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Epitope Conservation of AZD5148, a Broadly Neutralizing Anti-Toxin B Monoclonal Antibody, Among Diverse and Global Contemporary *Clostridioides difficile* Isolates

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Background. *Clostridioides difficile* toxin B is a virulence factor for *C. difficile* infections and a clinically validated target for prevention of *C. difficile* infection recurrence. AZD5148 is a toxin B–neutralizing human monoclonal antibody that binds to an epitope on the toxin B glucosyltransferase domain. Herein, we aim to evaluate the conservation of this binding epitope using a collection of 9134 global *C. difficile* genomes obtained from a public repository to confirm that residues that are crucial for neutralization are conserved.

Methods. Using isolates collected between 2015 and 2023 from 2 independent sources, we performed variant calling, sequence typing, and phylogenetic analysis. We tested in vitro neutralization of cytotoxicity by AZD5148 using 2 cell lines.

Results. Herein, we showed that the AZD5148 epitope is highly conserved across all geographic regions and sequence types and has a higher average conservation frequency in its binding site (99.58%) when compared with bezlotoxumab (82.47%). AZD5148 also exhibited broad neutralization in vitro against 8 recently circulating ribotypes.

Conclusions. Our comprehensive analysis of global sequences found the AZD5148 epitope to be highly conserved and thus unlikely to be affected by most genotypic variations in recently circulating *C. difficile* ribotypes and sequence types.

Keywords. *C. difficile*; *C. difficile infection*; monoclonal antibody; toxin b.

Clostridioides difficile is a gram-positive, anaerobic, spore-forming bacterium transmitted via the fecal-oral route [1]. *C. difficile* infections (CDIs) cause an estimated 462 100 cases in the United States annually, with about 12 800 resulting in death [2]. The median cumulative incidence of having a recurrent CDI after an initial CDI was reported at 17% in a large systematic review of diverse studies. However, the risk of subsequent recurrences after the first recurrence increases substantially with the recurrence number [3].

The virulence of CDI is primarily mediated by the effect of toxin A (TcdA) and toxin B (TcdB) [4]. TcdB is a potent virulence factor and a clinically validated target [5–9], which initiates a multistep process starting with the combined repetitive oligopeptide sequences (CROPs) domain binding to a specific receptor, endocytosis, pore formation in acidified endosomes involving the delivery domain, and translocation of the glucosyltransferase domain (GTD) and autoprotease domain into the cytosol [10]. It was recently uncovered that the CROPs domain is often only conformationally required and its direct binding to a cell receptor is not necessary for intoxication or cell entry [11–13]. An anti-TcdB monoclonal antibody (mAb), Zinplava (bezlotoxumab), was approved in 2016 and recently withdrawn from the market by the sponsor [5]. Newer therapies for reduction of CDI recurrence, such as fecal microbiota transplant products, were approved in 2023 [14, 15]. Despite these key advances, there still exists an unmet clinical need for treatments for primary prevention or reduction of CDI recurrences that have a favorable safety profile, are easy to administer, and spare the microbiome.

We recently identified AZD5148: a TcdB–neutralizing human mAb that binds to an epitope on the GTD between residues 290 to 360 and prevents GTD export from the endosomes to the cytosol [16]. In this study, we use *C. difficile* molecular

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epidemiology data from 38 countries across 5 continents to examine the geotemporal prevalence of subtypes and show that the AZD5148 binding site on TcdB had low genomic diversity in recent years (2015–2023). We further assessed the effect of amino acid (AA) substitutions of key ribotypes (RT) on the neutralization by AZD5148 and compared that with a licensed anti-TcdB mAb.

METHODS

Public Sequences From EnteroBase

As of January 2024, a total of 35 921 *C. difficile* sequences were deposited into the EnteroBase repository. Sequences were collected globally between 1980 and 2024 and accompanied by metadata fields such as collection date, geographic region, and host species, among others.

Contemporary Sequences From International Health Management Associates

A total of 312 *C. difficile* clinical isolates from Europe and Japan collected between 2019 and 2022, were obtained from International Health Management Associates (IHMA).

Genetic Analysis of Assembled Genomes

Assembled genomes were annotated with Prokka (version 1.14.6), followed by extraction of the *C. difficile* TcdB sequence [17]. Multilocus sequence type (MLST) classification was conducted per the MLST tool (version 2.23.0) with allelic profiles of sequence types (STs) defined by the PubMLST *C. difficile* database [18]. Toxin typing was conducted via the Diffbase ToxinB database. Alignment for phylogenetic analysis was done with ParSNP. The reference sequence selected was a hypervirulent strain collected in 2006 from the United Kingdom and was classified by polymerase chain reaction (PCR) as ribotype 027 (GenBank FN545816.1; NAP1/BI/027 R20291). Assessment of AA sequence variation at the AZD5148 and bezlotoxumab binding sites was done by aligning TcdB sequences to the strain reference. Sequences with >5% ambiguous variant calls at the AA level were removed. Conservation frequency was calculated as the number of AAs matching the reference over the total number of sequences. Residue regions for each domain in the TcdB gene were determined from Kroh et al, wherein the AZD5148 binding touchpoints were defined by x-ray diffraction of the GTD of TcdB in complex with PA41 (PDB ID: 5VQM [16]). Bezlotoxumab binding touchpoints were taken from the x-ray diffraction of bezlotoxumab in complex with the CROPs domain of TcdB (PDB ID 4NP4 [19]) (Supplementary Methods).

Toxin Neutralization Assay In Vitro

The toxins TcdB RT-002, RT-003, RT-014, RT-023, RT-036, RT-078, and RT-106 were purchased from TgcBIOMICS (Bingen). The toxins TcdB RT-017 and RT-027 were produced in-house by using *Bacillus megaterium* as an expression system [20]. Vero cells were seeded in a 96-well white wall clear bottom plate (Greiner) at 1.5×10^3 cells/well/100 µL in culture media and incubated overnight at 37 °C and 5% CO_2. The next day, mAbs were 2-fold serially diluted in 0.075 mL of culture media (starting concentration in Supplementary Table 1) and incubated with 0.075 mL of TcdB giving 90% cytotoxicity (LD_{90}) in a U-bottom 96-well plate (Nunc) for 30 minutes at 37 °C. Media on the Vero cell plate was aspirated, and 100 µL of the mAb/toxin B mixture was transferred to the plate. Cells were then incubated at 37 °C in a 5% CO_2 incubator for 48 hours. The plate was then placed at room temperature for 20 minutes and shaken for 2 minutes at 500 rpm before addition of Cell Titer Glo (Promega) for 10 minutes at room temperature. Data were acquired via a Spark Multimode Microplate Reader (Tecan) and plotted as percentage viability as compared with cells with media only. The experimental conditions were similar for Caco-2 cells, except that cells were incubated with the TcdB LD_{90} and mAbs for 72 hours before the addition of Cell Titer Glo. Prism version 9.4 (GraphPad) was used for data analysis. X values were transformed with the equation $X = \log(X)$, followed by nonlinear regression analyses based on log(agonist) vs response (3 parameters) where $y = \frac{bottom+(top-bottom)}{1+10^{(logEC50-x)}}$.

