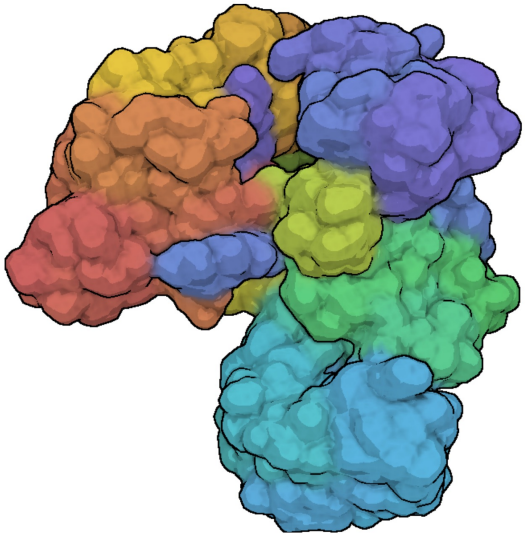
Leiden University
Medical Center

Structure of the replicative polymerase PolC in complex with the anti-CDI agent ibezapolstat and a related inhibitor

Mode of action and mechanism of resistance



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Conflict of interest statement

The POLSTOP2 project is co-funded by a PPP allowance made available by Health~Holland, Top Sector Life Sciences & Health, to stimulate public-private partnerships.

Health~Holland

Acurx Pharmaceuticals is a Consortium partner in this project.

ACURX PHARMACEUTICALS, INC.

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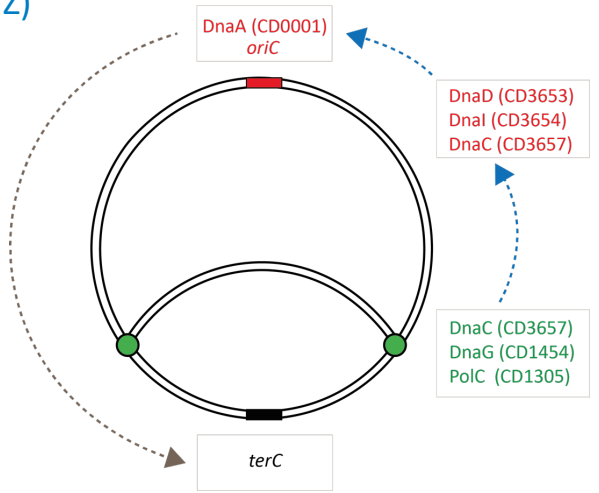
There is an urgent need for novel antimicrobials against *C. difficile*

- *C. difficile* epidemiology is influenced by antimicrobial resistance
  - Fluoroquinolone resistance in epidemic lineages
- Resistance to therapeutics is increasingly reported
  - Resistance to fidaxomicin, vancomycin (VAN) and metronidazole (MTZ)
- AMR in *C. difficile* is linked to decreased treatment success
  - Higher VAN/MTZ MICs are associated with reduced cure rates
- Pathogenesis of *C. difficile* is influenced by other organisms
  - *Enterococcus* spp. influence CDI development and outcomes

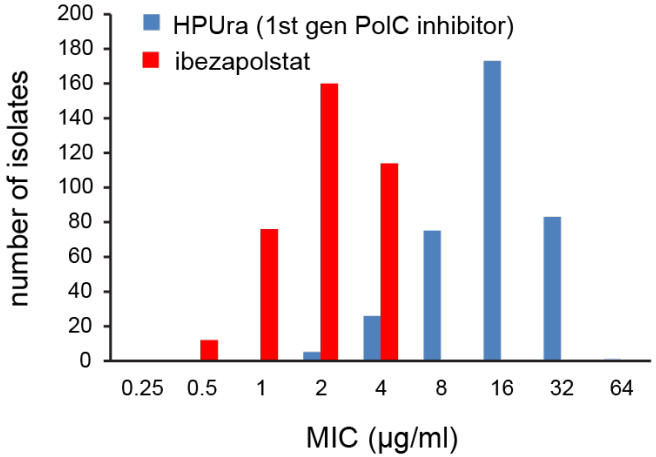
- DNA replication is a promising but underexplored target for antimicrobials



