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A Single Amino Acid Substitution in CspA Increases Germination Sensitivity and Broadens Bile Salt Germinant Specificity in *Clostridioides difficile* Spores

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ABSTRACT

Once regarded primarily as a healthcare-associated pathogen, *Clostridioides difficile* has increasingly been reported as a cause of community-acquired infection, raising questions about the contribution of animal and environmental reservoirs to persistence and transmission. Ribotype 033 (RT033) is predominantly associated with animal and environmental reservoirs, yet key traits underlying its ecology are poorly defined. Here we show that RT033 strains produce spores with enhanced germination sensitivity and expanded germinant specificity, enabling germination at low concentrations of bile-salts and in response to bile-salts that are typically inhibitory, including those more typical of animal hosts. Genetic analysis identified a single amino acid substitution (R1036I) in CspA, a component of the CspBAC germination apparatus, as the determinant of this phenotype. Expression of the RT033 *cspBAC* operon in a laboratory strain was sufficient to confer increased sensitivity and expanded bile-salt responsiveness. The R1036I substitution disrupts a conserved salt bridge at the CspA:CspC interface while not causing major destabilisation of the complex in the absence or presence of germinants and co-germinants. Together, our findings reveal a naturally occurring rewiring of bile-salt sensing in spores of RT033 strains that likely reflects adaptation to animal-associated bile-acid environments and may influence persistence, transmission, and zoonotic potential of this lineage.

1 | Introduction

Clostridioides difficile is a gram-positive, spore-forming, obligate anaerobe and a leading cause of antibiotic-associated diarrhoeal disease worldwide (Chilton et al. 2025; Smits, Garey, Riley, and Johnson 2025; Smits, Garey, Riley, et al. 2025). In high-income countries, *C. difficile* is the foremost cause of diarrhoea-related death and is estimated to account for ~15,600 deaths and 284,000 disability-adjusted life years globally each year (GBD 2021

Diarrhoeal Diseases Collaborators 2025). Its clinical impact reflects its capacity to withstand multiple broad-spectrum antibiotics and to persist in the intestinal environment, with recurrent infection occurring in approximately 25% of the cases (Berry and Khanna 2023; Finn et al. 2021). This is largely driven by the formation of spores, biofilms and biofilm-associated spores, which together promote survival and transmission across host and environmental niches (Serrano et al. 2024; Deakin et al. 2012; Dawson et al. 2012; Semenyuk et al. 2014; Tremblay and Dupuy 2022).

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The epidemiology of *C. difficile* has shifted in recent years, increasing recognition that *C. difficile* also causes community-associated infections (CDC 2025; Guh et al. 2020; Bloomfield and Riley 2016). This trend has intensified concerns about zoonotic transmission and the contribution of environmental and animal reservoirs (Janežic et al. 2016; Knetsch et al. 2014). Although foodborne outbreaks have not been reported to date, *C. difficile* is now considered a potential food-associated pathogen owing to its widespread detection in food animals, farm environments, and foods, and the recovery of strains genetically indistinguishable from those infecting patients (Knetsch et al. 2014, 2018; Rupnik et al. 2024; Rodriguez-Diaz et al. 2024; Alshannaq et al. 2025). Studies of food-producing animals and their associated environments have revealed a broad diversity of *C. difficile* PCR ribotypes. Many isolates belong to the livestock-associated clade 5/ST11 lineage (Knight et al. 2019), with RT078 being the most prevalent, followed by related ribotypes such as RT126 and RT033 (Rupnik et al. 2024; Alves et al. 2022; Dingle et al. 2019; Moloney et al. 2021). Ribotypes from other clades, however, such as clade 1 ribotypes including RT014/020 and RT106, have also been isolated from companion animals (Alves et al. 2023). The prominence of RT078 in both animal populations and hospitalised patients in Europe underscores the complexity of *C. difficile* transmission at the human-animal-environment interface and highlights the need to better understand the biology and epidemiology of other animal-associated lineages, such as RT033.

A recent investigation of *C. difficile* transmission dynamics on a Portuguese animal production unit revealed a predominance of RT033 (Alves et al. 2022). Classical RT033 strains carry the binary toxin genes (*cdtA/cdtB*), but they have traditionally been considered non-toxigenic. This is because insertion of a Tn6218-like element deleted key pathogenicity genes involved in toxin regulation (*tcdR*), secretion (*tcdE* and *tcdL*) and the TcdB toxin (*tcdB*) production. As a result, only a truncated *tcdA* pseudogene (normally encoding TcdA) remains, along with a disrupted *tcdC*, whose function is still unclear (Alves et al. 2022; Elliott et al. 2014; Geric Stare and Rupnik 2010). In the same study, however, a novel toxigenic RT033 variant was identified and designated PT-RT033 (Alves et al. 2022). This clone shares genetic features with clade 5 ST11 strains yet differs from classical RT033, carrying intact *tcdR*, *tcdB*, *tcdE*, *tcdL*, and a *tcdA* gene split into two pseudogenes, and with the prophage-like element *skin^{Cd}* inserted into the sporulation-specific *sigK* gene (Serrano et al. 2016). These distinctive features suggest that PT-RT033 may differ from classical RT033 in traits that influence persistence, environmental survival and the ability to cause disease.

Colonisation depends on spore germination in the gastrointestinal tract (Koenigsknecht et al. 2015; Lawler et al. 2020; Shen 2020; Francis, Allen, Shrestha, and Sorg 2013). Ingested spores survive passage through the stomach and reach the small intestine, where they germinate in response to bile salts, such as taurocholate (TCA), as well as amino acids, including glycine and histidine, and divalent cations such as calcium, which act as co-germinants (Heeg et al. 2012; Sorg and Sonenshein 2008). Bile salt-mediated germination helps explain the central role of the gut microbiota and its metabolome in *C. difficile* infection (CDI) (Theriot et al. 2016). Germinant and co-germinant signals are sensed by *C. difficile*'s subtilisin-like serine proteins encoded in the *cspBAC* operon. The first gene of the operon,

cspBA, codes for a fusion protein CspBA, which is subject to interdomain cleavage by the protease YabG to release CspB and CspA (Kevorkian et al. 2016; Marini et al. 2023). CspB has an intact catalytic triad, whereas CspA and CspC are two catalytically inactive pseudoproteases that are nevertheless essential for germination (Kevorkian et al. 2016; Adams et al. 2013; Donnelly et al. 2020). YabG also converts the cortex-lytic enzyme prepro-SleC into pro-SleC (Shrestha et al. 2019). Further processing of pro-SleC to produce the active SleC enzyme relies on CspB (Kevorkian et al. 2016). CspC functions as the primary germinant receptor, and mutations in *cspC* abolish germination and alter sensitivity to both germinants and co-germinants (Francis, Allen, Shrestha, and Sorg 2013; Bhattacharjee et al. 2016; Rohlfing et al. 2019). The role of CspA is more complex: deletion of *cspA* disrupts incorporation of CspC into spores and thereby impairs germination, while deletion of *yabG* or small deletions in *cspA* near the predicted YabG cleavage site generate spores that bypass the need for co-germinants in TCA-induced germination (Kevorkian et al. 2016; Shrestha et al. 2019; Kevorkian and Shen 2017). These findings led to the proposal that CspA functions as a co-germinant receptor. Recent work has shown that CspC and CspA form a heterodimer (CspC:CspA) and that residues at the CspC:CspA interface regulate the sensitivity of *C. difficile* spores to the germinant TCA (McNellis et al. 2026). The same study also showed that mutations disrupting heterodimer formation impair spore germination, suggesting that heterodimerisation is important for germinant sensing. Specifically, CspC and CspA likely act in a coordinated manner to control activation of CspB and, consequently, the conversion of pro-SleC to active SleC. Cortex degradation by SleC then triggers release of dipicolinic acid, spore-core rehydration and spore outgrowth (Burns et al. 2010; Francis et al. 2015).

During our characterisation of RT033/PT-RT033 strains, we found that RT033/PT-RT033 strains exhibit enhanced germination sensitivity and expanded germinant specificity. Notably, strains of this ribotype germinate in response to bile salts that are typically inhibitory and abundant in animals. By analysing the *cspBAC* operon of RT033/PT-RT033 strains, we identified a single amino acid substitution in CspA (R1036I) which we demonstrate is associated with the expanded germination phenotype. We propose that this expanded germination is likely to influence the colonisation by and the persistence of RT033/PT-RT033 strains. Given its strong association with animal hosts, this ribotype provides a unique opportunity to dissect how variation in bacterial traits contributes to host adaptation and potentially to zoonotic risk.

2 | Results

2.1 | Spores From RT033/PT-RT033 Germinate Under Sporulation Inducing Conditions

In a previous study, Alves and co-authors isolated *C. difficile* strains from a zootechnical station in Portugal with the goal of understanding the transmission dynamics of *C. difficile* within the One Health components (Alves et al. 2022). This study included samples from agricultural soil, water sources, pigs, cattle and other livestock. Ribotyping revealed that strains of RT033, and of a particular clone of this ribotype, termed