Ethical Approval Statement

This article does not contain any studies involving animals or humans performed by any of the authors.

RESULTS

Geotemporal Distribution of the Dataset

AZD5148 targets the GTD region of TcdB (Figure 1*A*). We queried EnteroBase for *C. difficile* genomes collected from January 2015 to December 2023. Out of the 11 691 genome sequences collected, 9285 sequences passed our predefined inclusion criteria (Figure 1*B*). The remaining strains were either TcdA+ or double-negative TcdB–/TcdA–. PCR-determined ribotyping is a traditional approach used for classifying *C. difficile* strains into subtypes; however, with recent advancements of sequencing technology, in silico MLST has become a standard for characterizing strains, especially where PCR ribotype is not possible. The majority of these sequences could be classified into an ST by using in silico approaches (98.4%, 9134/9285), although only 8.2% (762/9285) had associated PCR-determined ribotype information due to limited availability in this database.

The 9134 *C. difficile* sequences with an assigned ST were broadly distributed geographically and temporally (Figure 1*C* and 1*D*, respectively). North America represented the highest cumulative proportion of isolates over time (61.80%, 5645/9134). With respect to countries, the highest representation during this 9-year period was from the United States

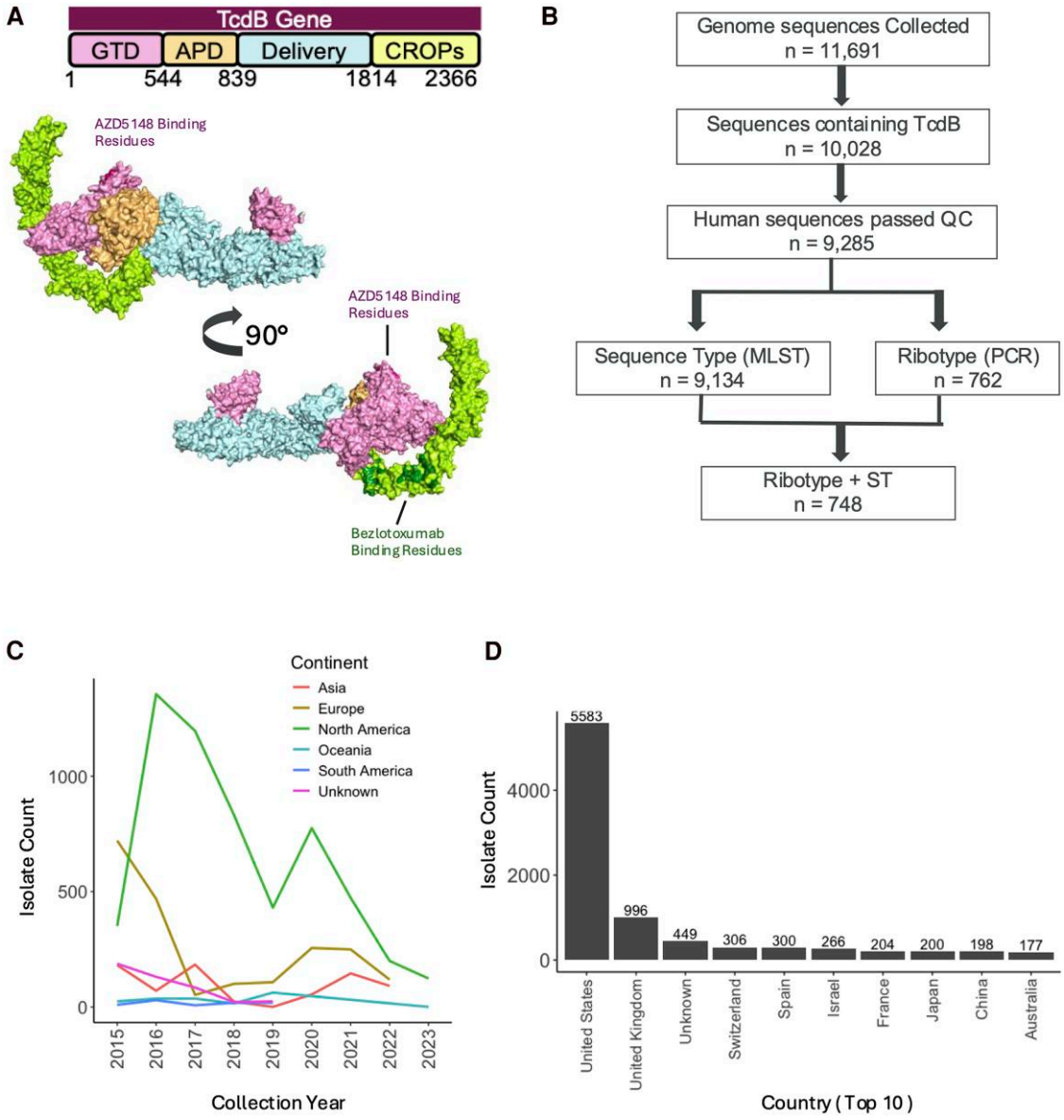

Figure 1. Geotemporal distribution of dataset. *A*, Genomic position of all TcdB gene domains (top)—including the glucosyltransferase domain (GTD; pink), autoprotease domain (APD; orange), delivery domain (blue), and a combined repetitive oligopeptide sequences (CROPs) domain (green)—and structural representation of each domain (bottom), including the AZD5148 binding epitope (dark purple) and the bezlotoxumab binding epitope (dark green). *B*, Schematic of the filtering parameters used when processing 11 691 total genomes. *C*, Line plot of the geotemporal distribution of 9134 *C. difficile* genomes collected in this dataset. Lines are colored by continent. *D*, Bar plot of the total isolate count for each country. Thirty-eight countries from North America, Europe, Asia, Oceania, and South America were represented in the dataset. The top 10 most representative countries are shown in this plot. MLST, multilocus sequence type; PCR, polymerase chain reaction; QC, quality control; ST, sequence type; TcdB, *C. difficile* toxin B. Panel *A* (top) adapted from: Kroh et al [16]. Licensed under the Creative Commons Attribution 4.0 International License (CC BY 4.0, http://creativecommons.org/licenses/by/4.0/). Disclaimer: This material has been adapted from the original publication. The adaptation does not imply endorsement by the original authors or publisher.