AMR: He (2013) Nat Genetics, Kolte (2024) J Antimicrob Chemother, Wickramage (2021) J Antimicrob Chemother
Interactions with *Enterococcus*: Smith (2022) Nature, Specker (2022) J Vis Exp, Smith (2024) mBio, Granata (2023) J Clin Microbiol

Ibezapolstat is a promising anti-CDI agent

- Ibezapolstat (IBZ, ACX-362E, GLS-362E)
- Novel target: replicative DNA polymerase (**PolC**; a.k.a. DNA pol IIIC)
- Improved activity towards *C. difficile* compared to "ancestral" HPUra
- No pre-existing (cross)resistance (new-to-nature)
- Phase 2: no recurrence and eradication of *C. difficile,* microbiome sparing



Van Eijk (2019) Antimicr Agents Chemother

Torti (2011) Curr Enzym Inhib
Dvoskin (2012) Antimicrob Agents Chemother
Xu (2019) Bioorg Med Chem
Garey (2020) J Antimicrob Chemother
Garey (2022) Clin Infect Dis
McPherson (2022) Antimicrob Agents Chemother
Bassères (2024) Antimicrob Agents Chemother

Ibezapolstat also has activity vs other G+ priority pathogens, suggesting broader utility for the class

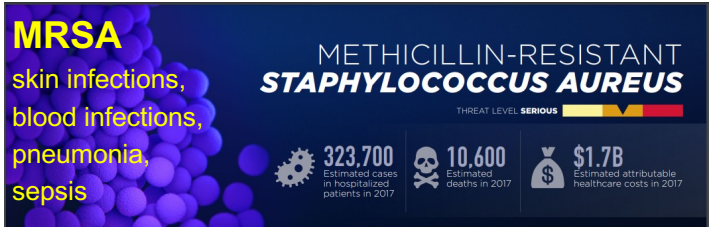
- PolC is conserved in Bacillota (Firmicutes)
- Similar minimal inhibitory concentrations
- No cross-resistance with existing classes of antimicrobials observed to date

Organism	Strain	IBZ minimal inhibitory concentration (mg/L) *
S. aureus (**MRSA**)	ATCC 29213	2
S. pneumoniae (**PRSP**)	ATCC 49619	8
E. faecium (**VRE**)	ATCC 700221	1
C. difficile	630	1
E. coli	ATCC 25922	>64

MIC testing performed by CRO, except for *C. difficile*
S. aureus also reported in Van Eijk (2019) Antimicr Agents Chemother

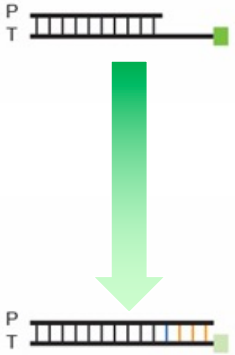
*because of low solubility and poor systemic absorption, IBZ is active only in the GI tract and will not be indicated to treat systemic infections

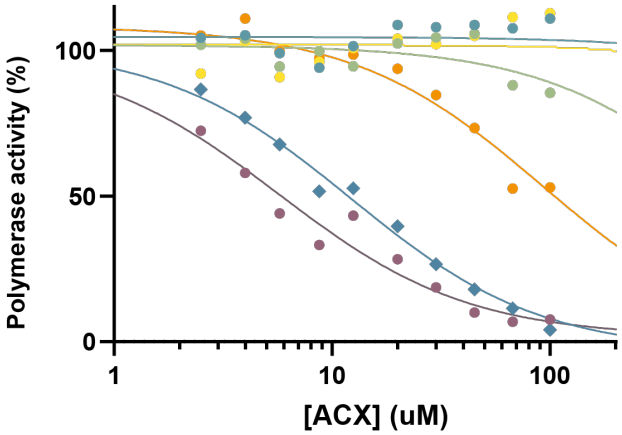
https://www.cdc.gov/antimicrobial-resistance/data-research/threats/index.html