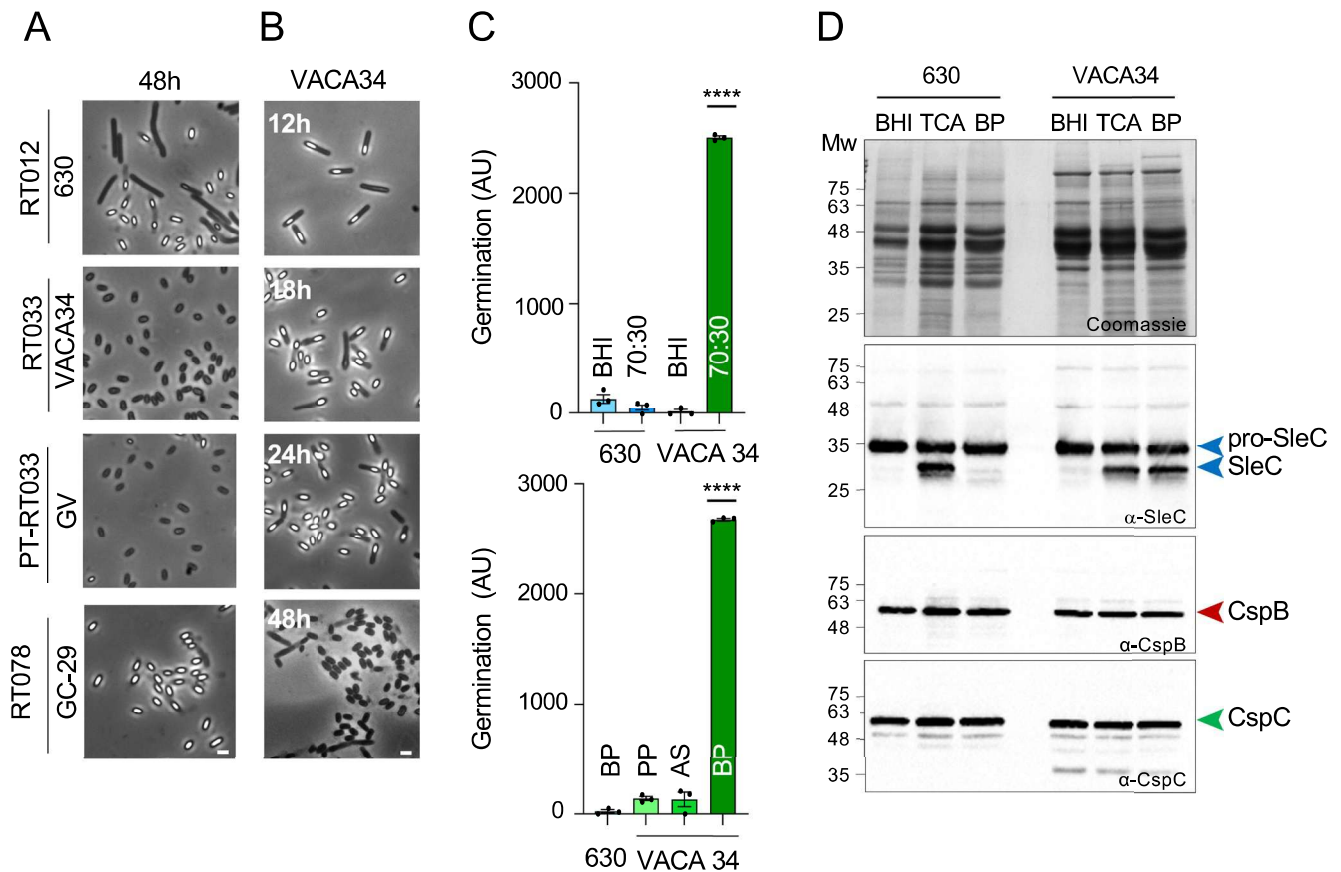


FIGURE 1 | Germination of *Clostridioides difficile* RT033/PT-RT033 strains in sporulation inducing conditions. (A) Phase-contrast microscopy of *C. difficile* strains from the indicated ribotypes after 48 h of incubation in 70:30 medium. Scale bar: 1 μ m. (B) Phase-contrast microscopy of a time-course for a strain from RT033 (VACA34) from 12 to 48 h in 70:30 media (right). Scale bar: 1 μ m. (C) Spores were incubated with BHI or 70:30 medium (top panel) and with the following 70:30 medium components (bottom panel): AS, ammonium sulfate; BP, Bacto-Peptone; PP, Proteose Peptone. Germination levels were monitored by following the decrease in OD₆₀₀ of the spore suspension and normalised to the initial value. Germination in arbitrary units (AU) was calculated using the area below inverted OD₆₀₀ curves (see [Material and Methods](#)). In all graphs, the data shown indicate the mean of at least three biological replicates, the measured values (dots) and the standard deviation of the mean (error bars). Asterisks indicate statistical significance determined with a one-way ANOVA with Tukey's multiple comparisons test (**** $p \leq 0.0001$, BHI vs. 70:30 in the upper panel, VACA34 BP vs. 630 BP in the lower panel). (D) Spores produced by strains 630 Δ erm (RT12) and VACA 34 (RT033) were purified and incubated in BHI, Taurocholic acid (TCA) or BactoPeptone (BP) for 2 h. The spores were then collected and the surface proteins extracted. The proteins in the extracts were resolved by SDS-PAGE and the gels stained with Coomassie of subject to immunoblotting with anti-SleC, anti-CspB or anti-CspC antibodies.

PT-RT033, were predominant among the isolates. Here, we further characterised the RT033/PT-RT033 isolates to identify traits that could contribute to host adaptation, persistence, environmental survival and potential for transmission. We first assessed biofilm formation, sporulation efficiency, and spore germination efficiency across a panel of RT033/PT-RT033 isolates. For reference, we used the laboratory strain 630 Δ erm (RT012) and strain GC-29. GC-29 belongs to the epidemic ribotype 078, which is commonly found in livestock and associated with community-acquired CA-CDI in humans (Rupnik et al. 2024; Knight et al. 2019). GC-29 belongs to clade 5 ST11, along with RT033 and the PT-RT033 clone, making it an appropriate reference strain within the same clade. Consistent with observations for other epidemic lineages, RT033/PT-RT033 isolates formed more robust biofilms and sporulated more efficiently than the laboratory strain (Figure S1A; see [Supporting Information](#) for additional details on sporulation and biofilm assays).

With the aim of assessing germination efficiency, spores were purified 48 h after inoculation in the sporulation-inducing medium 70:30 (Putnam et al. 2013). In parallel, samples from the cultures were imaged by phase-contrast microscopy as a qualitative assessment of spore formation. In samples from the cultures of strain 630 Δ erm and GC-29, free phase-bright spores were readily seen, while in RT033/PT-RT033 strains (GV and VACA34) most of the spores were phase-dark (Figure 1A). Phase-dark spores were observed in all strains from RT033/PT-RT033 that were examined (Figure S1B). The phase-brightness of spores is a result of their dense, dehydrated core and the thick protective cortex peptidoglycan layer, formed at a late stage in morphogenesis, overall resulting in a high refractive index (Kong et al. 2011). Conversely, phase-dark spores can be the result of either a morphological block during sporulation that arrests development prior to cortex synthesis or that specifically affect its synthesis (Martins et al. 2024). Phase-dark spores can also result from germination. During germination, the cortex

is hydrolysed, Ca^{2+} -dipicolinate (CaDPA, a compound characteristic of spores) is released and the spore core undergoes rehydration, leading to a low refractive index and the phase-dark appearance under phase-contrast optics (Kong et al. 2011; Vinay et al. 2025).

To determine whether the accumulation of phase-dark spores in RT033 cultures resulted from a defect in spore formation or from premature spore germination, we monitored the appearance of phase-bright spores in cultures of strain VACA34 in 70:30 medium, over time, using phase-contrast microscopy (Figure 1B). Sporangia containing phase-bright spores were detected at 12 h, and free spores were observed at 18 h, with a higher fraction present by hour 24. Strikingly, by 48 h most spores were phase-dark. These observations indicate that the formation of phase-bright spores was not impaired and that the accumulation of phase-dark spores in the RT033 strain most likely resulted from spore germination in 70:30 medium.

To independently test this inference, spores from strain VACA34 were purified at 20 h, a time at which only phase-bright spores were seen by phase-contrast microscopy, and optical density-based germination assays were conducted in the presence of 70:30 liquid medium (Figure 1C, top panel). In all subsequent experiments VACA34 was used as the reference RT033/PT-RT033 strain. Spores of strain 630 Δ erm, used as the reference for a standard laboratory strain, did not germinate in the presence of 70:30; in sharp contrast, purified spores of strain VACA34 germinated in the presence of 70:30 (Figure 1C, top panel). To our knowledge, this is the first report of spore germination in 70:30 medium. To understand which of the components of the 70:30 medium induced germination of VACA34 spores, we conducted germination assays in the presence of its individual components, that is, Bacto-Peptone (BP), Proteose-Peptone (PP) and ammonium sulfate (AS) (Putnam et al. 2013). We found that the spores produced by strain VACA34 germinated in response to Bacto-Peptone (Figure 1C, bottom panel) (see [Material and Methods](#)) (Rohlfing et al. 2019; McNellis et al. 2026).

To investigate the basis of the 70:30-induced germination, we first evaluated whether germination followed the canonical pathway involving the pro-SleC to SleC conversion (Kevorkian et al. 2016; Shrestha et al. 2019; Burns et al. 2010). Purified spores of 630 Δ erm and VACA34 were incubated in BHI or in BHI supplemented with TCA 0.5% and in the Bacto-Peptone for 2 h. Following incubation, the spores were collected, the surface proteins were extracted and the presence of mature SleC, CspC and CspB was assessed by immunoblotting (Figure 1D). Cleaved, active SleC was detected in 630 Δ erm only after incubation in TCA and in VACA34 spores incubated with either TCA or with Bacto-Peptone. Importantly, the levels of the CspB, CspC and SleC did not differ between the spores of VACA34 and 630 Δ erm (Figure 1D). This shows that the Bacto-Peptone-mediated germination is not due to increased levels of CspC or CspB and proceeds via the normal pathway in which SleC activation triggers cortex peptidoglycan degradation. Although we did not assess CspA levels directly, CspA is co-translated with CspB, and therefore comparable levels of this protein are expected.

2.2 | Spores From RT033 Strains Display Enhanced Germination Sensitivity

In *C. difficile*, germination is triggered by bile salts, particularly by derivatives of cholic acid (CA) such as taurocholic acid (TCA) (Sorg and Sonenshein 2008). Bacto-Peptone contains small amounts of bile salts, namely glycocholic acid (GCA), taurocholic acid (TCA), and cholic acid (CA) (Kamekura et al. 1988; Bina et al. 2021). In contrast, bile salts are present at 10–100-fold lower concentrations in peptone from OXOID (Kamekura et al. 1988). Accordingly, when sporulation was induced in 70:30 medium containing OXOID peptone, VACA34 spores did not germinate (Figure S1C).

We reasoned that spores produced by RT033 strains are more sensitive to bile salts, enabling them to germinate in response to the low bile-salt concentrations present in Bacto-Peptone (Kamekura et al. 1988; Bina et al. 2021). To test this, we performed germination assays in a range of concentrations, from 0% to 0.5% of GCA, TCA, and CA (Figure 2A) using 630 Δ erm and GC-29 as references. Since GC-29 spores do not germinate in response to 70:30 medium (Figure 1A), they provide a useful comparison to distinguish germination phenotypes while maintaining an epidemic background that is closer to RT033 than the laboratory strain.