(61.12%, 5583/9134). Most sequences were collected before 2019 (67.30%, 6147/9134; Supplementary Figure 1).

Next, we sought to understand the genetic diversity of the *C. difficile* strains by determining their molecular STs and the associated clades. In our dataset, 290 unique STs were identified, representing 5 ST clades. Overall, most sequences belonged to clade 1 (75.16%, 6865/9134) and clade 2 (13.07%, 1194/9134; Figure 2*A*). In terms of STs, most sequences belong to ST-002 (12.57%, 1148/9134; Figure 2*B*). Due to limited availability of PCR-determined ribotype information from EnteroBase, only 748 isolates could be fully characterized with their ribotype, ST, and clade, representing 8.12% of the selected strains from the database. Overall, the ribotypes with the highest representation were RT-078 (12.86%, 98/762), RT-014 (8.79%, 67/762), and the hypervirulent RT-027 (7.74%, 59/762; Figure 2*C*).

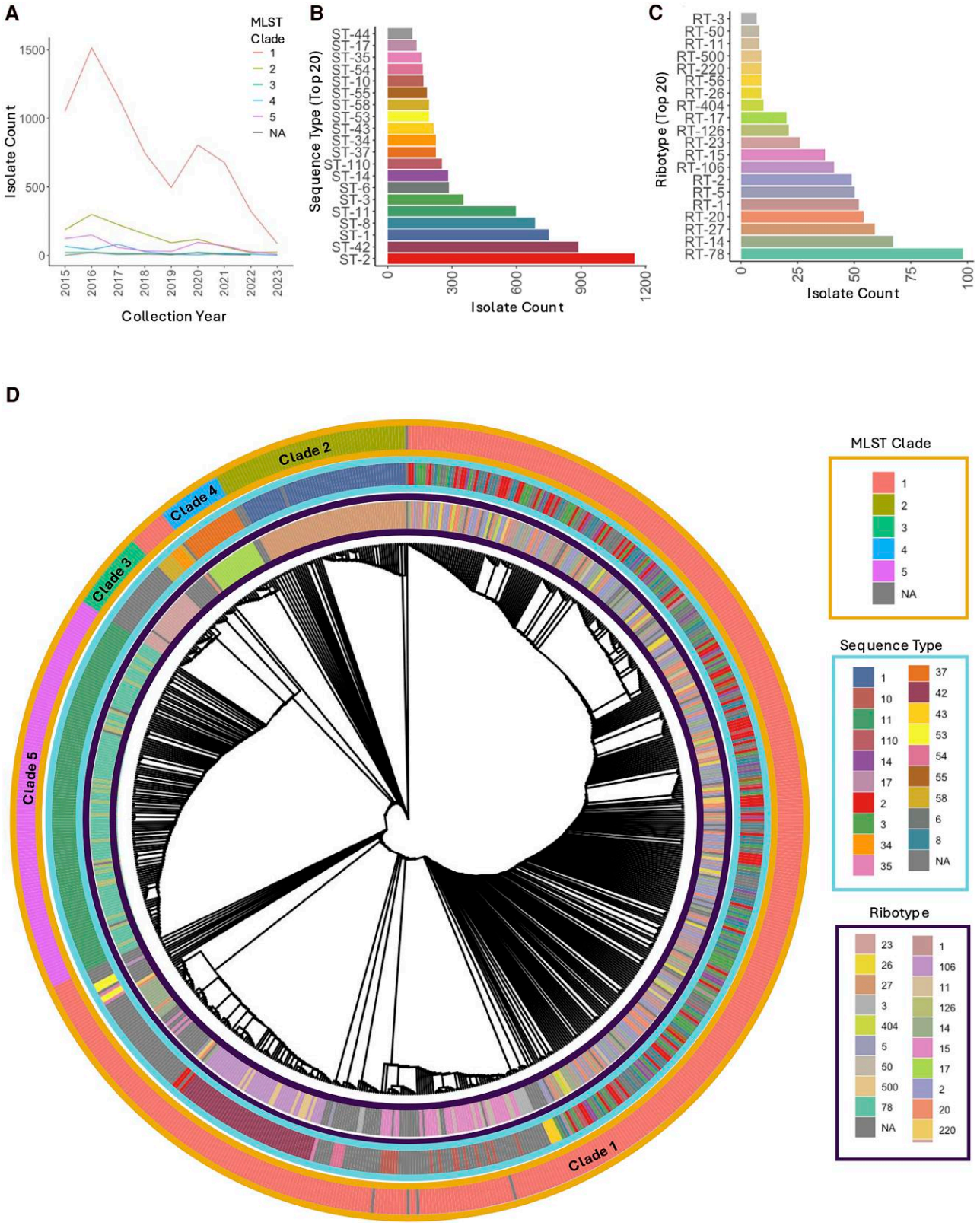

Figure 2. Molecular epidemiology of frequently occurring ribotypes (RTs) and sequence types (STs) in *Clostridium difficile* EnteroBase sequences. *A*, Line plot of the temporal distribution of 9134 *C. difficile* genomes collected in this dataset. Lines are colored by MLST clade. *B*, Bar plot of the total isolate count for each ST. The top 20 most representative STs are shown in this plot. *C*, Bar plot of the total isolate count for each RT. The top 20 most representative RTs are shown in this plot. *D*, Phyologenetic tree of the 748 sequences with RT and ST information without branch length. Annotation row: orange, MLST clade; blue, ST; purple, RT. The top 20 RTs and STs are included, and *NA* indicates others that are not in the top 20. MLST, multilocus sequence type.

