glycogène	Amidon	Réaction CAMP
C. diphtheriae												
Biovar Gravis	−	+	+	−	+	−	+	+	−/+	+	+	−
Biovar Mitis	+/−	+	+	−	+	−	+	+	−/+	−	−	−
Biovar Intermedius	−	+	+	−	+	−	+	+	−/+	−	−	−
Biovar Belfanti	−	+	+	−	−	−	+	+	−/+	−	−	−

Cysteinase positive, Milieu de Tinsdale

Gravis sur gélose TSA

Mitis sur gélose TSA

Nitrate réductase et glycogène

biovar Mitis

nitrate réductase et glycogène

Biovar Gravis

Mitis − Gravis +

Caractère glycogène en Hiss serum

Biovars of *C. diphtheriae*

Gravis etymology: severe cases

- Relative death rates in humans: Gravis 13.3%, Mitis 2.3%

- Paralytic phenomena were more common in Gravis infections

- In animal models (Guinea pig, rabbit): Gravis type always virulent; Mitis only 84%

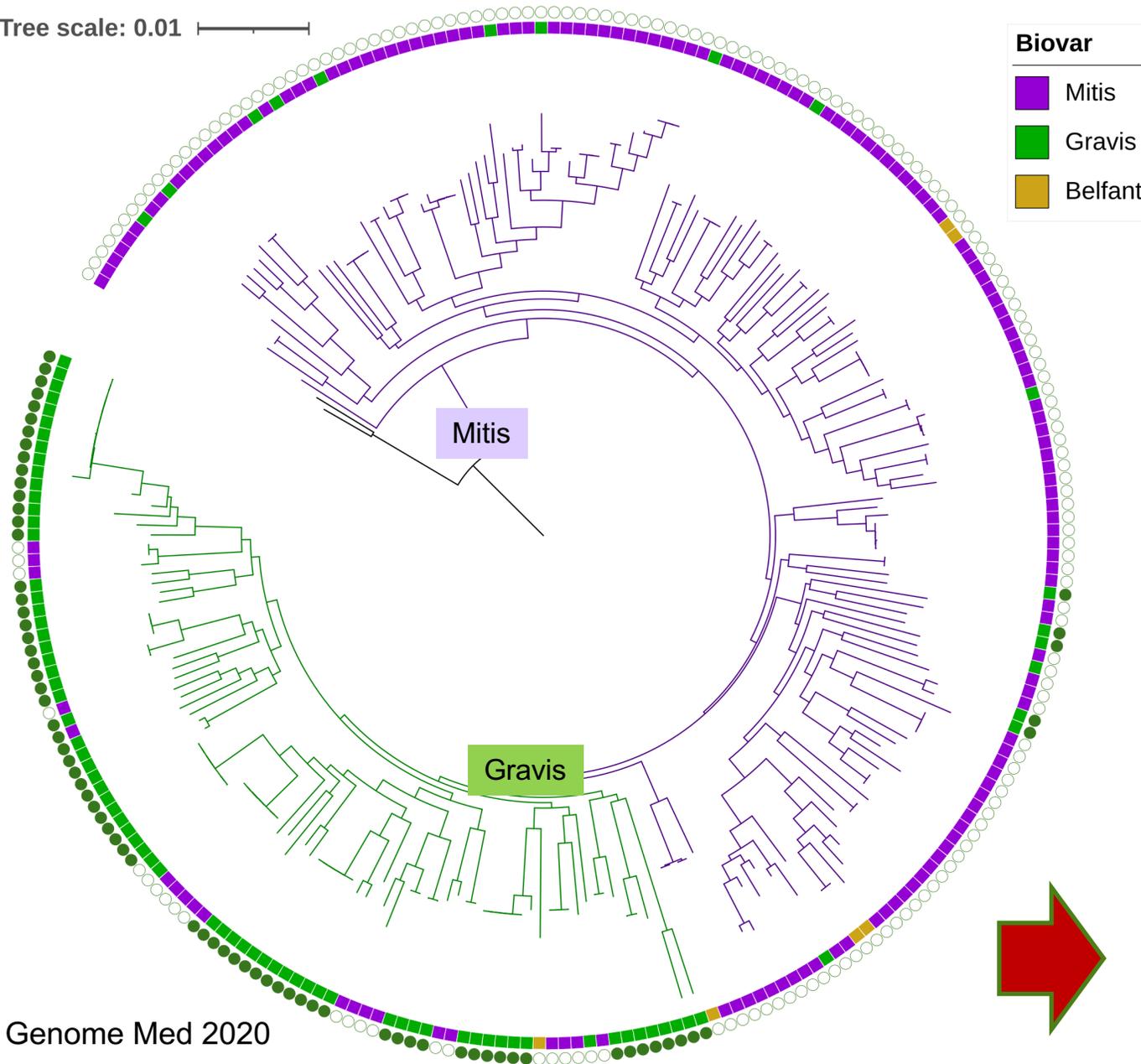McLeod, 1943

Biovars and phylogenetic structure

Tree scale: 0.01

Biovar

- Mitis
- Gravis
- Belfanti

Mitis

Gravis

Major dichotomy:
Mitis / Gravis

Hennart *et al*., Genome Med 2020

Biovars and phylogenetic structure

Tree scale: 0.01

Biovar
- Mitis (purple)
- Gravis (green)
- Belfanti (yellow)

Gravis markers
- spuA

spuA: alpha-1,6-glycosidase
(Santos *et al*., 2018)

