



A *Clostridium difficile* alanine racemase affects spore germination and accommodates serine as a substrate

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Clostridium difficile has become one of the most common bacterial pathogens in hospital-acquired infections in the United States. Although *C. difficile* is strictly anaerobic, it survives in aerobic environments and transmits between hosts via spores. *C. difficile* spore germination is triggered in response to certain bile acids and glycine. Although glycine is the most effective co-germinant, other amino acids can substitute with varying efficiencies. Of these, L-alanine is an effective co-germinant and is also a germinant for most bacterial spores. Many endospore-forming bacteria embed alanine racemases into their spore coats, and these enzymes are thought to convert the L-alanine germinant into D-alanine, a spore germination inhibitor. Although the *C. difficile* Alr2 racemase is the sixth most highly expressed gene during *C. difficile* spore formation, a previous study reported that Alr2 has little to no role in germination of *C. difficile* spores in rich medium. Here, we hypothesized that Alr2 could affect *C. difficile* L-alanine-induced spore germination in a defined medium. We found that *alr2* mutant spores more readily germinate in response to L-alanine as a co-germinant. Surprisingly, D-alanine also functioned as a co-germinant. Moreover, we found that Alr2 could interconvert L- and D-serine and that Alr2 bound to L- and D-serine with ~2-fold weaker affinity to that of L- and D-alanine. Finally, we demonstrate that L- and D-serine are also co-germinants for *C. difficile* spores. These results suggest that *C. difficile* spores can respond to a diverse set of amino acid co-germinants and reveal that Alr2 can accommodate serine as a substrate.

Clostridium difficile infection (CDI)² has become one of the most common hospital acquired infections in the United States (1, 2). Antibiotic use is the major risk factor associated with CDI (2). Treatment of potential hosts with broad spectrum antibiotics leads to major alterations in the normally protective colonic microbiota and permits colonization of that host by

C. difficile spores (2). Although antibiotics (e.g. vancomycin) are available to treat CDI, the continued perturbation to the colonic microbiota by these antibiotics leads to multiple rounds of recurring infections (3). It is for this reason that the Centers for Disease Control and Prevention have listed *C. difficile* as “an urgent threat” regarding the antibiotic-associated threats to the United States (1).

Although *C. difficile* is a strict anaerobe, it survives in aerobic environments and transmits between hosts in the spore form (4). Spores are metabolically dormant forms of bacteria and formed from vegetatively growing cells, often upon nutrient limitation. During spore formation, a vegetative cell asymmetrically divides into a mother cell compartment and a forespore compartment (5). Through coordinated gene expression, the two compartments mature the forespore into a multilayered spore that can resist harsh conditions (e.g. radiation, heat, antibiotics, etc.) (6). Spores are composed of a DNA-containing core where much of the water has been replaced with dipicolinic acid (often as a calcium chelate (CaDPA)), an inner membrane, a thin peptidoglycan layer, a thick specialized peptidoglycan-containing cortex layer, an outer membrane, a coat layer, and, in some organisms, an exosporium layer (5–7).

In a host, these spores must exit dormancy (germinate) to generate the actively growing, toxin-producing cells that elicit the primary symptoms of disease. *C. difficile* spore germination is stimulated by the combinatorial action of certain bile acids and certain amino acids (8–11). *C. difficile* spore germination is activated by cholic acid-class bile acids, whereas chenodeoxycholic acids competitively inhibit cholic acid-mediated germination (9, 10). Although bile acids are important for germination, they are not sufficient to activate germination on their own. Amino acids are also required for germination, and the most effective co-germinant is glycine (10). However, other amino acids can function as co-germinants with varying efficiencies (e.g. alanine) (11).

In most organisms, germinants interact with their cognate receptors at the inner membrane of the spore (7). These *ger*-type germinant receptors bind to germinants and lead to the release of CaDPA from the core in exchange for water (7). Subsequently, the spore cortex is degraded through the action of spore cortex lytic enzymes. *C. difficile* does not encode orthologues of the classical *ger*-type germinant receptor and instead uses a novel pathway for initiating spore germination where the CspC pseudoprotease is the bile acid germinant receptor (5, 12, 13). We hypothesized that CspC transmits a bile acid signal to the CspB protease, which then cleaves the spore cortex lytic

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This article contains supplemental Figs. S1–S4.

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² The abbreviations used are: CDI, *C. difficile* infection; TA, taurocholic acid; CaDPA, calcium dipicolinic acid; BHIS-TA, brain-heart infusion broth supplemented with taurocholic acid; ITC, isothermal titration calorimetry; PLP, pyridoxal 5'-phosphate; FDAA, 1-fluoro-2-4-dinitrophenyl-5-L-alanine amide.