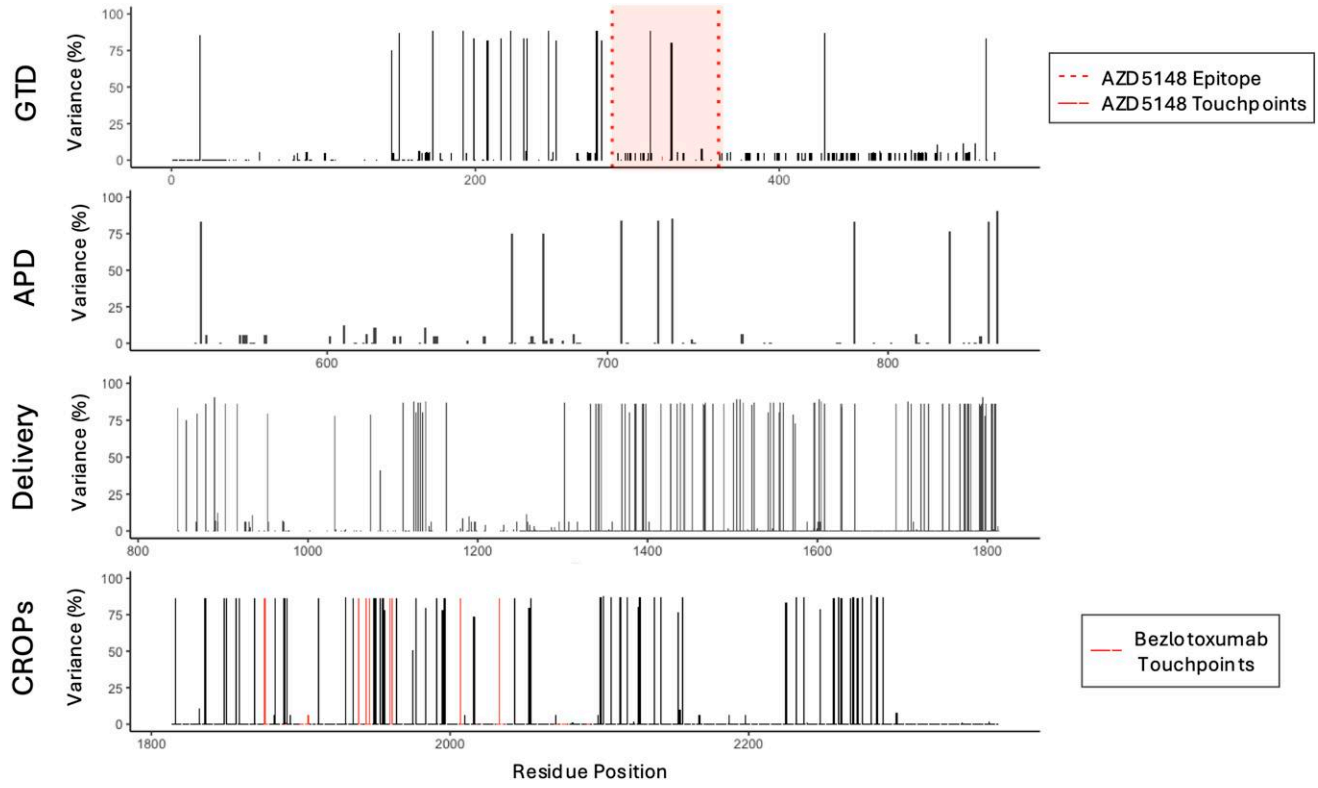

Figure 3. TcdB domain variance in *Clostridium difficile* EnteroBase sequences. Data show the variance percentage of each genomic position across the TcdB gene separated by the 4 domains. The AZD5148 epitope is highlighted in red in the GTD, and bezlotoxumab touchpoints are highlighted in red in the CROPs domain. APD, autoprotease domain; CROPs, combined repetitive oligopeptide sequences; GTD, glucosyltransferase domain; TcdB, *C. difficile* toxin B.

Next, we investigated the geotemporal distribution of STs to elucidate outbreaks of ST clades over the last 9 years and found that clade 1 was again the most prevalent in all years. In all clades, apart from clade 4, prevalence decreased between 2016 and 2019 and increased in 2020, before declining from 2020 to 2023. Clade 4 prevalence decreased between 2015 and 2016 from 0.73% to 0.47% and increased in 2017 to 0.91%, before declining in 2023 to 0.02%. Of all clade 4 isolates, Asia had the highest prevalence (1.41%; Supplementary Table 2).

To increase representation of contemporary isolates from geographic regions not well represented in this dataset, we obtained recently circulating *C. difficile* isolates across Europe and Asia from IHMA (Supplementary Figure 2A). Out of the 312 sequences, 237 contained the TcdB gene and all were classified into an ST. In the isolate subset, 57.38% of the isolates were from 6 European countries (136/237; Supplementary Figure 2B), and 42.62% (101/237) were from Asia, specifically Japan. We observed good agreement between the STs identified in both datasets, and 6 of the top 10 STs were identical. Consistent with the EnteroBase dataset, most isolates belong to clade 1 (78.48%, 186/237; Supplementary Figure 2C). ST-002 accounted for 14.77% of all STs (35/237; Supplementary Figure 2D).

Phylogenetic Analysis

To elucidate the phylogenetic distribution of strain subtypes, we built a whole genome phylogenetic tree using the 748 isolates with ribotype, ST, and clade information (Figure 2*D*). In most cases, strains with PCR-determined ribotypes are associated with 1 ST clade. Most STs and ribotypes were also associated (Supplementary Table 3, Supplementary Figure 3A). All the isolates from clade 4 belong to RT-017 (Figure 2*D*). Isolates from clade 1 belong predominately to RT-020 (9.67%, 50/517) and RT-005 (9.67%, 50/517). Similarly, a phylogenetic tree, constructed with all 9134 sequences, showed that the sequences grouped in a similar manner by ST clade (Supplementary Figure 3B). This information could be used with known ST to infer the ribotypes, where unknown.

TcdB Domain Variance and Conservation in *C. difficile* Isolates

To estimate the degree of conservation of the TcdB sequence among all 9134 isolates, the percentage of variance was calculated for all AA residues within each TcdB domain by using the R20291 strain as the reference (hypervirulent RT-027; GenBank FN545816.1). The GTD was the most conserved domain (96.75% average conservation), and the CROPs domain was the least conserved (90.08% average

conservation; Figure 3). The percentage of conservation was then calculated for each domain and stratified by ST clade (Supplementary Figure 4). In the GTD, clades 1, 2, 3, and 5 have conservation >96% in both datasets, with clade 4 showing the lowest conservation (78.87%). In the CROPs domain, clade 2 has the highest conservation (98.64%). This analysis concludes that TcdB conservation is dependent on strain subtype.

AZD5148 Binding Site Is Highly Conserved

Although the overall conservation was high, we still observed some genomic variations within the AZD5148 binding epitope (Figure 4*A*). The binding epitope of AZD5148 was defined by an x-ray–determined crystal complex, which can be visualized within the TcdB structure in Figure 1*A* (PDB ID 5VQM). The epitope has an average conservation frequency of 96.57%, and the binding residues have an average conservation frequency of 99.73%. Of these changes, clade 4 substitutions at residue 323 are present in a binding residue but at a low frequency. A global conservation frequency of 97.05% was observed at binding site residue 323 (Supplementary Table 4), 100% of which was represented in clade 4 (Supplementary Table 5). At residue 323, a low frequency of isolates has tyrosine substituted by histidine (2.95% of all isolates). In 1 sequence from clade 1, we found a substitution from leucine to isoleucine at position 350 (Figure 4*B*). AZD5148 conservation was also analyzed for each toxin type and is included in Supplementary Table 6. These data are recapitulated in the IHMA dataset, and a tyrosine-to-histidine change occurred in only 3.38% of isolates (Figure 4*B*). Lastly, of the isolates with a Y323H change within clade 4, most belong to ST-037 (n = 223, EnteroBase; n = 3, IHMA) or ST-081 (n = 36, EnteroBase; n = 5, IHMA; Figure 4*C*).