Mitis

Gravis

Mitis − Gravis +

Glycogen test in Hiss serum
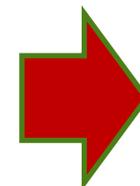
spuA, marker of biovar Gravis

Hennart *et al*., Genome Med 2020

Some conclusions on *Corynebacterium diphtheriae* biovar-lineages

- Phylogenetic dichotomy, strongly associated with biovars Mitis and Gravis

- The ancestral status of the biovar is uncertain

- Biovar Belfantii is observed in all *C. rouxii* and *C. belfantii* isolates, but also rarely in *C. diphtheriae* (results from the loss of nitrate reduction by Mitis isolates)
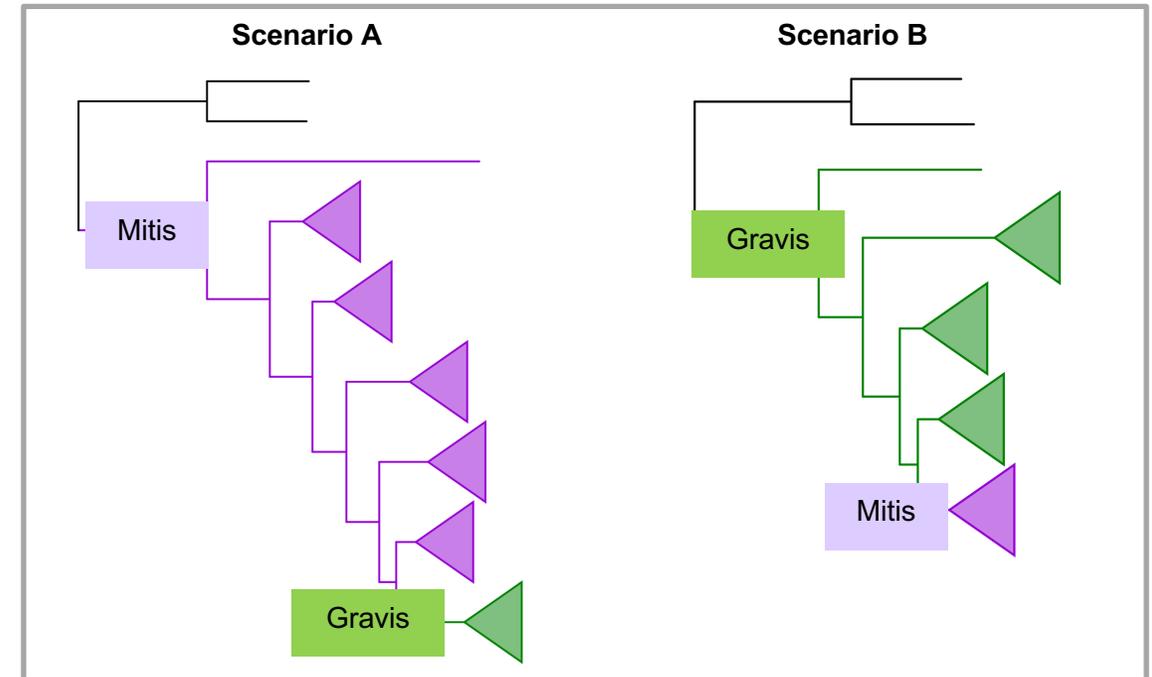
Melanie Hennart[1,2], Leonardo G. Panunzi[1,3], Carla Rodrigues[1], Quentin Gaday[4], Sarah L. Baines[5], Marina Barros-Pinkelnig[1], Annick Carmi-Leroy[1,6], Melody Dazas[1,6], Anne Marie Wehenkel[4], Xavier Didelot[7], Julie Toubiana[1,6,8], Edgar Badell[1,6] and Sylvain Brisse[1,6*]

C. diphtheriae:

Antimicrobial resistance & genetic mechanisms

- What are the main genetic mechanisms of resistance?

- How prevalent are these mechanisms?

- How is AMR distributed in *C. diphtheriae* sublineages*?*

- How is it associated with diphtheria toxin?