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enzyme, pro-SleC, to an active form (14–16). Activated SleC degrades the cortex leading to CaDPA release, because of osmotic changes in the core resulting from cortex degradation (15, 17).

Recently, GerG and GerS were identified as important players in *C. difficile* spore germination. Encoded downstream of *gerS* is *alr2*, coding for an alanine racemase (18, 19). Alanine racemases enzymatically convert L-alanine to the D-alanine stereoisomer and are known to be involved in spore development and germination (20, 21). During spore development, the alanine racemase converts the L-alanine germinant to the D-alanine inhibitor to prevent germination of the developing spore within the mother cell (21–24). During *Bacillus subtilis* spore germination, the alanine racemase converts L-alanine to D-alanine to suppress spore germination, a process termed “autoinhibition” (24, 25); D-alanine does not inhibit germination by other *B. subtilis* germinants and is specific to the GerA germinant receptor (22). Autoinhibition of spore germination occurs, presumably, until the abundance of L-alanine accumulates to sufficient levels to overwhelm the racemase and stimulate spore germination (21). In *C. difficile* only the L-form of alanine can trigger spore germination when added with taurocholic acid (TA) (11). Unlike what is seen for *B. subtilis* spore germination, D-alanine does not inhibit germination by *C. difficile* spores (11).

Interestingly, in the same study that identified GerS as a novel regulator of spore germination, inactivation of *C. difficile* *alr2* had minimal impact on *C. difficile* spore germination when analyzed in rich medium (18). However, we hypothesized that *alr2* may play an important role during spore germination in defined medium in the presence of alanine and TA as germinants. Indeed, we found that *C. difficile* *alr2* mutants play an important role during spore germination by converting D-alanine to the L-alanine co-germinant. Interestingly, we found that Alr2 also interconverts L- and D-serine and that these amino acids are germinants for *C. difficile* spores. Finally, we determine that the affinity of Alr2 for amino acids may be destabilized by the bound co-factor because an Alr2 mutant that does not bind to its co-factor has an affinity for L/D-alanine that is ~80× greater than that of the wild-type protein. Our results shed new light on mechanisms of *C. difficile* spore germination and suggest that *C. difficile* spores are equipped to recognize more amino acids as co-germinants than previously thought.

Results

C. difficile *alr2* is not required for spore germination in rich medium

C. difficile *alr2* (encoding an alanine racemase) is the sixth most expressed sporulation protein (26). Prior work has shown that although highly expressed, Alr2 seems to play little to no role during *C. difficile* spore germination in rich medium supplemented with TA germinant (18). However, given the diverse signals present in rich medium, we hypothesized that *C. difficile* *alr2* may play a role similar to what is observed in other spore-forming bacteria by affecting germination in response to L-alanine. Toward this goal, we generated a TargeTron mutation in *C. difficile* UK1 *alr2*. When suspended in rich BHIS medium

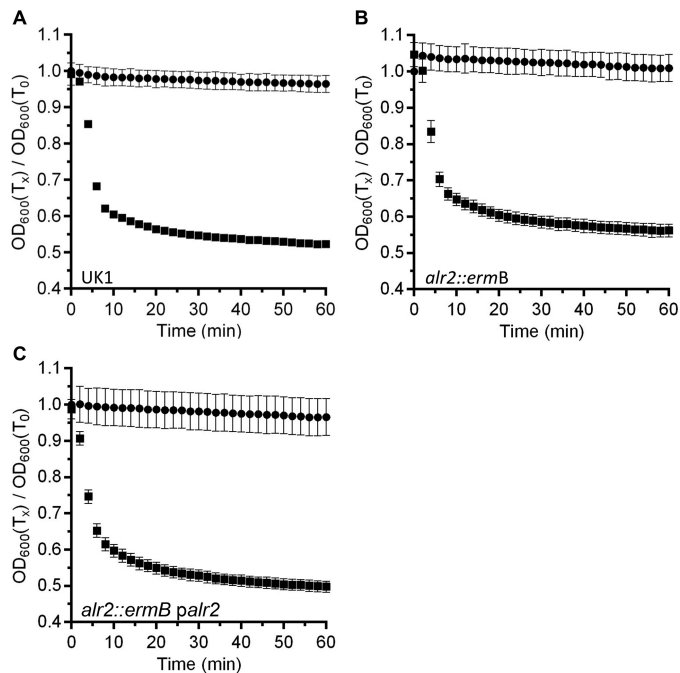


Figure 1. Alr2 does not contribute to germination by *C. difficile* spores in rich medium. Purified spores from wild-type *C. difficile* UK1 (A), *C. difficile* RS07 (*alr2::ermB*) (B), or *C. difficile* RS07 pRS89 (*palr2*) (C) were suspended in rich BHIS medium (●) or BHIS supplemented with 10 mM TA (■) at 25 °C. The A_{600} of the suspension was monitored over time. The data points represent the averages from three independent experiments, and the error bars represent the standard deviation of the mean.