A medium-throughput screen identified novel PolC-inhibitors with activity against PolC of VRE and MRSA

- Based on quenching of fluorescence in a primer extension reaction
- Screen of >50 ACX inhibitors (guanine analogs) selected for inhibitory activity against MRSA/VRE/PRSP
- IC_{50} – IBZ: 9 μM; ACX-801: 5 μM

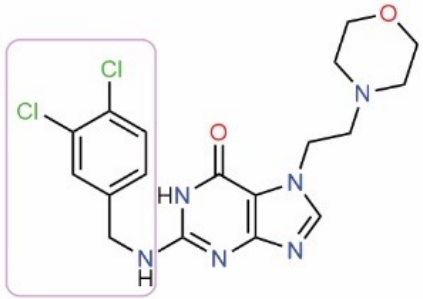




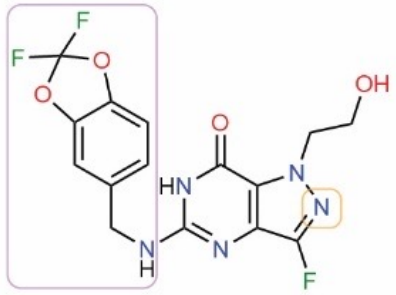
poor inhibition
- ACX
- ACX
- ACX

moderate inhibition
- ACX

good inhibition
- ACX-801
- IBZ



Ibezapolstat (IBZ)



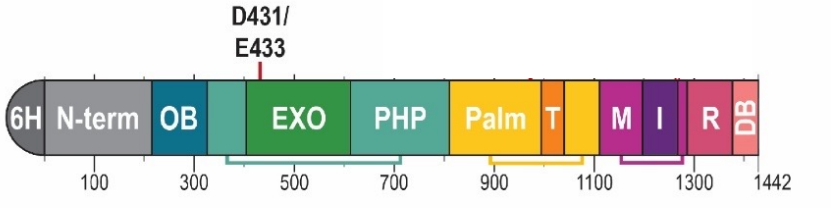
ACX-801

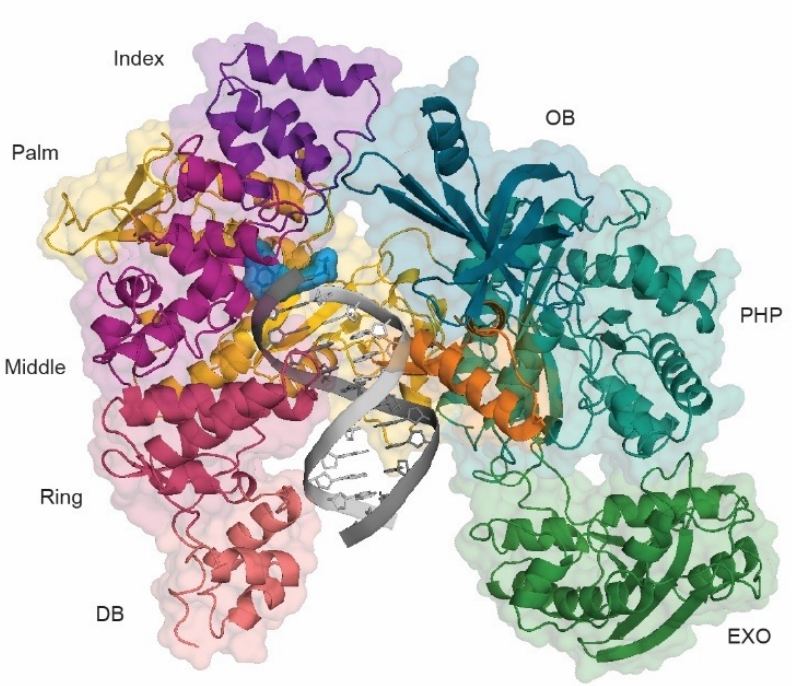
Toste Rego (2013) EMBO J
Song (2009) Biosens Bioelectron