Among the three bile salts TCA, GCA, and CA, only TCA induced germination of 630 Δ erm spores at TCA concentrations of 0.1% or higher (Figure 2A). For this bile salt, both GC-29 and VACA34 spores showed a similar phenotype, initiating germination at a concentration of TCA equal to or greater than 0.05% (Figure 2A, upper panel). Thus, GC-29 and VACA34 spores show increased sensitivity to TCA when compared to spores of the laboratory strain 630 Δ erm. For CA, both GC-29 and VACA34 spores displayed comparable germination efficiency and, although not statistically significant, VACA34 spores germinated at lower bile salt concentrations (0.005%) when compared to GC-29 (0.01%) (Figure 2A, lower panel). Germination in GCA showed significant differences between the two strains, with strain VACA34 consistently exhibiting greater sensitivity, corresponding to at least a 50-fold lower concentration of the bile salts (GC-29, 0.05%; VACA34, 0.005%) (Figure 2A, middle panel). Overall, RT033 spores were more sensitive to CA derivatives and initiated germination at germinant concentrations much lower than spores of the laboratory strain.

2.3 | Heterologous Expression of the *cspBAC* Operon From RT033 Strains Is Sufficient to Increase the Sensitivity of 630 Δ erm Spores to TCA

Given the role of the *cspBAC* operon in germination (Francis, Allen, Shrestha, and Sorg 2013; Kevorkian et al. 2016; Adams et al. 2013; Shrestha et al. 2019; Rohlfing et al. 2019; Kevorkian and Shen 2017; McNellis et al. 2026), we hypothesised that the germination profile of RT033 strains could depend on mutations in the *cspBAC* operon. To test this, we constructed a plasmid, pMTL84121 (Heap et al. 2009), carrying the *cspBAC* operon from RT033 (033-*cspBAC*) and introduced it into the laboratory strain 630 Δ erm. As a control for copy number effects, a strain carrying the *cspBAC* operon from 630 Δ erm (630-*cspBAC*) in pMTL84121 was also constructed, and as a control for the presence of the

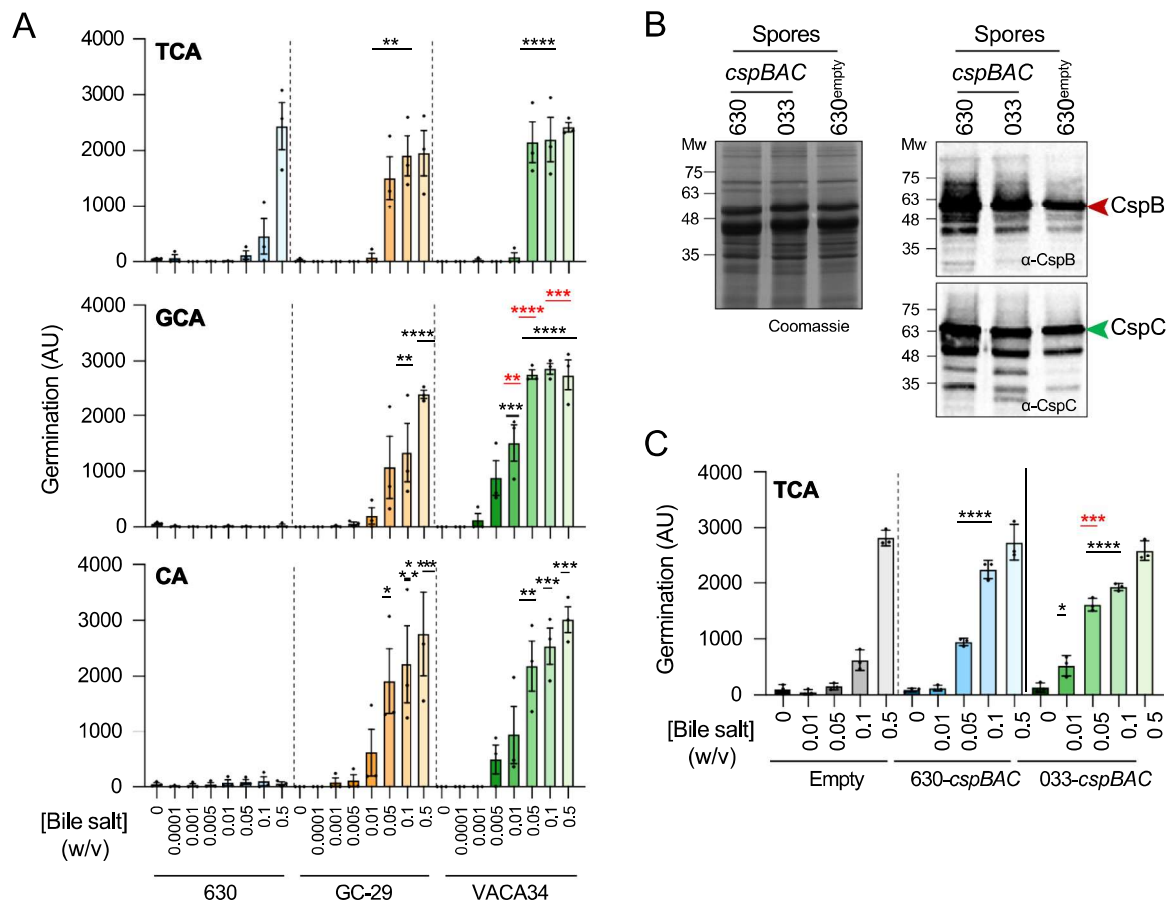


FIGURE 2 | The *cspBAC* operon from RT033 confers increased germination sensitivity to 630 Δ erm. (A) Spores produced by strains 630 Δ erm (RT012), GC-29 (078) and VACA34 (RT033) were purified and incubated in BHI with the indicated bile salts at different concentrations. Germination levels were monitored by following the decrease in OD₆₀₀ of the spore suspension and normalised to the initial value. Germination in arbitrary units (AU) was calculated using the area below inverted OD₆₀₀ curves (see [Material and Methods](#)). CA, cholic acid; GCA, glycocholic acid; TCA, taurocholic acid. Asterisks indicate statistical significance determined with a one-way ANOVA with Tukey's multiple comparisons test (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$, VACA34 vs. 630 Δ erm in black, VACA34 vs. GC-29 in red). (B) Spores were purified from strain 630 Δ erm carrying plasmids with its own *cspBAC* operon, the *cspBAC* operon from RT033 or an empty plasmid. The spore surface proteins were extracted, resolved by SDS-PAGE and the gels stained with Coomassie or subject to immunoblotting with anti-CspB or anti-CspC antibodies. (C) Spores purified from the 630 Δ erm strain carrying a plasmid-borne copy of the *cspBAC* operon, or the *cspBAC* operon from the RT033 strain or an empty plasmid, were incubated in BHI with the indicated bile salts at different concentrations. TCA, taurocholic acid. Asterisks indicate statistical significance determined with a one-way ANOVA with Tukey's multiple comparisons test (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$, 630 Δ erm (033-*cspBAC*) vs. 630 Δ erm (empty) in black and 630 Δ erm (033-*cspBAC*) vs. 630 Δ erm (630-*cspBAC*) in red). In all graphs, the data shown indicate the mean of at least three biological replicates, the measured values (dots) and the standard deviation of the mean (error bars).

plasmid, the empty vector was also introduced into 630 Δ erm. Spores were purified from these strains, the surface proteins were extracted and resolved by SDS-PAGE, and the gels stained with Coomassie or subject to immunoblotting with anti-CspC and anti-CspB antibodies. The profile of the extractable coat/exosporium proteins did not show major differences among strains, indicating that neither the presence of the vector nor the *cspBAC* alleles interfered in a visible way with the assembly of the surface layers (Figure 2B). However, the presence of the plasmids resulted in slightly increased levels of CspB and CspC in 630 Δ erm strains expressing either the multicopy 630-*cspBAC* or 033-*cspBAC* allele (Figure 2B). Germination in TCA revealed that the 630 Δ erm carrying the multicopy 630-*cspBAC* allele showed increased germination sensitivity compared to the strain carrying the empty plasmid (0.01% for the strain carrying the empty plasmid, 0.05% for the strain producing 630-*cspBAC*; Figure 2C). Thus, in 630 Δ erm, additional

copies of the native *cspBAC* operon are sufficient to increase germination sensitivity. Strikingly, however, this increase in germination sensitivity was even higher for the 630 Δ erm expressing the 033-*cspBAC* multicopy allele (0.01% for TCA; Figure 2C). Similar results were obtained when GCA or CA were used to induce spore germination (Figure S3). Overall, these results show that expression of 033-*cspBAC* is sufficient to enhance the germination sensitivity of 630 Δ erm spores, phenocopying the RT033 phenotype.

2.4 | Enhanced Germination Is Driven by a Single Amino Acid Substitution at the CspC:CspA Interface

In earlier studies, mutations that increase spore sensitivity to the TCA germinant have been mapped to the *cspBAC* operon