pbp2m, a gene associated with beta-lactam resistance

Methods: Pangenome analysis and Genome-wide association study (TreeWAS: Collins & Didelot *PLoS Comput Biol 2018*)
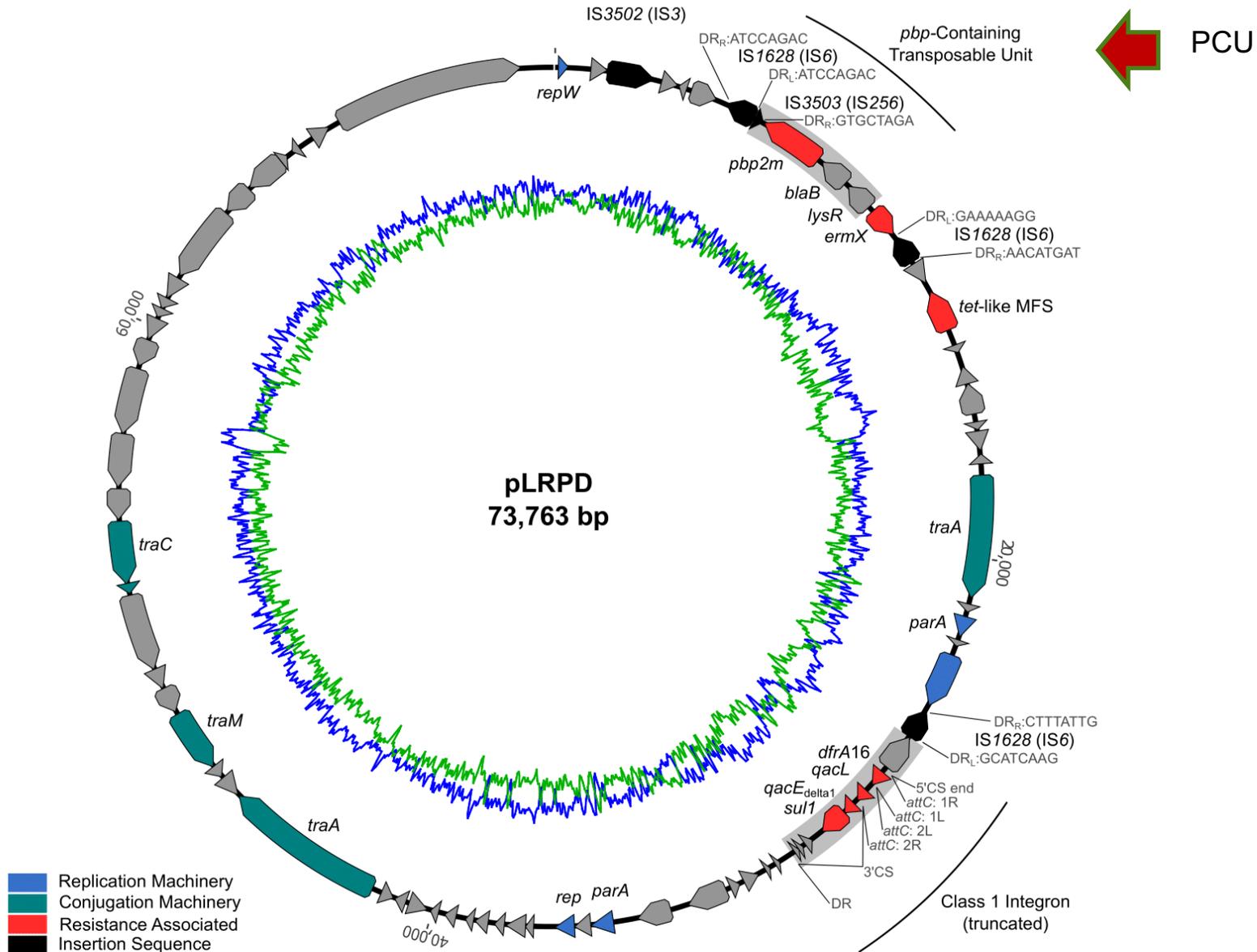
Results:

- *pbp2m* strongly linked with PEN-R

- Codes for a low-affinity penicillin-binding protein

- Confers low-level resistance (CMI 1 – 2 mg/L)

- *pbp2m* does not explain all PEN-R: Mutations in chromosomal *pbp* genes?