3D structure of PolC:inhibitor complex was resolved by CryoEM at 2.8Å resolution

- Exonuclease-inactivated PolC, N-terminal 6xHistag
- Fork-mimicking oligonucleotide as DNA

- First PolC structure with an intact exonuclease domain
- Similar to Xray structure of PolC catalytic domain from *Geobacillus kaustophilus*

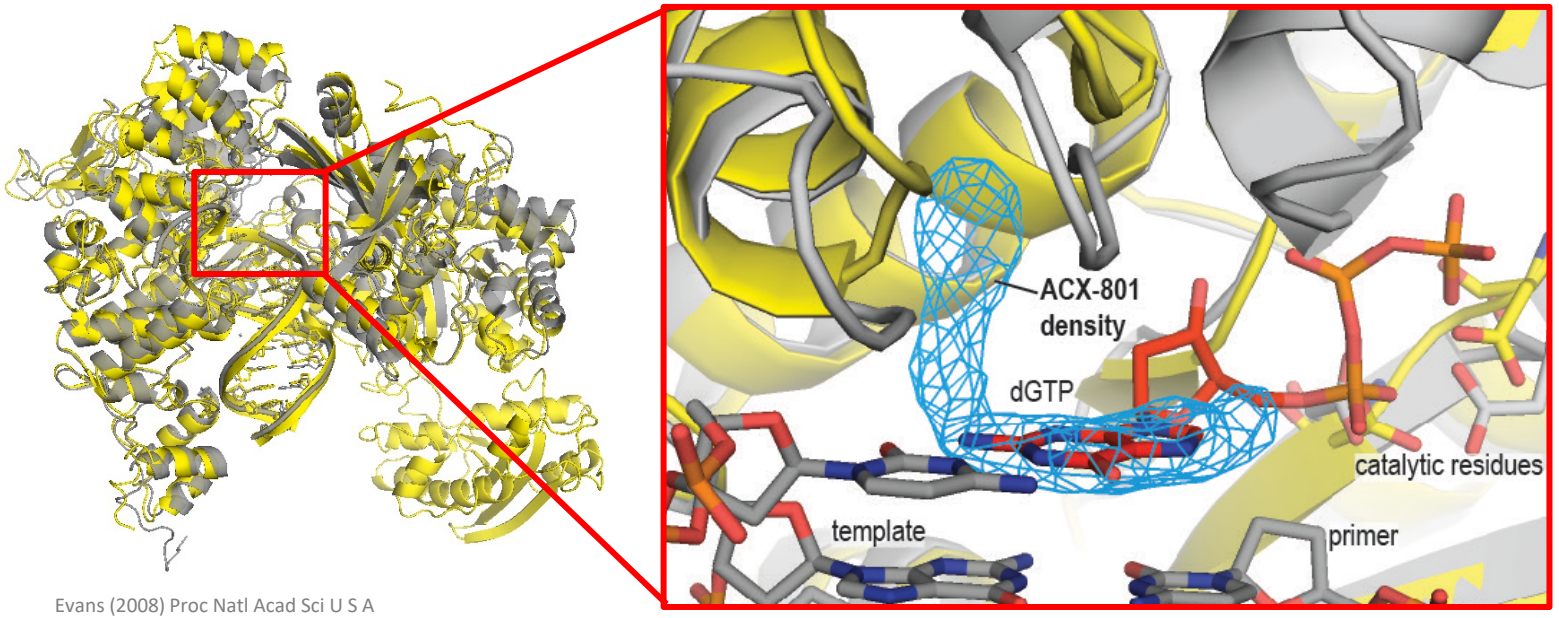


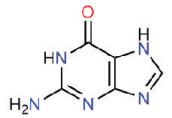


Evans (2008) Proc Natl Acad Sci U S A

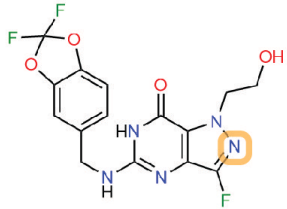
PolC:inhibitor complex provides direct evidence for a mechanism as guanine analog