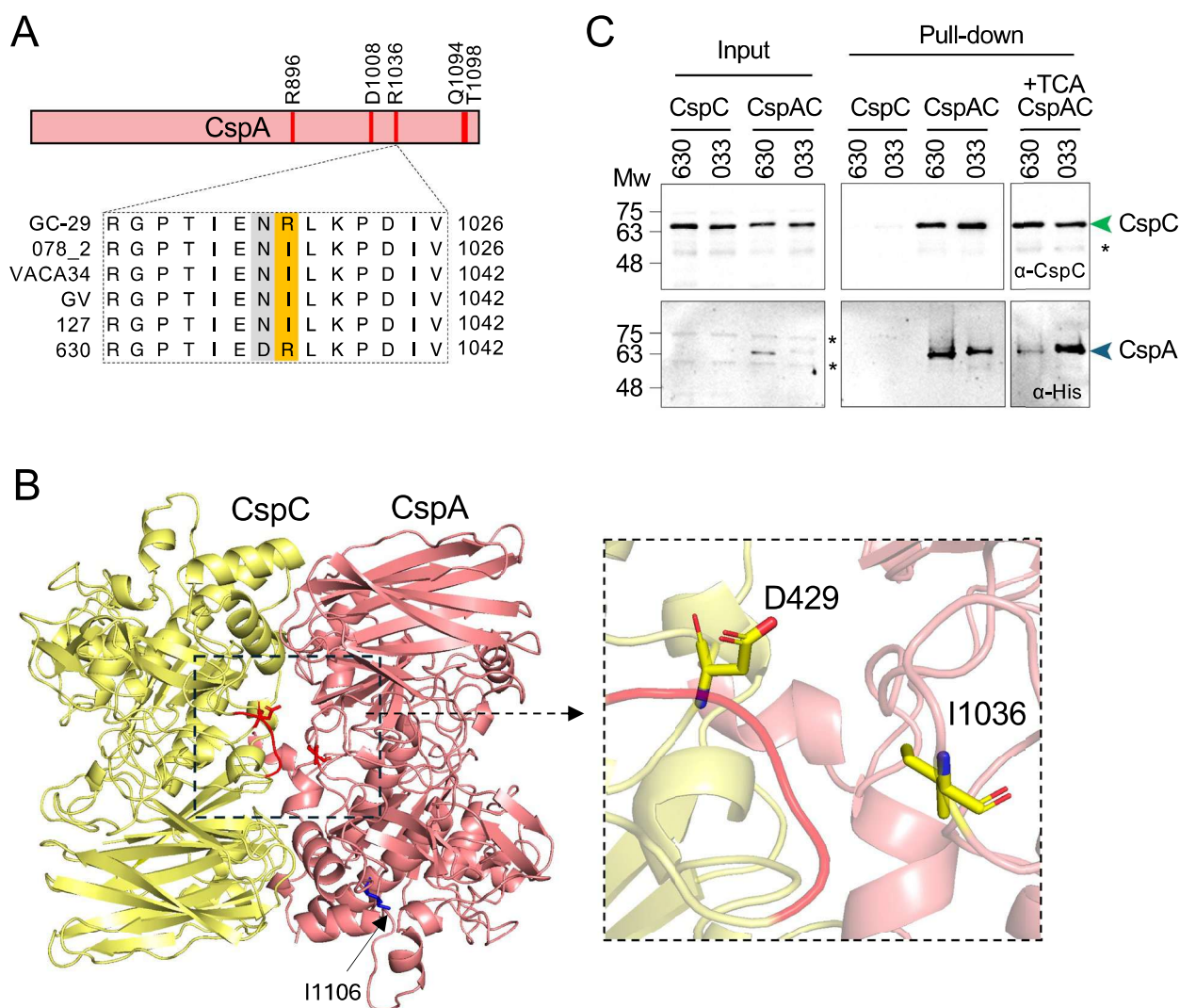


FIGURE 3 | CspC and CspA from RT033 form a stable complex even in the presence of bile salt germinants. (A) Amino-acid alignment of CspA sequences from different *C. difficile* strains. The isoleucine residues specific to RT033 strains, RT127, and strain 1645 are highlighted in yellow. Highlighted in red are the residues in CspA found to enhance germination sensitivity: R896, D1008, R1036, Q1094 and T1098. (B) AlphaFold3 model of the complex formed by the CspC and CspA proteins from RT033. In the CspC protein of laboratory strain 630Δ*erm*, D429 forms a salt bridge with R1036 from CspA which inhibits germinant and co-germinant signalling in dormant spores (McNellis et al. 2026). R1036 is replaced by an Isoleucine in the protein from the RT033 strain. Residues D429 in CspC and I1036 in CspA are highlighted in red; also highlighted is a loop (G455 to I458) in CspC thought to be important for germinant and co-germinant sensitivity (Rohlfing et al. 2019). Highlighted in blue is residue I1106, present in RT033/PT-RT033/RT127 strains. The right panel shows an expansion of the interface between CspC and CspA, with the D429 in CspC and I1036 in CspA represented in stick mode. (C) His₆-tag pull-down assays for CspA interactions. *E. coli* extracts (input, left panel) expressing his₆-tagged CspA and untagged CspC from either strain 630Δ*erm* or RT033 were incubated with Ni²⁺-Sepharose HP beads. Unbound proteins were removed by washing, and bound proteins were eluted in loading buffer, resolved by SDS-PAGE and analysed by immunoblotting (pull-down, middle and right panels). When indicated the cell extracts were incubated with TCA for 30 min before the pull-down (right panel). The asterisk refers to an unspecific band.

(Francis, Allen, Shrestha, and Sorg 2013; Rohlfing et al. 2019; McNellis et al. 2026). The *cspC* D429K, R456G, or G457R alleles and the *cspA* alleles D1008K, R1036A, R896E/Q1094E, or R896A/Q1094A/T1098A confer increased sensitivity to TCA as compared to the wild-type *cspC* or *cspA* genes (Francis, Allen, Shrestha, and Sorg 2013; Rohlfing et al. 2019; McNellis et al. 2026). Notably, residues D429 and R456 of CspC form salt bridges with CspA R1036 and D1008, respectively, at the CspC:CspA heterodimer interface (McNellis et al. 2026). In contrast, no specific point mutations have been linked to this

phenotype in *cspB*, which encodes the protease responsible for processing pro-SleC (Adams et al. 2013).

In an effort to identify the determinants underlying the enhanced germination phenotype, the CspBA and CspC sequences from strains VACA34, GC-29, and 630Δ*erm* were aligned (Figures 3A and S5). This analysis revealed only two amino-acid substitutions in CspA, specific to strain VACA34, at positions 1036 and 1106 (numbering according to the 630Δ*erm* CspA sequence). At both positions, strains GC-29

and 630 Δ erm encode arginine, whereas VACA34 encodes isoleucine. Notably, these substitutions are conserved across all RT033 and PT-RT033 strains examined (see also below). These observations suggest that the enhanced germination phenotype observed in spores of this lineage may be driven primarily by the R1036I substitution in CspA, consistent with the increased germinant sensitivity recently reported for the *cspA*^{R1036A} allele in a 630 Δ erm background (McNellis et al. 2026). By contrast, the functional consequences of substitutions at position R1106 have not yet been reported (see below).

In the structure of the CspC:CspA complex, CspA R1036 forms a salt bridge with D429 of CspC, and disruption of this interaction by substitution of R1036 with alanine increases spore sensitivity to TCA (McNellis et al. 2026). As we show here, in CspA from RT033 strains, R1036 is replaced by the hydrophobic residue isoleucine, which similarly precludes formation of a salt bridge with the conserved D429 residue in CspC (Figure 3B). Thus, it is likely that, as observed for R1036A, the R1036I substitution at the CspA:CspC interface underlies the enhanced germination phenotype of RT033 spores. By contrast, I1106 lies near the C-terminus of CspA, on the protein surface within a region of unknown function (Figure 3B, highlighted in blue), and its potential contribution to germination remains to be determined.

2.5 | The CspC:CspA Complex Formed by the RT033 Proteins Is Maintained in the Presence of Germinant and Co-Germinant

To test whether the CspC:CspA complex is also formed in RT033 strains, we co-expressed the CspC and His₆-tagged CspA proteins from 630 Δ erm and from VACA34 (RT033) in *E. coli*. His₆-tagged CspA was immobilised on a Ni²⁺-NTA column and the ability of untagged CspC to be retained was assessed (Figure 3C). Although His₆-tagged 033-CspA accumulated at lower levels than the protein from 630 Δ erm, similar amounts were bound to the resin. Western blot analysis revealed that CspC was specifically recovered with His₆-tagged CspA from both strain backgrounds (Figure 3C), demonstrating that CspA and CspC from RT033 strains also form a complex, similar to that observed in 630 Δ erm (McNellis et al. 2026). No CspC signal was detected in control pull-downs from extracts lacking His₆-tagged CspA (Figure 3C), confirming the specificity of the interaction.

Prior work showed that the presence of the germinant TCA and co-germinant glycine does not affect the stability of recombinantly produced CspC:CspA complex derived from a RT027 strain (McNellis et al. 2026). Because RT033 spores are more sensitive to germinants, we considered the possibility that the 033-CspC:CspA complex is less stable in the presence of germinants and co-germinants. To test this, we repeated the pull-down assays using extracts pre-incubated with glycine and TCA (Figure 3C). The 630-CspC:CspA complex remained stable in the presence of germinant and co-germinant (McNellis et al. 2026). Likewise, we observed no change in the ability of His₆-tagged 033-CspA to pull down 033-CspC after pre-incubation with germinant and co-germinant (Figure 3C). Together, these results indicate that germinants and co-germinants do not

measurably affect the stability of the recombinantly produced 033-CspC:CspA complex.

2.6 | RT033 Spores Display Expanded Germinant Specificity, Enabling Germination in the Presence of Bile Salts That Are Normally Inhibitory

Germination of *C. difficile* spores can be induced not only by cholate (CA) derivatives but also by deoxycholic acid (DCA), taurodeoxycholic acid (TDCA) and taurochenodeoxycholic acid (TCDCA) (Theriot et al. 2016; Wang et al. 2015; Howerton et al. 2011) (Figure 4A). In contrast, other bile salts, including chenodeoxycholic acid (CDCA) and lithocholic acid (LCA), are well-established inhibitors of spore germination (Heeg et al. 2012; Sorg and Sonenshein 2008, 2010; Theriot et al. 2016; Bhattacharjee et al. 2016; Wang et al. 2015; Francis, Allen, and Sorg 2013) (Figure 4A). We therefore asked whether, in addition to exhibiting an increased germination sensitivity, RT033 spores also display expanded germinant specificity and respond to a broader spectrum of bile salts, including those normally considered inhibitory.

To address this, we assayed spore germination in the presence of DCA, TDCA, TCDCA, CDCA and LCA, as well as glycochenodeoxycholic acid (GCDCA) and glycodeoxycholic acid (GDCA), for which germination activity in *C. difficile* has not previously been reported (Figures 4B and S2). Due to solubility constraints, the maximum usable concentration of GCDCA and CDCA was limited to 0.1%, whereas LCA was limited to 0.005% (Rohlfing et al. 2019). As in previous assays, spores from strains 630 Δ erm and GC-29 were included as references.

Spores of 630 Δ erm did not germinate in response to any of the bile salts tested (Figures 4B and S2). In contrast, spores from GC-29 and VACA34 germinated in response to GDCA, TDCA and DCA, displaying overall comparable germination efficiencies (Figure S2). Although these differences did not reach statistical significance, VACA34 spores consistently germinated at lower bile salt concentrations than GC-29 spores: 0.001% versus 0.005% for TDCA, 0.005% versus 0.01% for DCA, and 0.001% versus 0.005% for GDCA, respectively (Figure S2).