supplemented with TA (BHIS-TA), wild-type *C. difficile* UK1 spores rapidly germinate as shown as a decrease in the optical density of the suspension (Fig. 1A). Similarly, *C. difficile* RS07 (*alr2::ermB*) spores also rapidly germinated in BHIS-TA (Fig. 1B), and when the *alr2* disruption was complemented by expressing *alr2* from a plasmid, no difference was observed compared with the other tested strains (Fig. 1C). These results confirm prior observations that Alr2 plays little role during germination of *C. difficile* spores in rich medium.

C. difficile *alr2* is dispensable for germination in response to L-alanine but essential for germination in response to D-alanine

To test the effects of an *alr2* disruption on *C. difficile* spore germination, we analyzed spore germination in defined medium. *C. difficile* UK1 spores were suspended in buffer supplemented with TA and glycine, TA and L-alanine, or TA and D-alanine, and germination was monitored at 25 °C (supplemental Fig. S1). As expected, wild-type *C. difficile* UK1 germinated in response to glycine and in response to L-alanine (supplemental Fig. S1A). *C. difficile* UK1 spores did not initiate germination in response to TA and D-alanine. When *C. difficile* RS07 was tested for germination in response to TA and glycine or TA and L-alanine or TA and D-alanine, we observed a decrease in the ability of the strain to germinate in response to L-alanine but not in response to glycine (D-alanine, again, had no effect on *C. difficile* spore germination) (supplemental Fig. S1B). This defect in germination in response to L-alanine could be complemented to near wild-type levels (supplemental Fig. S1C).

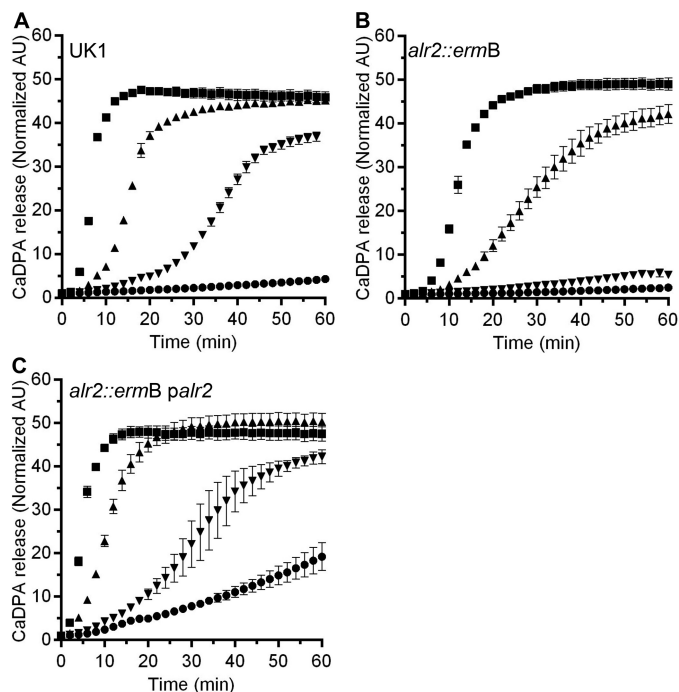


Figure 2. Alr2 affects L/D-alanine-mediated germination by *C. difficile* spores. Purified spores from wild-type *C. difficile* UK1 (A), *C. difficile* RS07 (*alr2::ermB*) (B), or *C. difficile* RS07 pRS89 (*palr2*) (C) were suspended germination buffer supplemented with TA alone (●) or supplemented with glycine (■), L-alanine (▲), D-alanine (▼). CaDPA release from the germinating spores was monitored at 37 °C as an increase in Tb³⁺ fluorescence over time. The data points represent the average from three independent experiments, and the error bars represent the standard deviation of the mean.