Hennart *et al*., Genome Med., 2020;
see also Forde et al., Clin Inf Dis, 2020
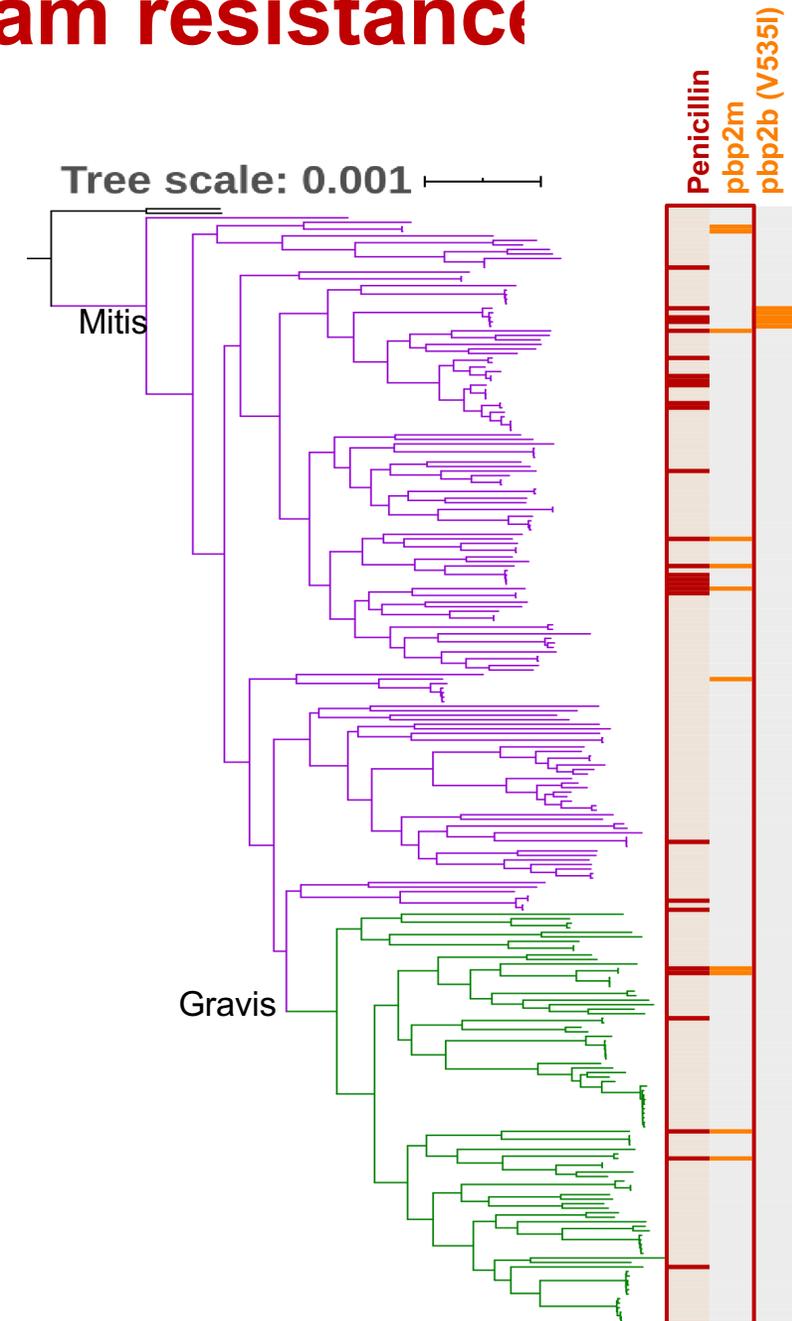
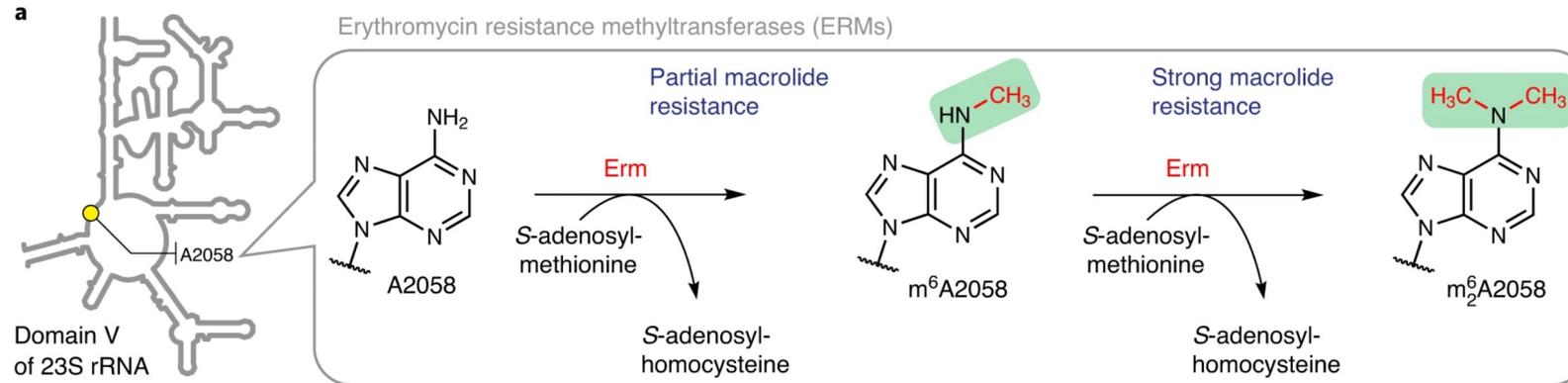# Multidrug resistance plasmid carrying pbp2m

PCU

Genomic contexts:

- *pbp2m* and the PCU are found in various genomic contexts and *Corynebacterium* species

- Sometimes in multi-copy

- Association with *ermX*
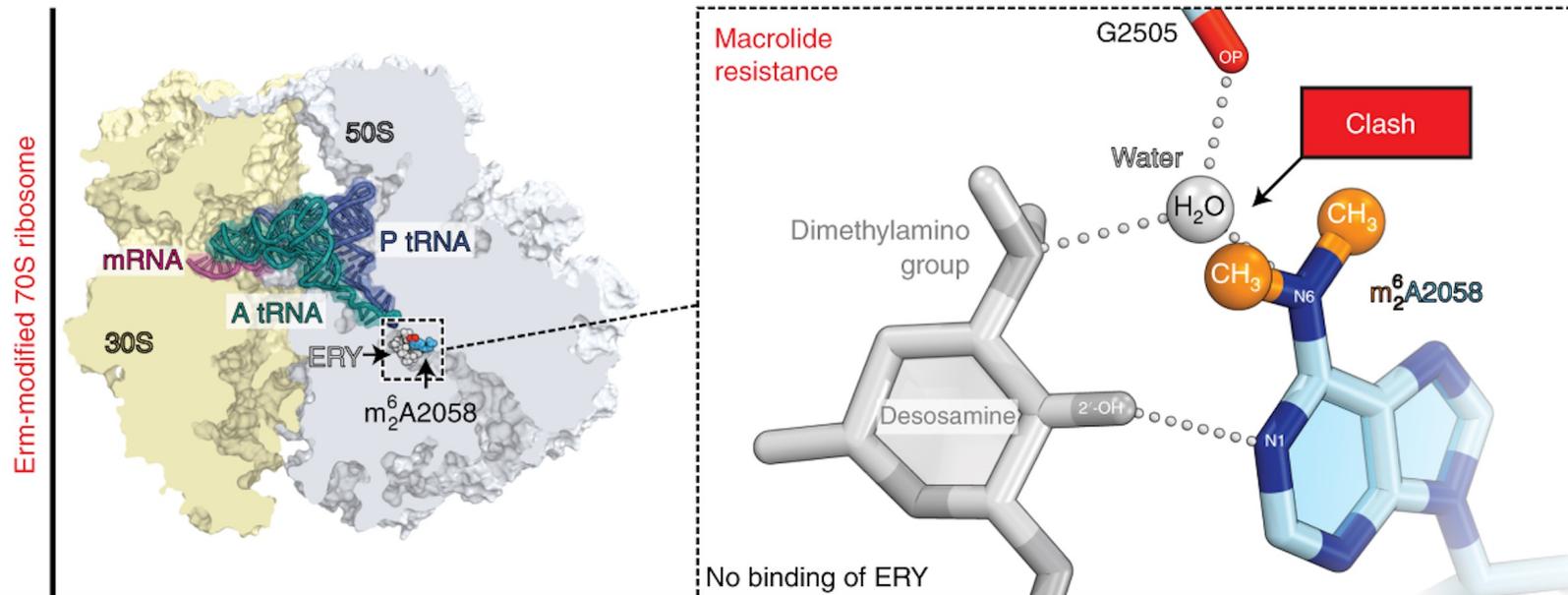
Hennart *et al*., Genome Med., 2020