More pronounced differences were observed for TCDCA and GCDCA, where VACA34 spores exhibited significantly enhanced sensitivity compared with GC-29 spores (Figure S2). In each case, germination occurred at concentrations at least 50-fold lower in VACA34: 0.001% versus 0.005% for TCDCA and 0.001% versus 0.1% for GCDCA (Figure S2). Strikingly, VACA34 spores germinated in response to both LCA (0.0001%) and CDCA (0.001%), bile salts that are normally inhibitory, whereas spores from 630 Δ erm and GC-29 failed to germinate under these conditions (Figure 4B). Similar germination responses were observed with spores from a PT-RT033 strain, PIG25 (Figure S1D). Importantly, spores from 630 Δ erm carrying an additional copy of 033-*cspBAC*, but not those carrying an extra copy of 630-*cspBAC*, also germinated in response to LCA (0.001%) and CDCA (0.001%), although at lower levels than VACA34 spores, suggesting that additional features of the RT033 ribotype may affect germinant sensitivity (Figure S3).

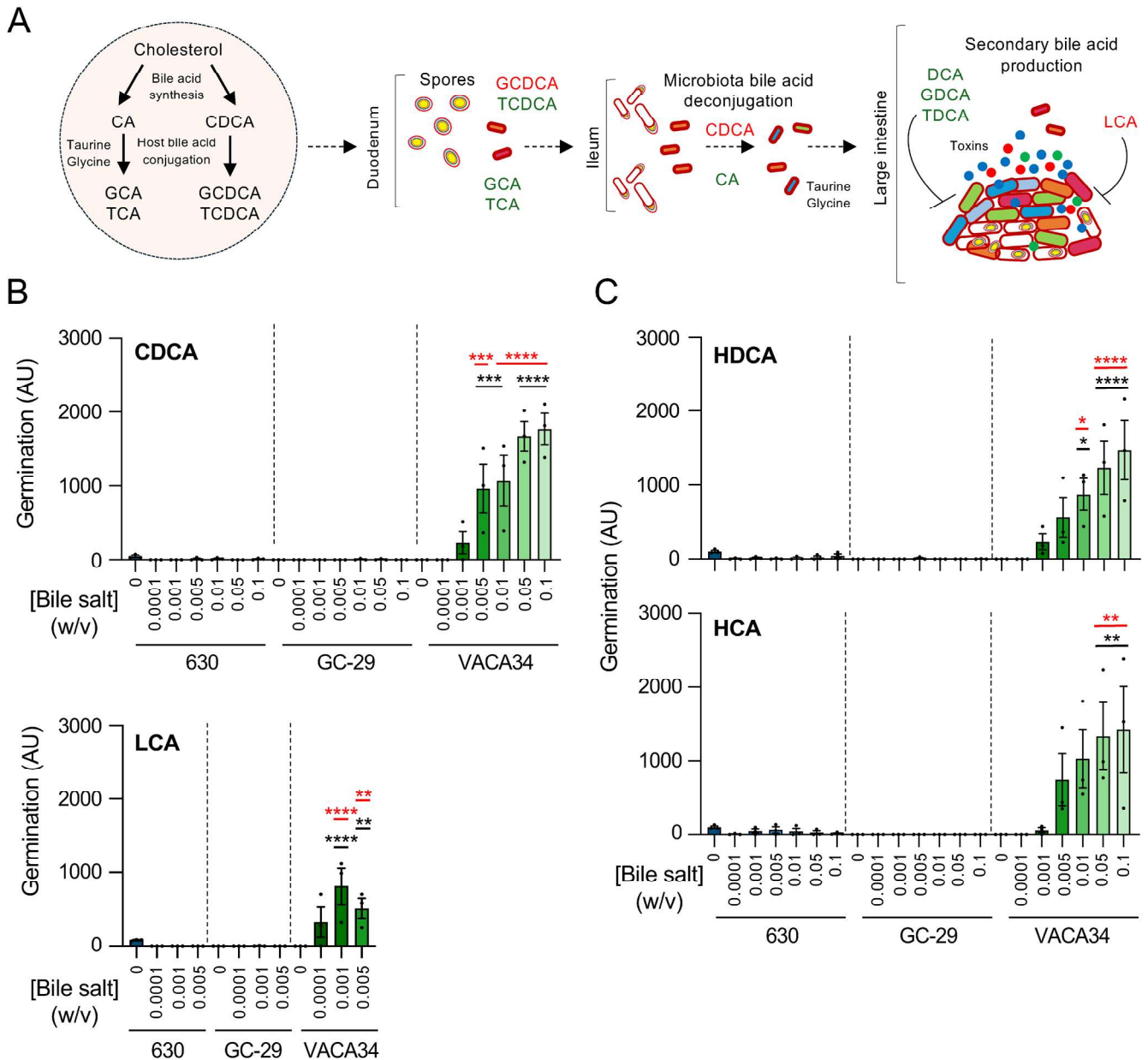


FIGURE 4 | RT033 spores germinate in response to animal-associated and normally inhibitory bile salts. (A) The role of bile acids in *C. difficile* life cycle (Shen 2015). Primary bile acids are synthesised in the liver from cholesterol, producing cholic acid (CA) and chenodeoxycholic acid (CDCA), which can subsequently be conjugated to taurine or glycine to form bile salts such as taurocholate (TCA), glycocholate (GCA), taurochenodeoxycholate (TCDCA) and glycochenodeoxycholate (GCDCA). These bile acids are secreted into the small intestine, where they facilitate lipid digestion. While the majority are reabsorbed and recycled back to the liver, a fraction enters the large intestine, where they are modified by the intestinal microbiota through deconjugation and 7α -dehydroxylation, giving rise to secondary bile acids such as deoxycholic acid (DCA) and lithocholic acid (LCA). Bile acids exert distinct and sometimes opposing effects on *C. difficile* spores and vegetative cells along the gastrointestinal tract. Bile acids shown in green are described as germination inducers, whereas those shown in red act as germination inhibitors. Blunt-ended arrows indicate bile acids that inhibit vegetative growth of *C. difficile*. Notably, some bile acids that promote spore germination can simultaneously inhibit vegetative growth, and the balance between bile-acid-mediated germination signals and growth inhibition is thought to be a key determinant of successful *C. difficile* infection. (B, C) Spores produced by strains 630 Δ erm (RT12), GC-29 (078), VACA34 (RT033) were purified and incubated in BHI with the indicated bile salts at different concentrations. Germination levels were monitored by following the decrease in OD₆₀₀ of the spore suspension and normalised to the initial value. Germination in arbitrary units (AU) was calculated using the area below inverted OD₆₀₀ curves (see Material and Methods). CDCA, chenodeoxycholic acid; HCA, hyocholic acid; HDCA, hyodeoxycholic acid; LCA, lithocholic acid. Asterisks indicate statistical significance determined with a one-way ANOVA with Tukey's multiple comparisons test (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$ VACA34 vs. 630 Δ erm in black and VACA34 vs. GC-29 in red). In all graphs, the data shown indicate the mean of at least three biological replicates, the measured values (dots) and the standard deviation of the mean (error bars).

Taken together, these results demonstrate that RT033 spores exhibit not only enhanced germination sensitivity but also expanded germinant specificity, enabling germination in response to bile salts that are typically inhibitory. Moreover, our genomic data indicate that this phenotype is most likely driven by the single R1036I substitution in CspA, which is sufficient to confer both increased sensitivity and expanded germinant range.

2.7 | RT033 Spores Germinate in Response to Animal-Associated Bile Salts

RT033 belongs to clade 5, ST11, which is considered the *C. difficile* evolutionary lineage with the highest zoonotic potential (Knetsch et al. 2014; Knight et al. 2019; Alves et al. 2023). The RT033 strains used in this work are mostly associated with environmental reservoirs, such as agricultural soil and water sources, and particularly with animal hosts, including pigs, cattle and dogs (Alves et al. 2022, 2023). We hypothesised that the germination properties of RT033 could represent an environmental adaptation of these strains. Hyocholic acid (HCA) and its secondary derivative hyodeoxycholic acid (HDCA) are major components of the bile acid pool in pigs, together accounting for over 70%–75% of the total bile acids, whereas in humans they are present only in trace amounts (Sun et al. 2022; Chong et al. 2025). HDCA was also reported as having an inhibitory effect on germination (Theriot et al. 2016).

Given the species-specific distribution of HCA and HDCA, we tested germination of RT033 spores in the presence of these bile salts (Figure 4C). Although the solubility of HCA and HDCA limited the maximum concentration tested to 0.1%, we again observed a distinct germination phenotype in VACA34, which was the only strain capable of germinating in both HCA, at a concentration as low as 0.005% and HDCA, at 0.001% (Figure 4C). Given the reported inhibitory nature of HDCA (Theriot et al. 2016), these results are consistent with the ability of RT033 spores to germinate in response to inhibitory bile salts (Figure 4B; see above). While HCA and HDCA are characteristic of animal hosts, CDCA is also present at high levels in these hosts (Mu et al. 2022). Similar germination responses were observed with spores from the PT-RT033 strain PIG25 (Figure S1D). Thus, RT033 spores can germinate in bile salts commonly found in animal hosts.

Notably, in the 630 Δ erm background spores were able to germinate in response to HDCA (0.005%) and HCA (0.005%) only when expressing the 033-cspBAC allele (Figure S3). Overall, these results show that expression of 033-cspBAC alone, even in the laboratory strain 630 Δ erm background, is sufficient to enhance germination sensitivity and alter germination specificity, phenocopying the RT033 phenotype.

2.8 | The cspA^{I1036} Allele in Non-RT033 Strains Recapitulates the RT033/PT-RT033 Germination Phenotype

To determine whether the enhanced germination phenotype is specific to RT033/PT-RT033 strains, we searched for the

cspA^{I1036} allele in strains belonging to other ribotypes. From a total of 5830 strains from 58 different ribotypes, besides the RT033/PT-RT033 strains, the cspA^{I1036} allele was identified in isolates from RT127 cluster I and cluster II. These RT127 strains also encode isoleucine at position 1106, mirroring the RT033 genotype, although only the RT127 cluster I is phylogenetically closer to RT033 (Figure 5A). This is consistent with a closer shared ancestor and the maintenance of these mutations due to a potential biological advantage. In contrast, strains from RT078 and RT126, which are phylogenetically more distant (RT078/RT126 cluster in Figure 5A), carry cspA^{R1036}, except for three human isolates from RT078 from Portugal (CD2465, CD2440, 1645) (see below), which have a hybrid genotype, harbouring both the cspA^{I1036} and cspA^{R1106} alleles (Figure 5A).

A closer look at the RT078/126 cluster, which was enriched with genomes from public databases, including 181 strains from diverse epidemiological backgrounds, revealed a clear separation into two evolutionary branches, distinguishing the cspA^{R1036/R1106} genotype from the cspA^{I1036/I1106} genotype (Figure S4). Interestingly, a smaller branch diverging from the larger cluster by 8 SNPs consisted exclusively of the three RT078 Portuguese isolates plus a human isolate from Australia (ERR2898867), exhibiting a hybrid genotype (I1036/R1106). This analysis confirms that the presence of the isoleucine substitution is not ribotype- nor origin-dependent.