- **ACX-801 density ~ guanine of dGTP, H-bond with template cytosine**
- Distant from catalytic residues for triphosphate hydrolysis





guanine



ACX-801

Evans (2008) Proc Natl Acad Sci U S A

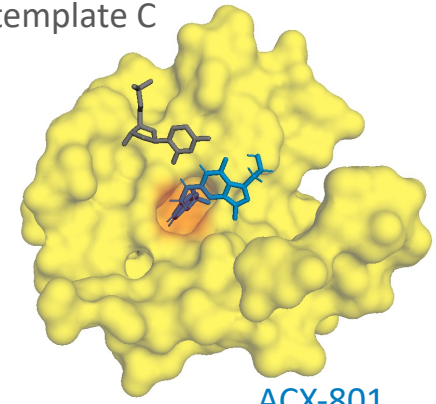
PolC:inhibitor complexes show an unusual conformation of the inhibitor at the binding site

- Pocket for moiety crucial for activity
- Strong bend in molecule
- Conservation in proximal residues
- Similar for ACX-801 (2.8Å) and IBZ (3.1Å)

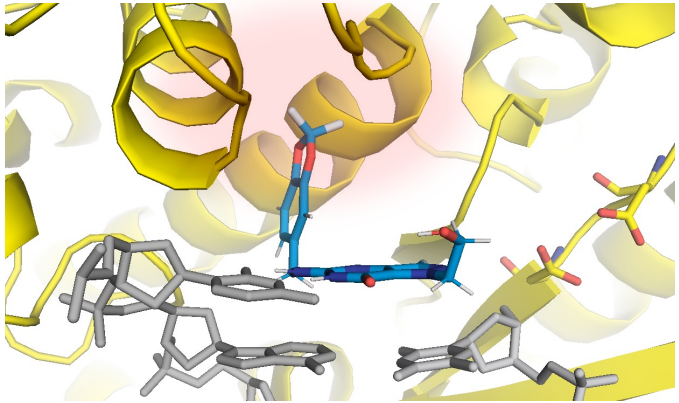
Residues < 4Å from PolC-inhibitor

892	1156	1181	1182	1185	1187	1274	1276	1277	1280	1281	1284	aa residue #
R	**E**	S	G	**H**	T	**Y**	**F**	P	**H**	**A**	**Y**	*E. faecium*
.	.	.	.	.	.	.	.	.	.	.	.	*S. aureus*
.	.	.	.	.	.	.	.	.	.	.	.	*S.pneumoniae*
.	.	.	.	.	.	.	.	.	.	.	.	*C. difficile*
.	.	.	.	.	.	.	.	.	.	.	.	*G. kaustophilus*