Focusing on Y323H, we found that the 268 sequences with this substitution were identified in 12 countries. The largest number of sequences with this substitution came from Asia, which represents 17.7% (129/730) of all Asia sequences, with very low frequencies observed in North America (1.8%) and Europe (1.7%). When normalized to the total sequences deposited, the top 2 countries represented were from China (26.3%, 52/198) and Thailand (93.0%, 40/43), consistent with previous observations that RT-017 is more predominant in Asia (Figure 4*D*).

Bezlotoxumab Binding Site Conservation in *C. difficile* Sequences Is Lower Than AZD5148

We next evaluated the conservation of the binding site of bezlotoxumab using a similar approach as AZD5148 (Figure 5*A* and 5*B*). The binding epitope of bezlotoxumab on TcdB was defined by an x-ray–determined crystal complex, as shown in Figure 1*A* (PDB ID: 4N4P). When compared with the R20291 strain, 9 of 40 residues show low conservation frequency with AA residues 1961 and 2033, with 13.66% conservation. The bezlotoxumab binding sites have an average conservation frequency of 82.47% when aligned to this reference (Supplementary Tables 7 and 8). Of the most variable residues, residue 1961 changes from glutamic acid to aspartic acid (13.66%, 1248/9134), and residue 2033 changes from glutamic acid to alanine (13.66%, 1248/9134) or serine (6.59%, 602/9134; Figure 5*B*). Bezlotoxumab conservation was also analyzed for each toxin type and is included in Supplementary Table 6.

AZD5148 Exhibited Broad Neutralizing Activity In Vitro

To understand whether the variation on TcdB GTD affects AZD5148 functionality, we next tested AZD5148 neutralizing activity against TcdB ribotypes in vitro using 2 cell lines. We used purified TcdB from the most clinically relevant *C. difficile* ribotypes: RT-002 (unknown clade, toxin type B1), RT-003 (clade 1, toxin type B1), RT-014 (clade 1, toxin type B1), RT-017 (clade 4, toxin type B3), RT-027 (clade 2, toxin type B2), RT-036 (unknown clade and unknown toxin type), RT-078 (clade 5, toxin type B5), and RT-106 (clade 1, toxin type B1) [10].

AZD5148 and bezlotoxumab exhibited binding affinity by enzyme-linked immunosorbent assay against TcdB from most ribotypes, with a few notable exceptions. AZD5148 showed no binding affinity against RT-017, whereas bezlotoxumab showed lower binding affinity against RT-027, RT-078, and RT-106 (Supplementary Figure 5). The binding interactions of AZD5148 and bezlotoxumab were validated by Octet biolayer interferometry. Notably, for RT-027, the affinity constant (KD) is higher for bezlotoxumab when compared with AZD5148 (Supplementary Table 9), suggesting that AZD5148 binds better.

AZD5148 exhibited potent neutralizing activity against different toxin B ribotypes, with mAb concentration corresponding to 50% inhibition (IC_{50}) ranging between 0.007 to 0.071 ng/mL in Vero cells and with up to 543-fold greater IC_{50} potentiation as compared with bezlotoxumab (Figure 6*A* and 6*B*, Supplementary Table 10). Similarly, AZD5148 exhibited IC_{50} ranging from 0.004 to 0.781 ng/mL in human colorectal cancer Caco-2 cells and up to 59.2-fold greater IC_{50} potentiation as compared with bezlotoxumab (Supplementary Figure 6A and 6B). In the Vero cell line, the maximum neutralization across all ribotypes in the presence of AZD5148 was consistently >80%, whereas in the Caco-2 cell line, AZD5148 exhibited a maximum neutralization of 60% for RT-027 at a concentration 100 times higher than for the Vero cells. Accordingly, the 2 cell lines exhibited different sensitivities to TcdB RT-027, with LD_{90} values of 5 ng/mL and 10 pg/mL for Caco-2 and Vero cells, respectively. This can be attributed to some GTD-independent cytotoxic effects that have been reported in vitro [21, 22]. Importantly, these differences in neutralizing activity across the cell lines may not be physiologically relevant, as we observed protective efficacy in preliminary preclinical analyses from a gnotobiotic piglet model of CDI induced by the NAP1/BI/027 hypervirulent strain [23].