Alignment of the primary sequence of CspBA from strains 127 (RT127, cluster II) and 1645 (RT078) revealed that, aside from the isoleucine substitution(s), their sequences were otherwise identical to those of RT033 (Figure S5). For CspC, the sequence from strain 1645 was identical to that of RT033, whereas strain 127 contained three additional substitutions (P38S, R289G and D340E) (Figure S5D). The hybrid genotype carried by strain 1645 provides a unique opportunity to assess the independent contribution of the I1036 substitution to the strain's phenotype. To functionally dissect the contribution of the individual substitutions, we purified spores from strains 1645 (R1036I only) and 127 (R1036I and R1106I) and performed germination assays using TCA, LCA, CDCA, HDCA and HCA (Figure 5B–D). Spores from the two strains germinated at lower concentrations of TCA, displaying a germination sensitivity comparable to RT033 (1645, 0.001% for TCA; 127, 0.005% for TCA). Strikingly, and as observed for RT033, spores from both strains also germinated in response to bile salts that are typically inhibitory, including LCA, CDCA, HDCA and HCA (1645: 0.0001% for all bile salts tested; 127: 0.001% for all bile salts tested). Strain 127, which carries both RT033-associated substitutions, exhibited a germination profile closely resembling that of RT033. For all bile salts tested, spores from strain 1645 germinated more efficiently than spores from strains 127 and VACA34.

Together, these data indicate that the R1036I substitution in CspA is the primary determinant of the RT033 germination phenotype and the CspC sequence variations present in 127 do not measurably affect spore germination under the conditions tested. The increased germination sensitivity to germinants observed for 1645 spores may reflect differences in genetic background and/or the absence of the additional CspA substitution at position 1106, which may exert an inhibitory effect on germination in RT033 and RT127 strains. Taken together, our results

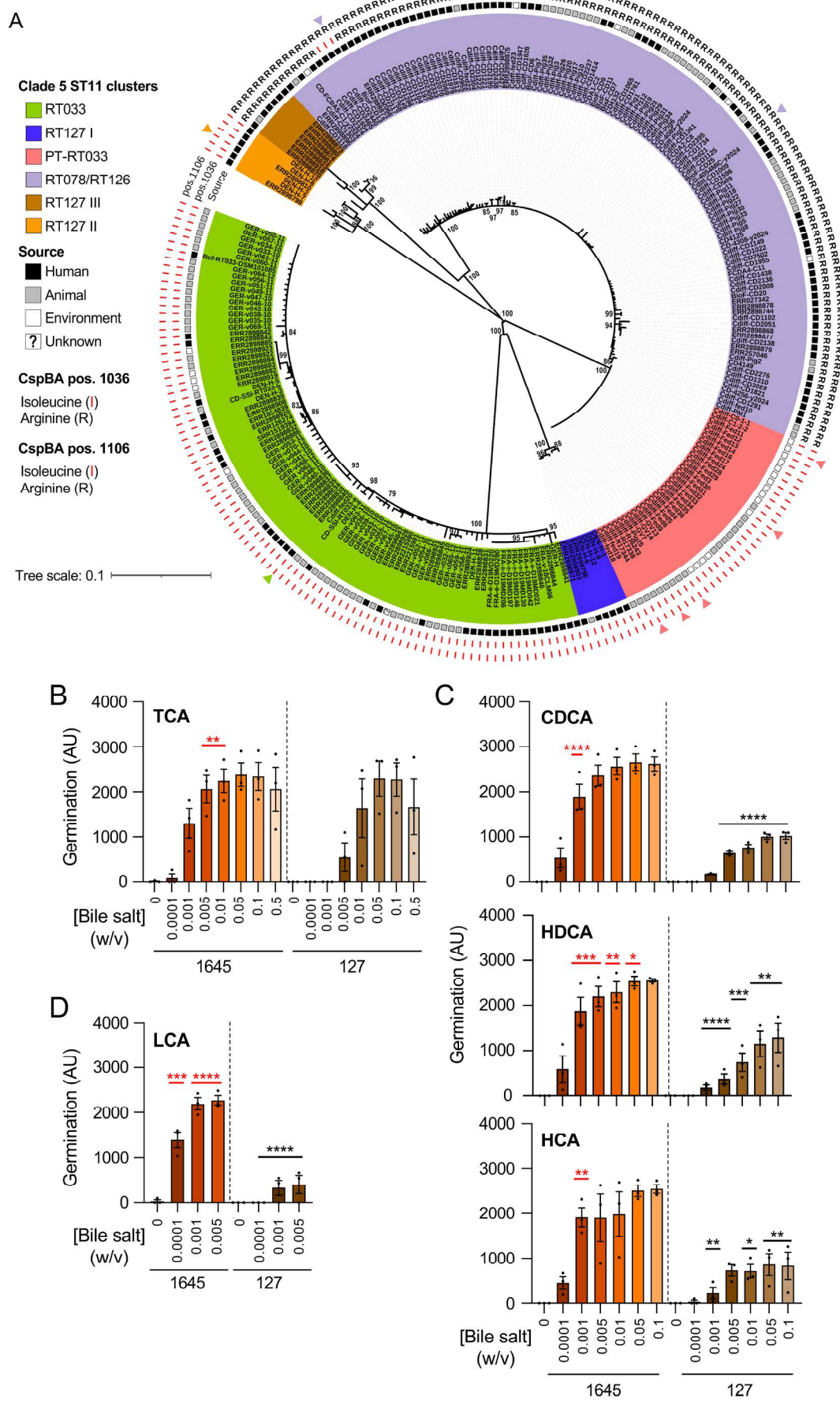


FIGURE 5 | Legend on next page.

FIGURE 5 | *C. difficile* strains from other ribotypes carrying the CspA^{R1036I} substitution display increased germinant sensitivity and expanded germinant response. (A) Phylogenetic positioning of *cspAB* isoleucine/arginine genotype in *Clostridioides difficile* clade 5 ST11 isolates from multiple RTs originating from environmental, animal and human sources. The tree was based on the core-SNP diversity found among 258 isolates relative to the reference genome DSM101085 (CP021319.1), after masking 10 regions totaling 6.66% of the genome (266,829 of 4,009,385 bp). Phylogenetic reconstruction was performed using the Maximum Likelihood (ML) method under the General Time Reversible model with Gamma-distributed rate variation among sites and a proportion of invariant sites (GTR+G+I). The final dataset comprised 926 positions. Bootstrap support values were estimated from 500 replicates and are indicated at the nodes; for clarity, only values ≥ 75 are shown. The tree was visualised using the iTOL website (<https://itol.embl.de>) (Letunic and Bork 2021). Arrowheads point to the strains used in this study. (B–D) Purified spores from 1645 and from strain from RT127, were incubated in BHI with the indicated bile salts at different concentrations and germination in arbitrary units (AU) was calculated (see [Material and Methods](#)). CDCA, chenodeoxycholic acid; HCA, hyocholic acid; HDCA, hyodeoxycholic acid; LCA, lithocholic acid; TCA, taurocholic acid. Asterisks indicate statistical significance determined with a one-way ANOVA with Tukey's multiple comparisons test ($*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$, $****p \leq 0.0001$, 127 vs. 1645 34 in black and VACA34 vs. 1645 in red). In all graphs, the data shown indicate the mean of at least three biological replicates, the measured values (dots) and the standard deviation of the mean (error bars).

demonstrate that a single amino acid substitution (R1036I) in CspA is required to confer enhanced germination sensitivity and expanded germinant specificity, rendering spores prone to germinate in bile salts that are typically inhibitory and commonly encountered in the animal host.

3 | Discussion

Spore production underpins *C. difficile* transmission, but successful infection also depends on how readily spores exit dormancy in response to host cues (Deakin et al. 2012; Moore et al. 2013). Our results show that RT033 strains are tuned to germinate at lower bile-salt concentrations and can respond to bile acids typically considered inhibitory (Figures 2, 4, S1D and S2). The genetic determinants of this phenotype map to the germination apparatus. Heterologous expression of the 033-*cspBAC* operon is sufficient to confer the phenotype to strain 630 Δ erm (Figures 2 and S3), supporting a model in which altered receptor function and/or signalling thresholds contribute to strain-specific behaviour (Bhattacharjee et al. 2016). The strong association between the RT033 phenotype and a single conserved CspA substitution (R1036I), shared by strains in other ribotypes (RT078 and RT127) (Figure 5), points to a favoured genetic route by which germination sensitivity and specificity can be re-wired. Although our data support a role for CspA R1036I, definitive attribution of the RT033-like germination phenotype will require future analysis of isogenic point mutants.

Recent work has shown that CspC and CspA form a heterodimer that integrates germinant and co-germinant signals during spore germination, and that interactions at this interface modulate the threshold for germinant responsiveness (McNellis et al. 2026). In this heterodimer, CspC residues D429 and R456, previously implicated in sensitivity to both germinant and co-germinant signals, form salt bridges with CspA residues R1036 and D1008, respectively (Rohlfing et al. 2019; McNellis et al. 2026). Consistent with the behaviour of the corresponding CspC interface residues, alanine substitutions of the salt-bridge-forming residues in CspA increase sensitivity to taurocholate (TCA) (McNellis et al. 2026), indicating that these interfacial contacts normally dampen germinant and co-germinant signalling in dormant spores. Notably, in one reported heterodimer structure only the CspC D429—CspA R1036 salt bridge is observed, raising the possibility that this interface is conformationally

dynamic and undergoes rearrangements during signal sensing/transduction.

We confirmed, using pull-down assays, that RT033 CspC and CspA also form a complex (Figure 3C). AlphaFold3 modelling of the RT033 heterodimer suggests that replacing R1036 with the hydrophobic residue isoleucine not only eliminates the salt bridge but also increases the distance to CspC D429 from ~ 3.6 to ~ 10 Å (Figure 3). Strikingly, RT033 spores are not only hypersensitive to TCA but also display increased responsiveness to all bile salts tested, including bile acids typically described as germination inhibitors. Together, these data extend the proposed role of the CspC D429–CspA R1036 interaction beyond dampening germinant signalling, suggesting that it may also contribute to discrimination among the bile salts that can trigger germination.