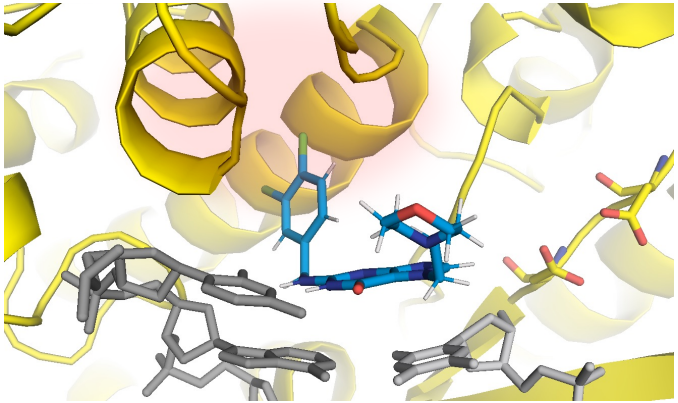
template C



ACX-801

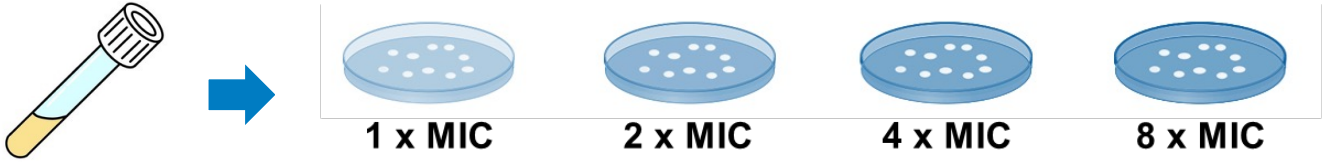


IBZ



Butler (1990) Nucleic Acids Res; Wright (2005 Bioorg Med Chem Lett ; Ruggieri (2023) Eur J Med Chem

polC mutations are identified in strains with reduced susceptibility to PolC inhibitors



- Multiple novel ACX compounds (CRO) or IBZ (LUMC) on multiple organisms
- Whole genome sequencing (PE150) + SNP calling
- The equivalent positions in *E. faecium* PolC: D1103Y, A1281T, F1276L/I/S
- Phenylalanine mutations also identified in study with ME-EMAU (another PolC inhibitor)

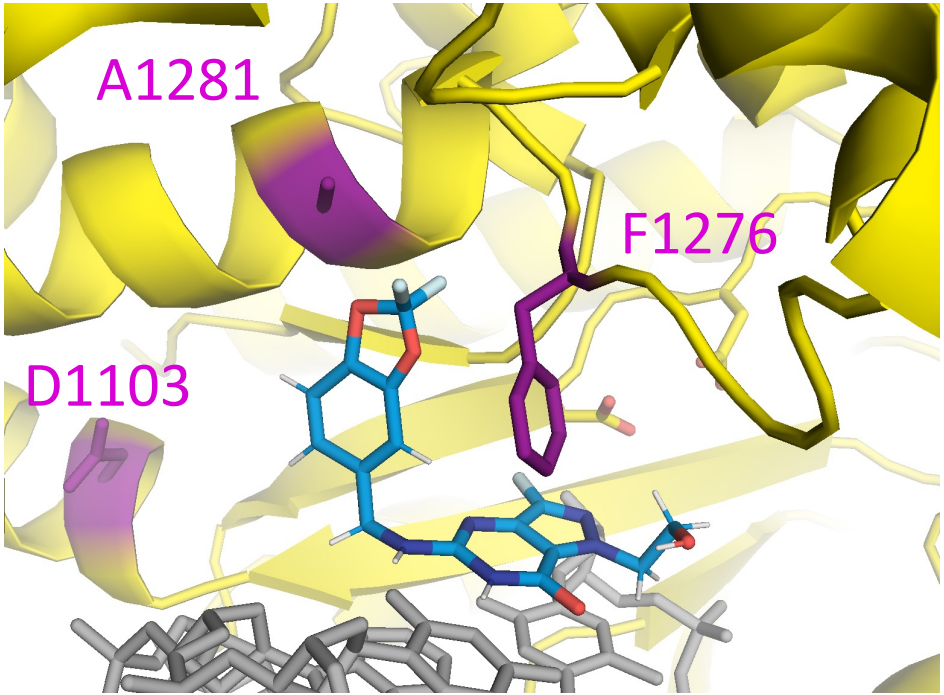
- PolC-inhibitor resistance determining region

  could be monitored for resistance mutations during clinical trials or clinical use of ibezapolstat

Nelson-Rigg (2023) Antimicrob Agents Chemother.