Macrolide resistance in *C. diphtheriae*: ermX gene

ErmX codes for a methyltransferase that targets the 23S rRNA

ermX confers high-level resistance in *C. diphtheriae*

(CMI > 256 mg/L)

Svetlov *et al*., Nat Chem Biol., 2020

Resistance mechanisms in *C. diphtheriae*

| Class	Genes
Cyclins	*tet(33); tet(O); tet(W); tet(Z)*
Macrolides	*erm(X); erm(C)*
Phenicol	*cmx; cmlA4*
Sulfonamides	*sul1*
Trimethoprim	*dfrA1; dfrA15; dfrA16*
(Quaternary ammonium)	*qacEdelta1; qacL*
Aminoglycosides	*aadA1; aadA2; aadA5; aadA13; aadA15; aph(3')-Ia; aph(3")-Ib; aph(6)-Id; aac(3)-XI*
Quinolones	*gyrA* mutations
Rifampicin	*rpoB* mutations

- Resistance to penicillin & other beta-lactams: *pbp2m*
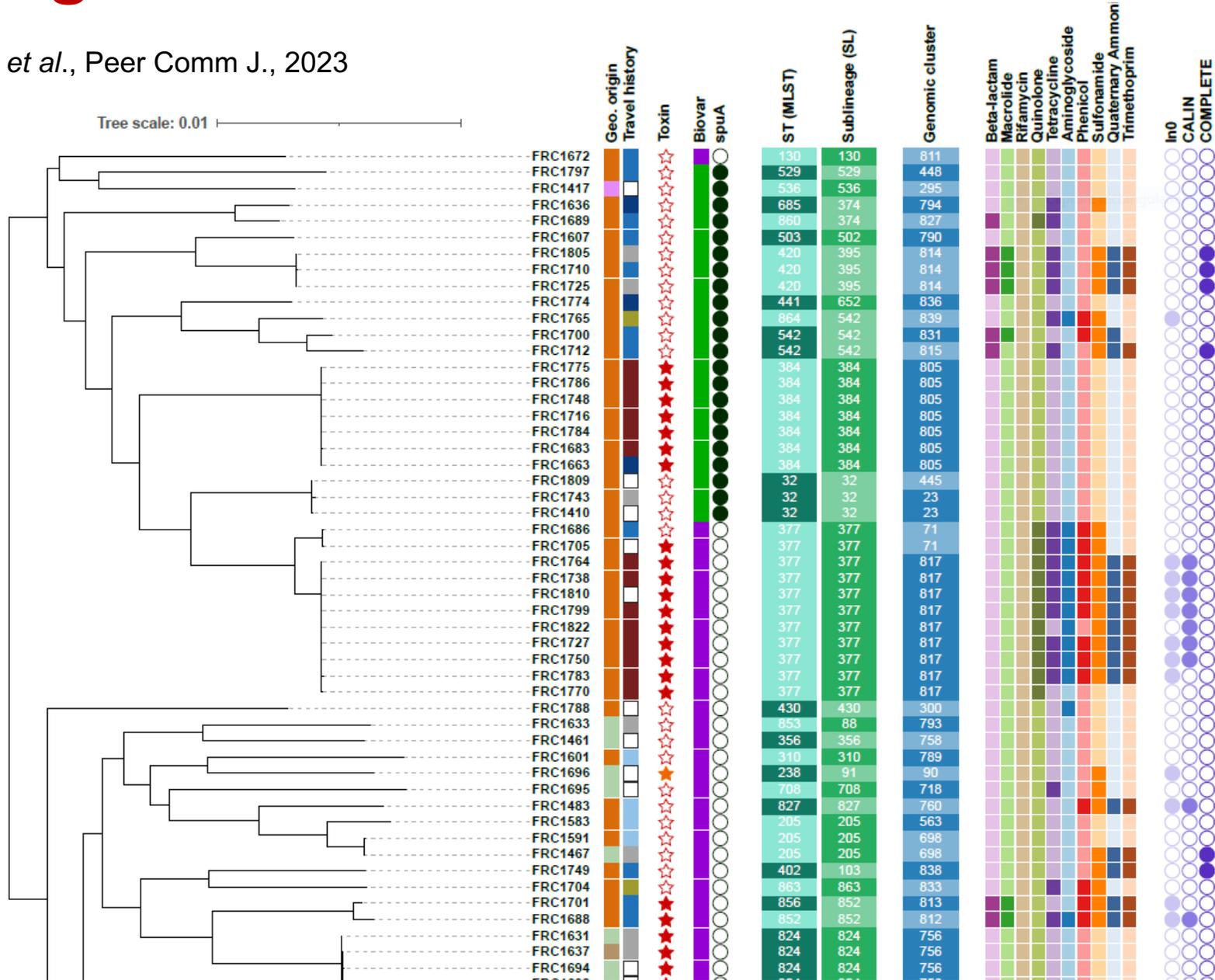
Antimicrobial resistance genes in *C. diphtheriae*