Current models place the CspC:CspA heterodimer at the centre of germinant and co-germinant signal integration, but the mechanisms by which signals sensed by this complex are transmitted to activate the CspB protease remain unclear. Germinant-induced conformational changes within the CspC:CspA complex have been proposed to regulate activation of CspB and subsequent SleC-mediated cortex degradation, yet this has not been demonstrated directly. Moreover, structures of the complex bound to a germinant or co-germinant are not currently available. Our findings identify a naturally occurring CspA substitution that perturbs the CspC:CspA interface, lowers the activation threshold, and broadens germinant specificity, providing a genetic entry point for dissecting signal transmission through the germination apparatus.

Earlier work suggested that *sleC*, *cspBA*, and *cspC* are restricted to the Peptostreptococcaceae lineage (Ramos-Silva et al. 2019). However, these genes may have a broader distribution among the Clostridia. In any case, germinant receptor specificity is both species- and strain-specific and likely reflects adaptation of endospore-forming bacteria to particular environmental niches (Paredes-Sabja et al. 2011; Shen et al. 2019; Setlow 2013). Their distribution, including that of specific alleles, may therefore represent adaptation to the gut environment, enabling germination in response to specific bile salts (Ramos-Silva et al. 2019). Gut bile acid composition varies widely among hosts and differs markedly between humans and livestock. The human bile acid pool is dominated by TCA and CDCA and their secondary derivatives, whereas livestock show more diverse, species-specific

profiles (Theriot et al. 2016; Sun et al. 2022; Chong et al. 2025; Guzik and Quinn 2021; Zheng et al. 2021). Because our strains were isolated from a zootechnical setting and share the CspA substitution that enables germination in bile acids characteristic of these hosts, our findings are consistent with adaptation to host-associated bile acid landscapes.

The broader germinant responsiveness of RT033 strains may expand the range of conditions permissive for germination, both in the gut and in other host associated environments. Bile acids in animal faeces can occur at concentrations comparable to those tested here and could therefore support spore germination (Sun et al. 2022; Porru et al. 2022). Accordingly, manure and animal waste may represent important reservoirs: they are anaerobic, enriched in host derived bile acids, and frequently contaminated with *C. difficile* spores (Usui et al. 2017; Trsinar et al. 2025). Expanded germinant specificity could therefore promote persistence and recycling within agricultural settings.

While increased germination sensitivity has been proposed to influence disease severity in epidemic *C. difficile* strains, the high genetic diversity of the species has made it difficult to establish direct links between germination efficiency and clinical outcome (Moore et al. 2013; Plaza-Garrido et al. 2015; Carlson et al. 2013; Beebe et al. 2025; Sulaiman et al. 2024). Nevertheless, broader germinant responsiveness may increase the likelihood that RT033 spores germinate across heterogeneous intestinal environments. Heightened sensitivity to bile acid germinants could lower the effective infectious dose by increasing the probability of spore germination, but tight control of germination is also thought to help constrain germination to permissive intestinal niches (Rohlfing et al. 2019; Vinay et al. 2025). In addition, germination alone is insufficient for infection: vegetative growth must be sustained, and several bile acids inhibit vegetative cells, potentially counterbalancing any advantage conferred by broader germinant responsiveness (see [Supporting Information](#); Figure S6). Nevertheless, we identified the same CspA R1036I substitution in two human-associated isolates (RT078 and RT127; TcdAB+CDT+) recovered from patients with active CDI, suggesting that germination traits selected in animal-associated settings remain compatible with colonisation, and potentially disease, in humans. Importantly, RT033 strains themselves have also been reported to cause symptomatic CDI in patients with established risk factors, including an atypical case of ileal CDI in a patient with prior total colectomy. This case is particularly notable because it suggests that RT033-related strains can cause disease in altered intestinal environments, where bile acid exposure and germinant availability may differ from those of the intact colon (Eckert et al. 2014).

Because RT033 strains carry an interrupted PaLoc and are likely impaired in TcdA/TcdB production, infections caused by these strains may be under-recognised in settings that rely on toxin-based diagnostic assays (Eckert et al. 2014; AM et al. 2017), potentially facilitating transmission outside routine clinical surveillance. Notably, TcdA/B-negative but CDT positive RT033 strains have been isolated from symptomatic patients (AM et al. 2017; Androga et al. 2015), and work with an isogenic TcdA/B–, CDT+ mutant in the hamster infection model indicates that binary toxin alone can contribute to disease (Kuehne et al. 2014).

In conclusion, our study identifies a naturally occurring substitution in CspA that lowers the threshold for germinant signalling and expands germinant specificity, enabling *C. difficile* spores to respond to bile acids previously regarded as inhibitory. By linking altered germination behaviour to host-associated bile acid environments and environmental reservoirs, these findings highlight germination as a flexible and adaptable trait. Although our study focused on bile acid-mediated germination, the effect of the R1036I substitution in CspA on co-germinant sensing remains an important question for future investigation. Defining how such germination phenotypes shape colonisation dynamics and disease outcome in vivo will be essential for understanding their epidemiological significance.

4 | Materials and Methods

4.1 | Growth Conditions and General Methods

The *C. difficile* strains used in this study were routinely cultured anaerobically at 37°C in brain heart infusion (BHI) medium (Oxoid) under an atmosphere of 5% H₂, 15% CO₂, and 80% N₂. When required, *C. difficile* cultures were supplemented with cefoxitin at 25 µg/mL and thiamphenicol at 15 µg/mL. Sporulation assays and spore purification was performed as described before (Martins et al. 2024). When necessary, thiamphenicol was added to 70:30 medium at a final concentration of 2 µg/mL. *E. coli* strains were routinely grown in Luria-Bertani (LB) medium, supplemented when necessary, with ampicillin (100 µg/mL) or chloramphenicol (15 µg/mL). For molecular cloning, *E. coli* DH5α (Bethesda Research Laboratories) was used. *E. coli* BL21(DE3) was used for protein production, whereas *E. coli* HB101(RP4) served as the donor strain in *C. difficile* conjugation experiments. All strains and primers used in this study are listed in Tables S1 and S2, respectively.

4.2 | Spore Purification

To induce sporulation, the strains were incubated in 70:30 medium at 37°C in anaerobic conditions for 48 or 20 h. The cells were scraped off from the medium with cold water, centrifuged at 4°C, 5752 × g for 10 min, resuspended in 50 mL of cold ddH₂O, and left at 4°C for 48 h. Spores were purified in a gradient of metrizoic acid (Gastrografin, from Bayer) (Martins et al. 2024). The purified spores were resuspended in 500 µL of ddH₂O and quantified by spectrophotometry measuring the OD at 580 nm.

4.3 | Germination Assays

The germination protocol was performed as described previously (Rohlfing et al. 2019). Spores were resuspended in BHI medium to a starting optical density (OD₆₀₀) of 0.8. A total of 180 µL of this spore suspension was dispensed into each well of a 96-well microplate (BRANDplates). Various concentrations of bile salts (0%, 0.0001%, 0.001%, 0.005%, 0.01%, 0.05%, 0.1%, and 0.5%) were added to reach a final volume of 200 µL per well. The OD₆₀₀ was monitored at 1.5-min intervals over a 60-min period using a Synergy Neo2 plate reader (BioTek), maintaining a constant temperature of 37°C and shaking for 5 s before each

reading. Background absorbance from media-only wells was subtracted from all values. Germination efficiency was calculated using the formula:

$$\text{Germination efficiency (\%)} = \frac{\text{OD}_{600} \text{ at } t_n}{\text{OD}_{600} \text{ at } t_0} \times 100$$

To visualise germination (AU), each value was subtracted from 100 to invert the kinetics curves. The area under these inverted curves was determined and presented as bar graphs for each strain (McNellis et al. 2026). For all germinants tested, the appearance of phase-dark spores after the assay was confirmed by phase-contrast microscopy.

4.4 | Coat Extraction

Coat proteins were extracted from purified spores by boiling the suspension for 8 min in loading buffer (125 mM Tris, 4% SDS, 10% (vol/vol) 2-mercaptoethanol, 1 mM DTT, 0.05% bromophenol blue, 10% glycerol at pH 6.8).

4.5 | Immunoblot Analysis

Samples were resolved by SDS-PAGE (12.5% gels). Gels were stained with Coomassie Brilliant Blue R-250, or subjected to immunoblot analysis with anti-CspB, anti-CspC, anti-SleC (Marini et al. 2023), each used at a dilution of 1:3000. A rabbit secondary antibody conjugated to horseradish peroxidase (Sigma) was used at a dilution of 1:5000. The monoclonal anti-His₆ tag primary antibody (Acrobiosystems) was used at a 1:1000 dilution, and an anti-mouse secondary antibody conjugated to horseradish peroxidase (Sigma) was used at a 1:2000 dilution. The immunoblots were developed with enhanced chemiluminescence reagents (Amersham Pharmacia Biotech).

4.6 | Overproduction of Proteins in *E. coli*

E. coli BL21(DE3) cells were grown in LB medium to an OD₆₀₀ of about 0.6 and then induced with 50 μM IPTG. Cells expressing CspC033 and CspAC033 were collected 30 min after induction, while cells expressing CspC630 and CspAC630 were collected after 90 min. The cultures were centrifuged at 4000 × g for 10 min at 4°C, and the cell pellets were resuspended in lysis buffer (100 mM NaCl, 10 mM Tris-HCl pH 8.0, 0.1% Triton X-100, 10% glycerol, 3.5 μL 2-mercaptoethanol, and 10 mM imidazole). The cells were broken using a FastPrep device with glass beads at 5 m/s for 1 min. The lysates were then centrifuged at 15000 × g for 1 min at 4°C to remove debris.

4.7 | Pull-Down Experiments

For His₆-tag pull-down assays, 250 μL of extract from each strain was incubated with and without bile salts (10 mM Tris-HCl, pH 8.0; 0.6 mM glycine; and 0.05% TCA when indicated) for 30 min at room temperature with shaking. The extracts were then incubated with Ni-Sepharose HP beads (GE Healthcare).

After 30 min at room temperature with shaking, unbound proteins were removed by washing the beads three times with wash buffer (VPEX-200: 200 mM NaCl, 10 mM Tris-HCl, pH 8.0, 0.1% Triton X-100, 10% glycerol, 3.5 μL 2-mercaptoethanol per 10 mL, 20 mM imidazole). Bound proteins were eluted with loading buffer, resolved on SDS-PAGE (12.5%) gels, and subject to immunoblot analysis.