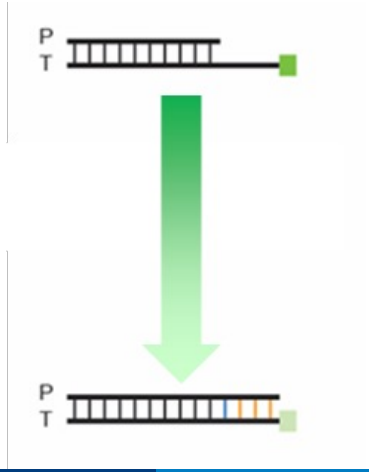
Structure shows that resistance positions are located adjacent to the PolC-inhibitor binding site

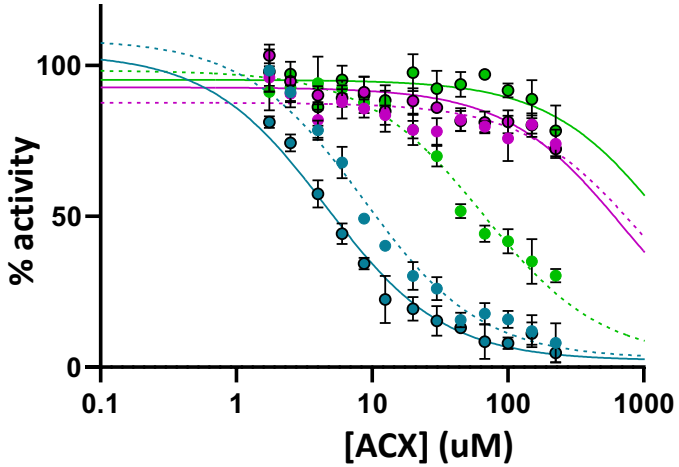
- Conserved A1281 and F1276 are within 4Å of ACX-801 binding
- Non-conserved D1103 is located further away (~9-10Å)
  - May affect DNA binding by PolC, necessary for drug interactions
- **Structural information provides an explanation for reduced susceptibility**



Mutations in PolC affect *in vitro* polymerase activity and inhibition by PolC-inhibitors

- A1281T: no activity in our real time assay; F1276L/S: clear polymerase activity (not shown)
- F1276L: poorly inhibited by IBZ and ACX-801
- F1276S: poorly inhibited by ACX-801, but modestly by IBZ
- Some mutations may confer more general resistance, whereas others may be more compound specific