Hennart *et al.*, Peer Comm J., 2023
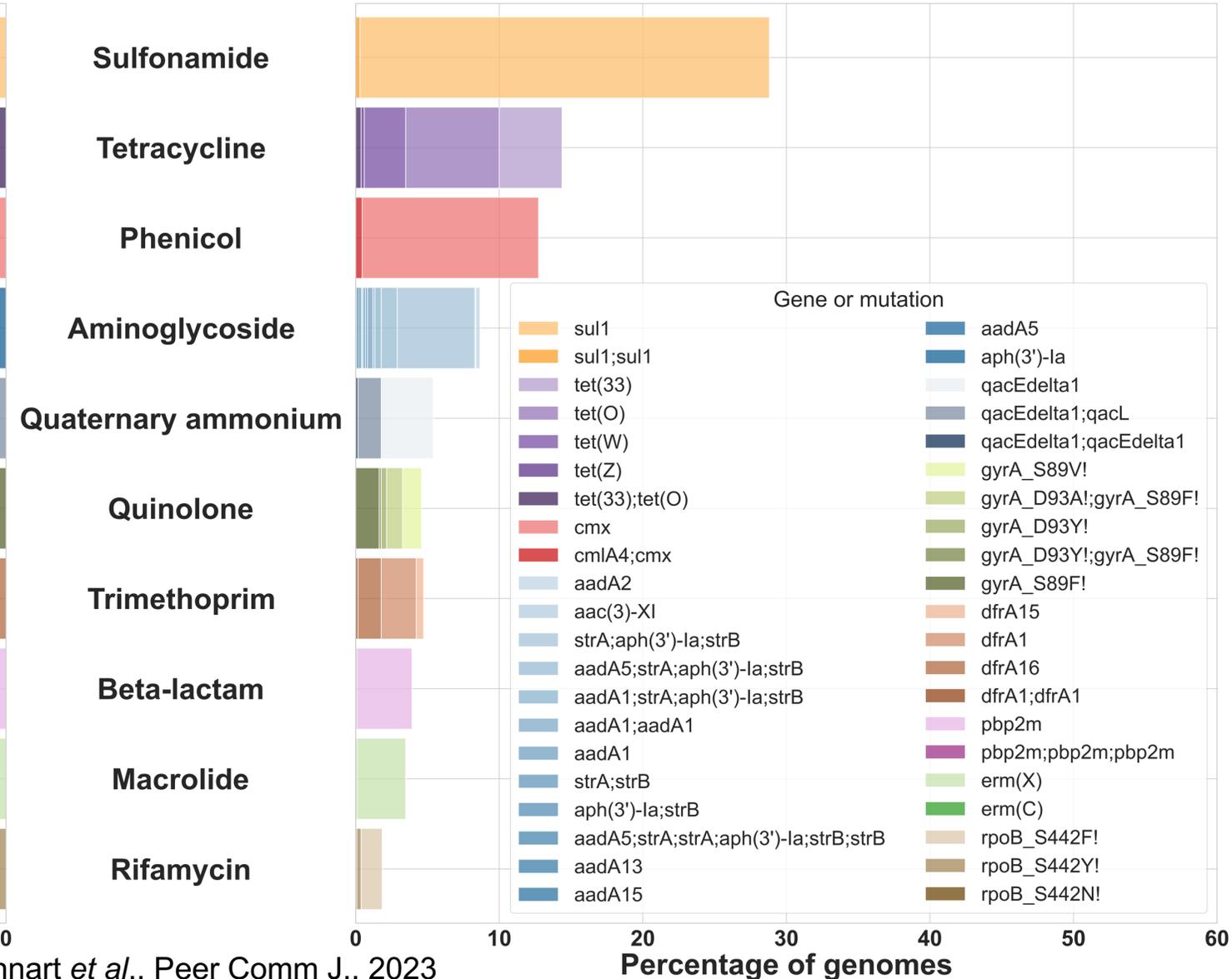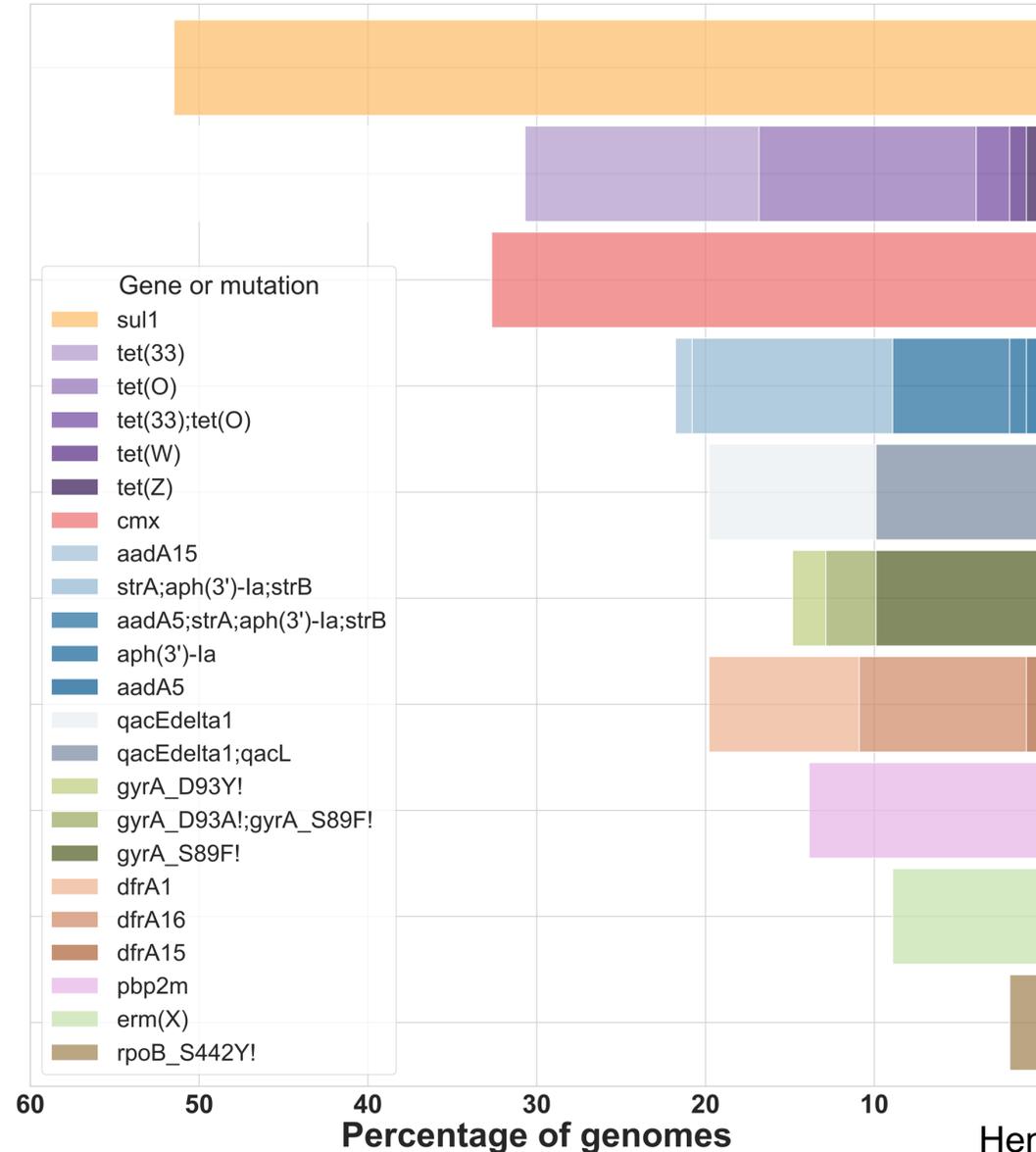
Emergence of PEN-R+MACRO-R+MDR C. diphtheriae