4.8 | Whole Genome Sequencing and Phylogenetic Analysis

Genomes included in the phylogenetic analysis ($n = 258$) were retrieved from the European Nucleotide Archive (ENA), following queries performed in the Enterobase platform, from previously published studies, and from INSA internal database. All reads generated for the present study were deposited in the ENA under the study accession number PRJEB4979.2. All genome sequences were *de novo* assembled using the INNUca v4.2.0 pipeline (<https://github.com/B-UMMI/INNUca>), as previously described (Zhou et al. 2020; Frentrop et al. 2020). Genome annotation was carried out using Prokka v1.14.5-2 software (<https://github.com/tseemann/prokka>) (Seemann 2014). Genetic relatedness among isolates was assessed using a reference-based mapping approach using Snippy v4.5.1 software (<https://github.com/tseemann/snippy>). For RT033, the genome of *C. difficile* DSM101085 (Genbank Accession number CP021319.1) was used, while for RT078 the *C. difficile* M120 (Genbank Accession number NC_017174.1). For both analyses, quality-processed reads were mapped against each reference, and single-nucleotide variant (SNV) calling was performed considering only variant sites that met the following criteria: (i) minimum mapping quality and minimum base quality of 30; (ii) read depth ≥ 10; and (iii) at least 70% of reads differing from the reference allele. Core-SNVs were extracted using Snippy's core module (snippy-core), by masking repetitive regions, mobile genetic elements (MGE) like transposons and prophages, recombination regions (including the PaLoc region, the S-layer cassette, the *skin*^{Cd} element and CRISPR regions) of each reference genome, as their inclusion would bias the phylogeny. All reported SNVs/indels were visually inspected and validated using the Integrative Genomics Viewer v2.11.0 (Robinson et al. 2011). Phylogenetic reconstructions were performed in MEGA7 software (<http://www.megasoftware.net>) (Kumar et al. 2016) using the Maximum Likelihood (ML) method (Felsenstein 1981). The General Time Reversible model with Gamma-distributed rate variation among sites and a proportion of invariant sites (GTR+G+I) was used as the nucleotide substitution model. Initial tree(s) for the heuristic search were generated automatically using NJ/BioNJ, and the ML tree was optimised using the Subtree-Pruning-Regrafting algorithm (SPR level 3) with a moderate branch swap filter. Branch support was assessed by bootstrapping with 500 replicates (Felsenstein 1985).

4.9 | AlphaFold3 Modelling

Structural models were obtained using the open-source AlphaFold3 server (Abramson et al. 2024), with the sequences

of the proteins as input and keeping all other parameters at their default settings. For each prediction, the top-ranked model was selected for further analysis. Structural models were visualised using PyMOL (Schrödinger LLC; www.pymol.org).

Author Contributions

Isabel Roseiro: conceptualization, investigation, writing – original draft, visualization, writing – review and editing, formal analysis, methodology. **Alexandra Nunes:** investigation, data curation. **Diogo Martins:** investigation, formal analysis, funding acquisition. **Frederico Alves:** investigation, formal analysis, writing – review and editing. **Søren Persson:** writing – review and editing, investigation. **Adriano O. Henriques:** funding acquisition, writing – review and editing, visualization, supervision, conceptualization, resources. **Mónica Oleastro:** investigation, writing – review and editing, supervision. **Mónica Serrano:** conceptualization, investigation, funding acquisition, writing – review and editing, visualization, supervision, formal analysis, methodology, project administration.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Data S1:** emi70332-sup-0001-Supinfo.docx. **Figure S1:** Phase contrast microscopy of *C. difficile* strains from different ribotypes. (A) Biofilm formation (top panel) was evaluated at 48 h using a crystal violet assay (see [Materials and Methods](#) section). Heat resistance tests (bottom panel) were performed to assess sporulation ratio. Strains were incubated in 70:30 media for 24 h, samples were collected, serially diluted aliquots spotted onto BHI agar plates supplemented with TCA 0.1% before (total viable titre) and after heat treatment 70°C for 30 min (heat resistant spore count). Plates were incubated under anaerobic conditions for 24 h. CFUs were counted for both conditions and the sporulation efficiency calculated (see the [Material and Methods](#) section). Asterisks indicate statistical significance

determined with a one-way ANOVA with Tukey's multiple comparisons test (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$ all strains vs. 630 Δ erm). In all graphs, the data shown indicate the mean of at least three biological replicates, the measured values (dots) and the standard deviation of the mean (error bars). (B) Phase contrast microscopy of cells from *C. difficile* strains from ribotypes 012, 078, 033 and PT-033 grown in 70:30 media for 48 h. Strains 630 Δ erm, A and B represent human isolates, while strains GC-29, VACA34, GV, R17344 and PIG25 represent animal isolates, and strains PT20 and SS2 represent environmental isolates. Scale bar 1 μ m. (C) Phase contrast microscopy of strain VACA34 grown in 70:30 media containing OXOID Peptone or Bacto-Peptone for 48 h. Scale bar, 1 μ m. (D) Purified spores from strain PIG25, were incubated in BHI with the indicated bile salts at different concentrations and germination in arbitrary units (AU) was calculated (see [Material and Methods](#)). CDCA, chenodeoxycholic acid; HCA, hyocholic acid; HDCA, hyodeoxycholic acid; LCA, lithocholic acid; TCA, taurocholic acid. Asterisks indicate statistical significance determined with a one-way ANOVA with Tukey's multiple comparisons test (** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$, PIG25 vs. strain 630). In all graphs, the data shown indicate the mean of at least three biological replicates, the measured values (dots) and the standard deviation of the mean (error bars). **Figure S2:** Strain from RT033 shows increased germination sensitivity. Germination levels were monitored by following the decrease in OD₆₀₀ of the spore suspension and normalised to the initial value. Germination in arbitrary units (AU) was calculated using the area below inverted OD₆₀₀ curves (see [Material and Methods](#)). Purified spores from strains 630 Δ erm (RT12), GC-29 (078), VACA34 (RT033) were incubated in BHI with the indicated bile salts at different concentrations. CA, cholic acid; CDCA, chenodeoxycholic acid; GCA, glycocholic acid; HCA, hyocholic acid; HDCA, hyodeoxycholic acid; LCA, lithocholic acid. In all graphs, the data shown indicate the mean of at least three biological replicates, the measured values (dots) and the standard deviation of the mean (error bars). Asterisks indicate statistical significance determined with a one-way ANOVA with Tukey's multiple comparisons test (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$ VACA34 vs. 630 Δ erm in black and VACA34 vs. GC-29 in red). **Figure S3:** Heterologous expression of the RT033 *cspBAC* operon in strain 630 Δ erm enhances germination sensitivity and expands the bile salt response. Spores purified from the 630 Δ erm strain carrying a plasmid-borne copy of the *cspBAC* operon, or the *cspBAC* operon from the RT033 strain or an empty plasmid, were incubated in BHI with the indicated bile salts at different concentrations. Germination was calculated as described in the [Material and Methods](#) section. CA, cholic acid; CDCA, chenodeoxycholic acid; GCA, glycocholic acid; HCA, hyocholic acid; HDCA, hyodeoxycholic acid; LCA, lithocholic acid. Asterisks indicate statistical significance determined with a one-way ANOVA with Tukey's multiple comparisons test [* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$, 630 Δ erm (033-*cspBAC* or 630-*cspBAC*) vs. 630 Δ erm (empty) in black and 630 Δ erm (033-*cspBAC*) vs. 630 Δ erm (630-*cspBAC*) in red]. **Figure S4:** Distribution of *cspA11036* and *csp11106* in *Clostridioides difficile* RT078/RT126 ST11 lineage. The tree was based on the core-SNP diversity found among 181 isolates relative to the reference genome DSM101085 (CP021319.1), after masking 16 regions totaling 8.52% of the genome (344,738 of 4,047,729 bp). Phylogenetic reconstruction was performed using the Maximum Likelihood (ML) method under the General Time Reversible model with Gamma-distributed rate variation among sites and a proportion of invariant sites (GTR+G+I). The final dataset comprised 618 positions. Bootstrap support values were estimated from 500 replicates and are indicated at the nodes; for clarity, only values ≥ 75 are shown. The tree was visualised using the iTOL website (<https://itol.embl.de/>) (Dingle et al. 2019). Arrowheads point to the strains used in this study. **Figure S5:** AlphaFold3 model of the Csp proteins. (A) AlphaFold3 model of the CspBA complex. The model of the CspBA protein is shown in grey, while blue and pink are used to show the superimposition of the individual proteins, CspB and CspA, respectively. The pro-domain (PD) of CspB is shown in green. (B) Domain organisation of CspBA. The inhibitory PD of CspB undergoes self-cleavage. The residues that form the catalytic triad (Asp976, His160 and S461) are indicated above the diagrams. The grey box with stripes represents the region proposed to be cleaved by YabG to release CspB and CspA.

Dotted lines highlight regions with amino acids substitutions in RT033 strains when compared to strains of RT078. The alignments show highly conserved residues in the vicinity of Iso1036 and Iso1106. (C) AlphaFold3 model of the CspC protein. Highlighted in red are residue D429 in CspC, found to inhibit germinant and co-germinant signalling in dormant spores (Moloney et al. 2021) and also residues G455 to I458 that form a loop previously described to be important for germinant and co-germinant sensitivity (Alves et al. 2023). (D) Representation of the CspC protein. Red lines represent residue D429 and the loop found to be important for germination. Dotted lines highlight regions with amino acids substitutions in strain 127 (see text for details). **Figure S6:** Bile salt sensitivity of *C. difficile* strains. *C. difficile* strains were grown in BHI to mid-exponential phase, back-diluted to OD₆₀₀ = 0.05, and incubated for ~18 h in the presence of increasing bile salt concentrations. Growth was measured by OD₆₀₀ and normalised to solvent-only controls for each strain. The colour scale indicates growth relative to the solvent control: green denotes growth similar to the control (> 0.8), yellow (0.7–0.4) and red (< 0.3) indicates growth inhibition. CA, cholic acid; chenodeoxycholic acid; DCA, deoxycholic acid; HCA, hyocholic acid; HDCA, hyodeoxycholic acid; LCA, lithocholic acid. CA was tested at 0.01%, 0.05%, 0.1% and 0.5%. CDCA, DCA, HCA and HDCA were tested at 0.005%, 0.01%, 0.05% and 0.1%. LCA was tested at 0.0001%, 0.001% and 0.005%. **Table S1:** Bacterial strains used in this study. **Table S2:** Oligonucleotide primers used in this study.