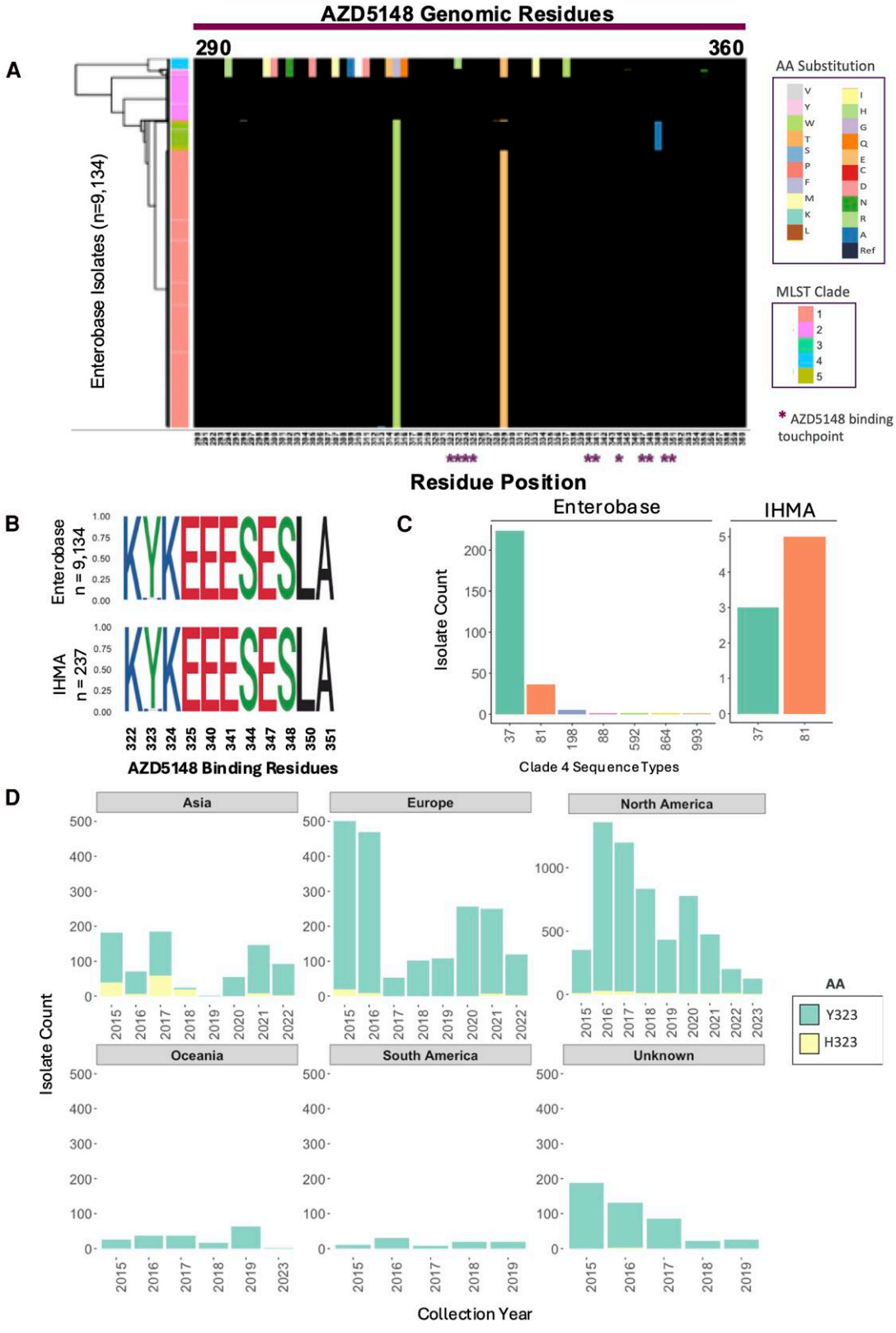

Figure 4. AZD5148 binding site conservation in *C. difficile* sequences from EnteroBase and IHMA. *A*, The heat map shows AA changes among EnteroBase isolates (y-axis) in each genomic residue of the AZD5148 binding epitope (x-axis). Each row represents an individual isolate and is hierarchically clustered in an unbiased fashion. The rows are annotated by MLST clade. Each color in the heat map represents an AA change, with black being the reference AA. The AZD5148 touchpoints are designated by an asterisk at each position. *B*, Logo plot of the AZD5148 touchpoints in the EnteroBase (top) and IHMA (bottom) datasets. *C*, Bar plot of the isolate counts for all clade 4 STs in the EnteroBase (left) and IHMA (right) datasets. *D*, Stacked bar plot of the isolate counts for position 323 across each year and separated by geographic region. Histidine (H323) is in yellow and tyrosine (T323) is in blue. AA, amino acid; IHMA, International Health Management Associates; MLST, multilocus sequence type.

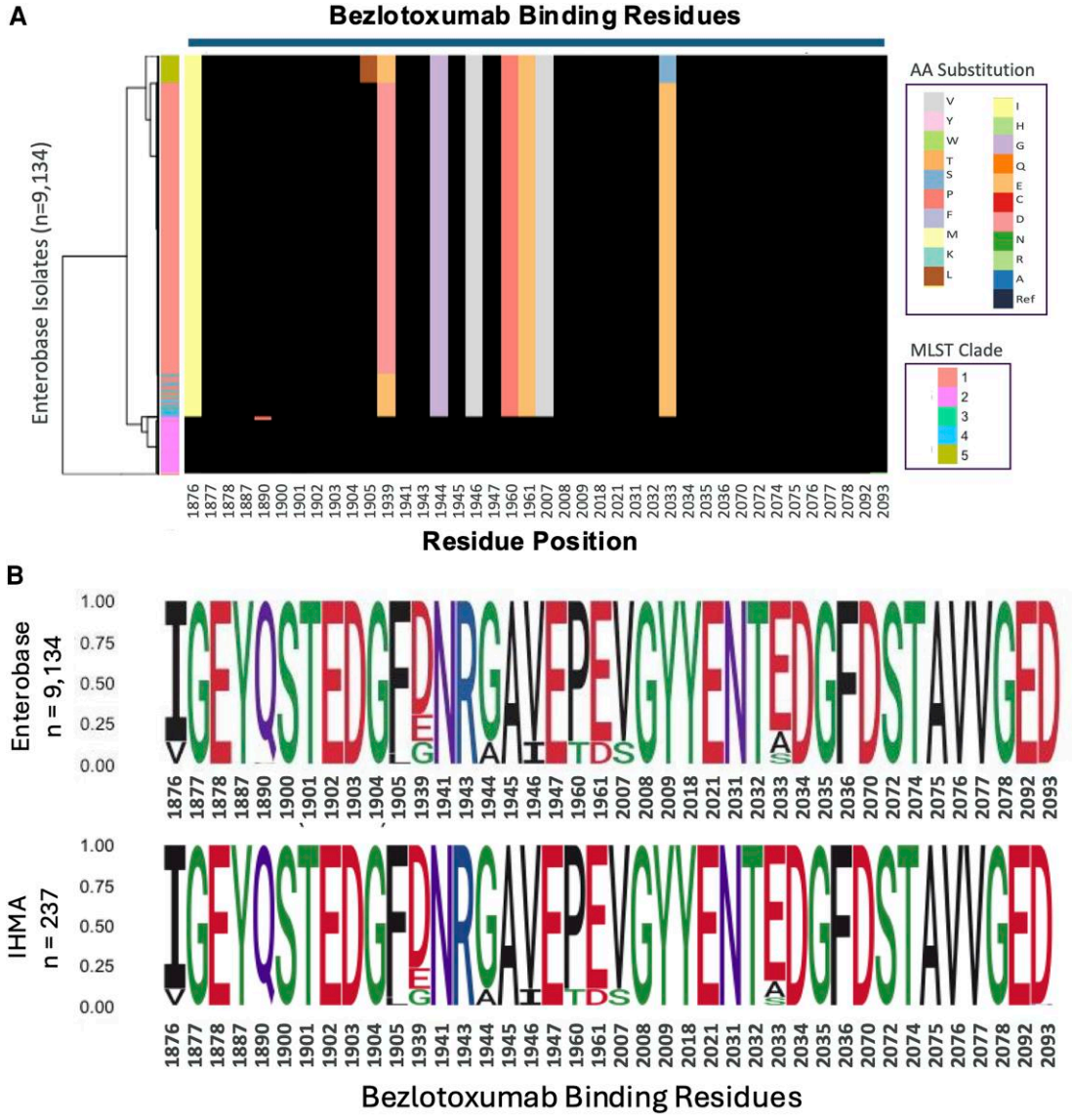

Figure 5. Bezlotoxumab binding site conservation for *Clostridium difficile* EnteroBase and IHMA sequences. *A*, Heat map shows AA changes among EnteroBase isolates (y-axis) in each genomic residue of the bezlotoxumab binding sites (x-axis). Each row represents an individual isolate and is hierarchically clustered in an unbiased fashion. The rows are annotated by MLST clade. Each color in the heat map represents an AA change, with black being the reference AA. *B*, Logo plot of the bezlotoxumab touchpoints in the EnteroBase (top) and IHMA (bottom) datasets. AA, amino acid; IHMA, International Health Management Associates; MLST, multilocus sequence type.