Hennart *et al*., Peer Comm J., 2023

- **France, 2022**
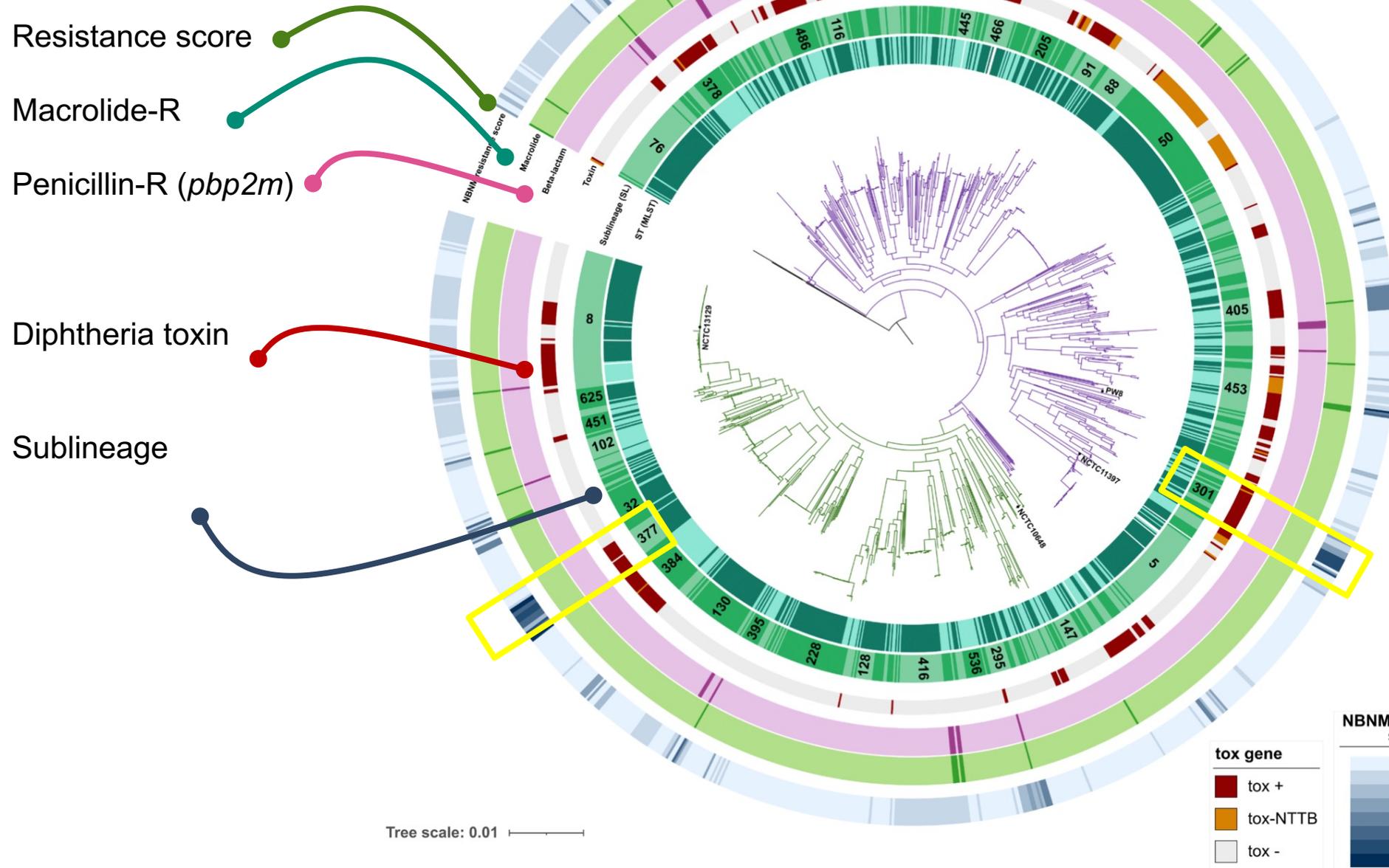
- **Origins: North/West Africa**