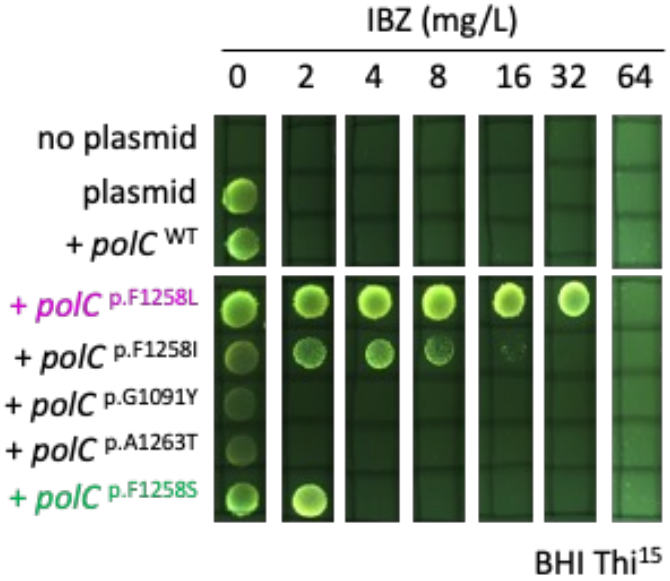




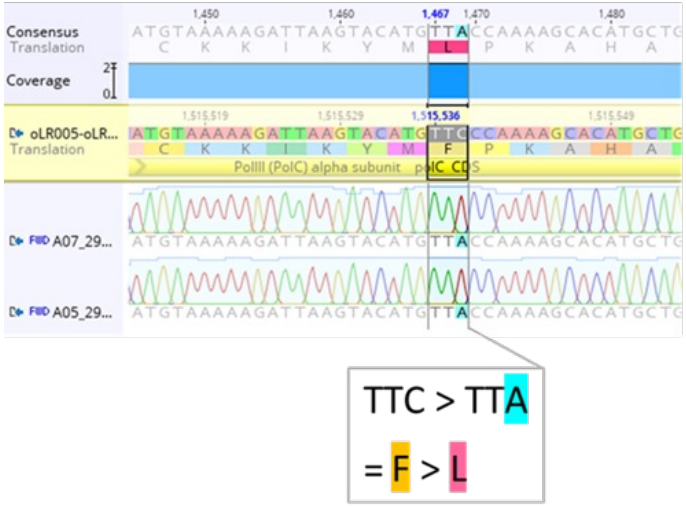
Condition	IC_{50} (uM)
PolC + IBZ	9
PolC + ACX-801	5
$PolC^{F1276L}$ + IBZ	993
$PolC^{F1276L}$ + ACX-801	717
$PolC^{F1276S}$ +IBZ	66
$PolC^{F1276S}$ + ACX-801	1545

Introduction of *polC* $^{p.F1258}$ mutations in *C. difficile* leads to reduced susceptibility towards ibezapolstat

- Plasmid carrying wild type or mutant *polC* alleles under native promoter
- Correlates with observed effect on *E. faecium* PolC inhibition by IBZ
- F1258L also identified in passage experiments



C. difficile polC $^{p.F1258L}$:



Key take-away points

- The active site in PolC is highly conserved between PolC's of Bacillota
- IBZ also inhibits *E. faecium* PolC (incl. VRE) -> IBZ does not foster VRE emergence
- CryoEM structures of PolC from *E. faecium* in complex with ACX-801 and IBZ show an unexpected conformation of the inhibitor
- Mutant strains with up to 16-fold higher MICs carry *polC* mutations in *C. difficile* and other Bacillota
- Introduction of *polC* $^{p.F1258}$ mutations in *C. difficile* leads to reduced susceptibility towards IBZ

Mode of action and the mechanism of reduced susceptibility to IBZ (and related compounds) are conserved within Bacillota

Structure data can guide rational design of new compounds with improved inhibitory activity and pharmacokinetic characteristics

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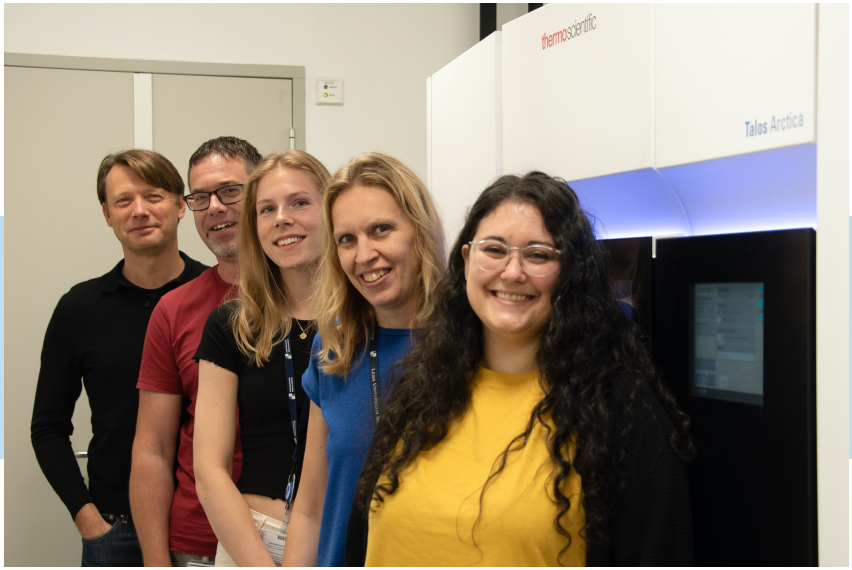

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- Netherlands Centre for Electron Nanoscopy

NeCEN