DISCUSSION

C. difficile continues to be a burden on health care, with global annual costs >$5 billion [24]. While the anti-TcdB mAb Zinplava (bezlotoxumab) has been licensed and showed partial reduction of CDI recurrence [5, 25], its supply has been discontinued in some European Union member states and in the United States [26]. Therefore, there still remains an unmet clinical need for primary prevention and reduction of recurrence. In the present study, we report that AZD5148, an anti-TcdB mAb, shows broad neutralizing coverage in vitro against clinically relevant *C. difficile* ribotypes and has a highly conserved binding epitope in 9134 *C. difficile* genomes obtained globally between 2015 and 2023. To our knowledge, this is the largest molecular surveillance study characterizing the variability of TcdB functional domains.

The TcdB gene contains 4 functionally distinct domains, and variations within these sequences could contribute to changes in toxigenic activity. Our analysis of variation within these domains revealed that the GTD was the most globally conserved domain and the CROPs was the least conserved in the evaluated sequences. The GTD showed high conservation in all clades regardless of strain ST.

One substitution found in the AZD5148 binding site, Y323H, was previously reported to occur in RT-017 strains [16, 27]. The

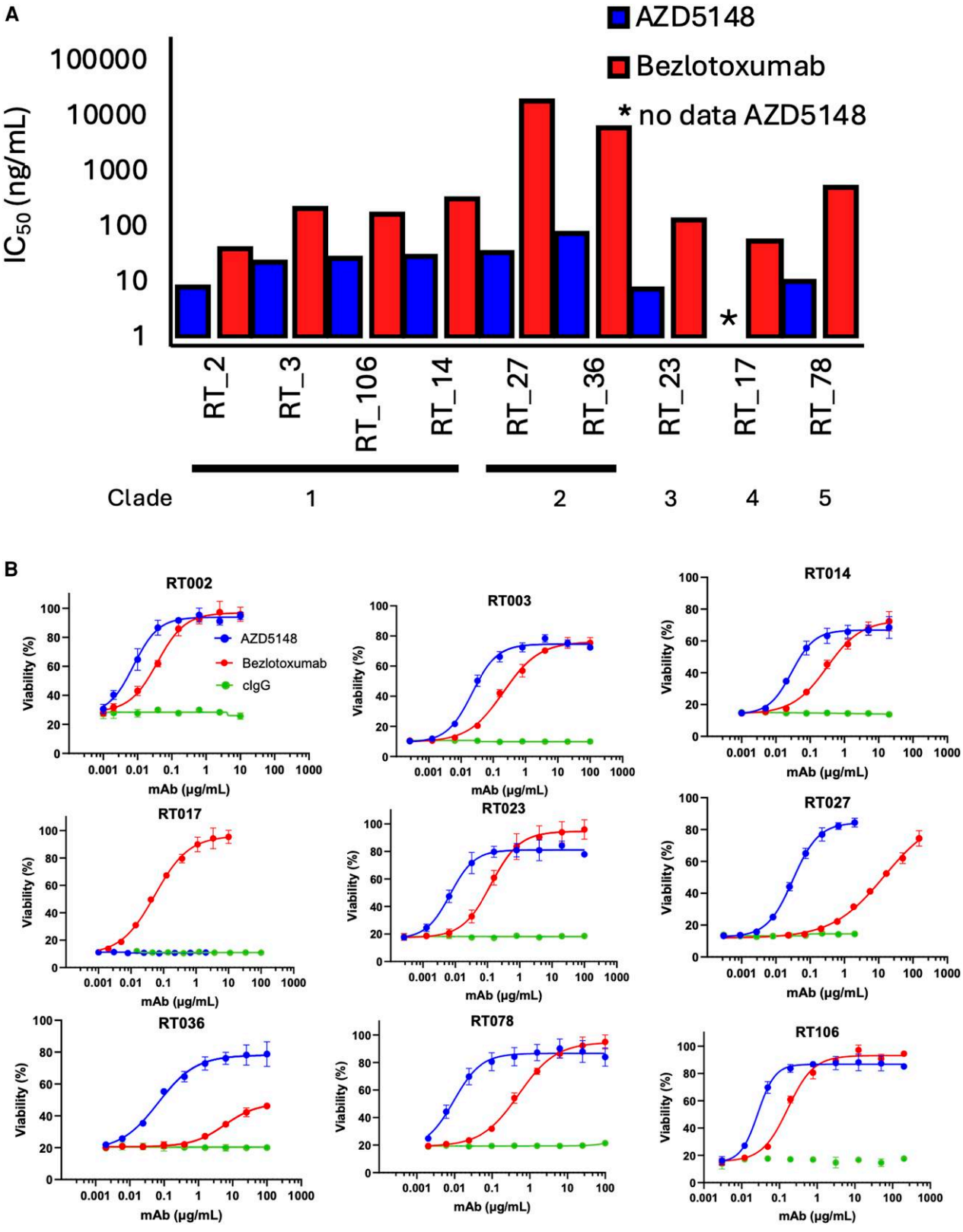

Figure 6. Comparison of AZD5148 and bezlotoumab potency. *A*, Bar plot of the $\log_{10}(IC_{50})$ of 9 TcdB ribotypes comparing AZD5148 (blue) with bezlotoxumab (red) in the Vero cell line. Asterisk indicates no neutralization data for AZD5148. *B*, Line plot of the $\log(IC_{50})$ of 9 TcdB ribotypes comparing AZD5148 (blue) with bezlotoxumab (red). Serial dilutions of each mAb incubated with Vero cells and RT-002 (1 ng/mL), RT-003 (2 ng/mL), RT-014 (20 ng/mL), RT-017 (10 pg/mL), RT-027 (10 pg/mL), RT-036 (150 pg/mL), RT-078 (50 pg/mL), and RT-106 (100 ng/mL). Each TcdB ribotypes was tested in duplicate in two different experiments. Each point represents the mean percent viability for 4 values with error bars representing the standard error of mean. Methods for binding enzyme-linked immunosorbent assays and measurement of kinetic rate and binding constants are provided in the supplementary material. IC_{50}, concentration corresponding to 50% inhibition; mAb, monoclonal antibody; TcdB, *C. difficile* toxin B.

sequences with this substitution were characterized as 1 ST, ST-037, which was found to be associated with RT-017 strains. The Y323H substitution occurred predominantly in Asia and prior to 2019 but at a low frequency. This is in agreement with recent studies identifying a higher prevalence of RT-017 sequences in Asia, predominantly in East and South East Asia [28, 29].