Peni-R, Ery-R, MDR (tox-neg.)

Peni-R, Ery-R, MDR

Travel history

- Near and Middle East
- North Africa
- West Africa
- Southern Africa
- Southern and South-Eastern Asia
- North America
- None
- Not documented

Population structure of *C. diphtheriae*

Resistance score

Macrolide-R

Penicillin-R (*pbp2m*)

Diphtheria toxin

Sublineage

MDR
+
tox

Dual-risk lineages

Macrolide-R
+
Penicillin-R (*pbp2m*):

Treatment response?

Tree scale: 0.01

tox gene
- tox +
- tox-NTTB
- tox -

NBNM resistance score
0
1
2
3
4
5
6
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diphtOscan: scanning *C. diphtheriae* genomes

- Taxonomic check / species identification

- Biovar-associated genes (*spuA* and *nar* clusters)

Mélanie Hennart *et al.*, 2023

- Virulence genes detection

Peer Community In Infections

- *tox* gene, and its possible truncations

- Resistance features (genes and mutations, and their co-localization)

- Integrons

- Phylogenetic tree (MASH)

DIPHTOSCAN

https://gitlab.pasteur.fr/BEBP/diphtoscan

# Conclusions: Antimicrobial agents and diphtheria

- Penicillin, or erythromycin, are recommended as first-line treatment

- Amoxicillin, clindamycin, vancomycin as alternative.

- There are few reports of penicillin resistance, always low level so far.

- There is 5-15% resistance to erythromycin, high-level

- Wound cultures positive for *C. diphtheriae* often are concurrently positive for group A *Streptococcus*, for which penicillin is the optimal antimicrobial agent
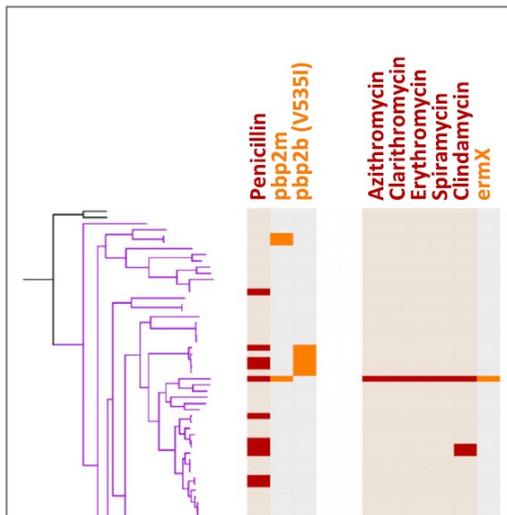
C. diphtheriae in the genomics era
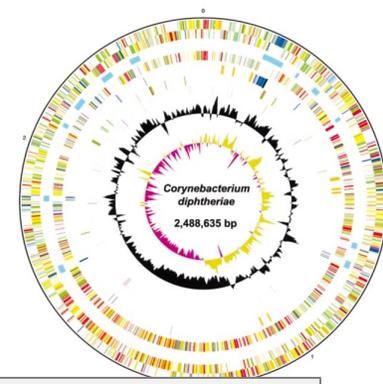
Phylogenetic diversity
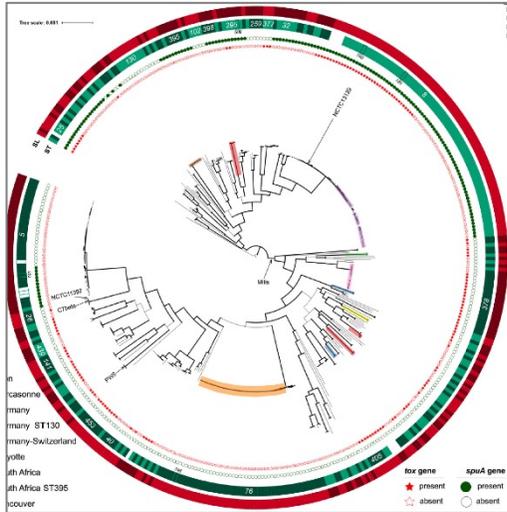
**Gene content
(phenotypic diversity)**

- Taxonomy of diphtheria agents

- Extended definition (*C. ulcerans*, skin infections): predominant in HICs

- Emergence of antimicrobial resistance & virulence

- Links genotype – clinical phenotype?

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