The epitope of Zinplava (bezlotoxumab) spans AA positions 1876 to 1961 and 2007 to 2094 in the CROPs domain [19]. Previous studies showed that the binding affinity of Zinplava is negatively affected in 5 ribotypes with variations within this binding epitope, including 2 of the most prevalent ribotypes reported in our analysis (RT-027 and RT-078) [30]. Mansfield et al identified 7 to 8 destabilizing substitutions using >8839 *C. difficile* genomes [27]. Here, we report that the CROPs domain and specifically the Zinplava binding sites show greater variation when compared with the GTD and AZD5148 binding sites in 2 independent datasets. Substitutions within the CROPs domain occurred at a higher frequency and were distributed across 4 clades. This genomic variation in the domain targeted by Zinplava may have contributed to some treatment failures in the pivotal clinical trial [30]. Additionally, Zinplava (bezlotoxumab) is a human IgG1 with a 19-day half-life in humans [31]. The absence of a half-life extension modification in Zinplava could have precluded it from sustaining a protective dose in humans beyond 12 weeks, the clinical endpoints of the MODIFY I and II phase 3 clinical trials [5].

Some TcdB receptors, such as CSPG4 and PVRL3, are known to bind outside the CROPs domain, so it is possible that some toxin could have escaped neutralization by Zinplava (bezlotoxumab) [32]. Contrastingly, the strategy of targeting the GTD, as employed by AZD5148, is independent of the receptor expressed on the target cell surface and showed potent neutralization, except for RT-017, in vitro against both cell lines in our study. The mechanism of blocking toxin translocation from within the endosome by targeting the GTD has proven to be an effective strategy in neutralizing TcdB activity. Therefore, AZD5148 outperformed in 2 cell types as compared with an alternative receptor blocking strategy, used by CROPs-targeting bezlotoxumab.

Consistent with our findings, epidemiologic reports have shown that RT-017 is prevalent in Asia but observed at low global frequency, and it is unknown if prevalence has decreased since this was reported in 2019 [28, 33]. Although there are limited data on CDI prevalence in Asia, RT-017 has been reported at low frequencies in Japan over the past 28 years [34]. Specifically, RT-017 was reported to be 6% between 1996 and 1999 [35], 1% between 2003 and 2007 [36], and 2% between 2014 and 2015 [37]. Although not detected in this study, RT-369 is another clade 4 strain that has recently shown prevalence in Japan [36, 38].

Understanding the epidemiology of pathogen subtypes is essential for mitigation strategies and outbreak response. To assess this in *C. difficile* strains, we performed geotemporal analysis of the STs in our dataset. Our analysis revealed clade 1 to be the most prevalent and clade 4 to be the least prevalent each year between 2015 and 2023. The AZD5148 epitope was conserved in all clades, with the exception of 1 Y323H substitution occurring exclusively in ST-037 (clade 4), representing only 2.9% of all global sequences. Clade 4 was predominately found in Asia (1.41%) with a frequency decreasing over time. While it is important to note that the number of sequences deposited in recent years was low, the frequency of clade 4 has been consistently low over the last 9 years. These results suggest that that low global occurrence of clade 4 and subsequent Y323H substitution do not significantly affect the overall conservation of AZD5148, making it a promising candidate for prevention of CDI.

Taken together, this study has revealed that AZD5148 is expected to neutralize >97% of recently circulating *C. difficile* strains in 2 cell lines. Recent epidemiologic studies have identified several circulating ribotypes occurring globally at high frequencies. However, the binding sites for AZD5148 are highly conserved among 9134 *C. difficile* sequences from various clades and STs. Our data show, however, that bezlotoxumab has lower neutralizing activity on most currently circulating ribotypes and has more variation in its binding site. This indicates that *C. difficile* is susceptible to the AZD5148 mAb, thus making it a candidate for prevention of CDI.

CONCLUSIONS

CDI remains a burden on the health care system, and despite recently developed therapeutics, recurrence still occurs. We have identified AZD5148, a TcdB-neutralizing human mAb that binds to a unique epitope on the GTD region, and we have shown that the AZD5148 epitope is highly conserved across all TcdB sequences, geographic regions, and STs in 2 independent datasets. The epitope of a currently approved mAb, bezlotoxumab, was found to be less conserved in comparison. AZD5148 also exhibited broad neutralization in 8 recently circulating ribotypes across 2 cell lines.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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Author contributions. K. A. M., A. M. S., C. T., V. Gopalakrishnan, B. R. S. , and J. G. conceptualized the analysis. K. A. M., A. M. S., C. T., and V. Gopalakrishnan were the investigators and interpreted the data. K. A. M., A. M. S., C. T., and T. B. gathered the data. K. A. M., E. N., V. Godfrey, K. R., and A. G. performed formal analysis of the data. All authors had full access to the analyses and data and granted their final approval of the manuscript before submission. All authors had final responsibility for the decision to submit for publication. All authors reviewed and provided substantive revisions to subsequent drafts. All authors approved the final draft and the decision to submit for publication.

Data sharing. Data underlying the findings described in this article may be obtained in accordance with AstraZeneca's data-sharing policy described at https://www.astrazenecaclinicaltrials.com/our-transparency-commitments/. Data for studies directly listed on Vivli can be requested through Vivli at www.vivli.org. Data for studies not listed on Vivli could be requested through Vivli at https://vivli.org/members/enquiries-about-studies-not-listed-on-the-vivli-platform/. The AstraZeneca Vivli member page is also available outlining further details: https://vivli.org/ourmember/astrazeneca/. The 9134 sequences described in this analysis are available from the EnteroBase *Clostridioides* public database. The 312 sequences for the isolates purchased from the IHMA collection will be made available upon request.

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Potential conflicts of interest. K. A. M., A. M. S., C. T., V. Godfrey, T. B., A. G., J. G., O. P., B. S., and V. Gopalakrishnan are/were employees of and hold stock or stock options in AstraZeneca. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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