To date, most germination assays in our lab and others were performed at room temperature. Although germination often proceeds quickly at this temperature, we reasoned that some *C. difficile* spore enzymes may best function at 37 °C (a physiologically relevant temperature). Therefore, we again tested germination of wild-type *C. difficile* spores in response to TA and glycine or TA and L-alanine or TA and D-alanine. As expected, wild-type *C. difficile* UK1 spores germinated in response to TA and glycine or TA and alanine (Fig. 2A). Surprisingly, *C. difficile* UK1 spores also germinated using D-alanine as a co-germinant with TA (Fig. 2A). *C. difficile* RS07 spores germinated similar to wild-type UK1 spores in TA and glycine or TA and L-alanine (Fig. 2B). Interestingly, *C. difficile* RS07 spores were unable to respond to D-alanine as a co-germinant with TA at the concentration tested (Fig. 2B). This defect in germination could be restored by expressing *alr2* from a plasmid (Fig. 2C). These results suggest that the Alr2 alanine racemase has a role in recognizing D-alanine as a co-germinant or that it converts D-alanine to L-alanine, which then functions as a co-germinant with TA.

C. difficile RS07 spores more readily germinate in response to L-alanine as a co-germinant with TA

Alanine racemases are known to be involved in spore germination, but all studied to date are thought to convert L-alanine to D-alanine, which then acts as an inhibitor of L-alanine-mediated germination, and it is unclear whether D-alanine conversion to L-alanine is important in these organisms. During germination, *C. difficile alr2* may convert D-alanine to L-alanine, which

Table 1

EC₅₀ values (mM) for amino acids and the *C. difficile* spore

The averages and standard deviations for three biological replicates are shown. CND, could not determine; EC₅₀, concentration that achieves the half-maximum germination rate.

	<i>C. difficile</i> strain		
	UK1 (wild type)	RS07 (<i>alr2::ermB</i>)	RS07 pRS89 (<i>alr2::ermB palr2</i>)
L-Alanine	5.5 ± 1.1	2.7 ± 1.8	10.7 ± 2.5
D-Alanine	11.5 ± 2.3	24.2 ± 6.6	6.6 ± 1.0
L-Serine	8.2 ± 0.8	2.5 ± 1.7	4.9 ± 0.5
D-Serine	7.8 ± 1.2	19.1 ± 5.0	CND

would stimulate spore germination in combination with TA. However, because L-alanine is a germinant (and D-alanine does not inhibit *C. difficile* spore germination), if Alr2 converts L-alanine to D-alanine during germination, *C. difficile* RS07 spores may be more sensitive to the L-alanine co-germinant. To quantify this, we determined the EC₅₀ values for L-alanine in *C. difficile* UK1 spores, *C. difficile* RS07 spores, and *C. difficile* RS07 *palr2* spores. Although spore germination is a multienzyme process, these kinetic measurements allow for the quantitative interaction of the spores with activators and inhibitors of germination (27–34). When *C. difficile* UK1 spores were suspended in buffer supplemented with TA and increasing concentrations of L-alanine, we observed an increase in the germination rate. Using the rates and concentrations tested, we found that wild-type *C. difficile* spores had an EC₅₀ of ~5.5 mM for L-alanine (Table 1). *C. difficile* RS07 spores were more sensitive to L-alanine as a co-germinant with an EC₅₀ value of 2.7 mM, suggesting that Alr2 is able to convert L-alanine to D-alanine (Table 1). When the complemented strain was analyzed, the EC₅₀ value was higher than wild-type, 10.7 mM, suggesting that overexpression of Alr2 drives L-alanine to D-alanine more than what is observed in wild type (Table 1).

Next, we determined the EC₅₀ values for D-alanine during spore germination. *C. difficile* UK1 demonstrates an EC₅₀ of 11.5 mM for D-alanine. If Alr2 is converting D-alanine to L-alanine during germination, the EC₅₀ value should increase in *C. difficile* RS07. Indeed, *C. difficile* RS07 spores were less sensitive to D-alanine as a germinant and yielded an EC₅₀ value of 24.2 mM, weaker than wild-type. When *alr2* was expressed from a plasmid, the EC₅₀ decreased to 6.6 mM because of its conversion to L-alanine. The EC₅₀ of *C. difficile* RS07 *palr2* with D-alanine was similar to the EC₅₀ of wild-type spores and L-alanine. Importantly, because Alr2 affected germination in response to L-alanine and D-alanine, the EC₅₀ values for *C. difficile* UK1 are an approximation for these amino acids (some of the L-alanine is converted to D-alanine and vice versa during germination). Taken together, these results suggest that D-alanine can be converted to L-alanine, which functions as a co-germinant. Moreover, these results demonstrate that D-alanine itself is a co-germinant for *C. difficile* spores.

Alr2 interconverts L-serine and D-serine

A prior study determined a crystal structure of a *C. difficile* alanine racemase (35). Although not known at the time, this racemase is Alr2. When recombinantly expressed and purified, Alr2 (supplemental Fig. S2) was suspended in reaction buffer and reacted with L- or D-alanine before labeling and separating

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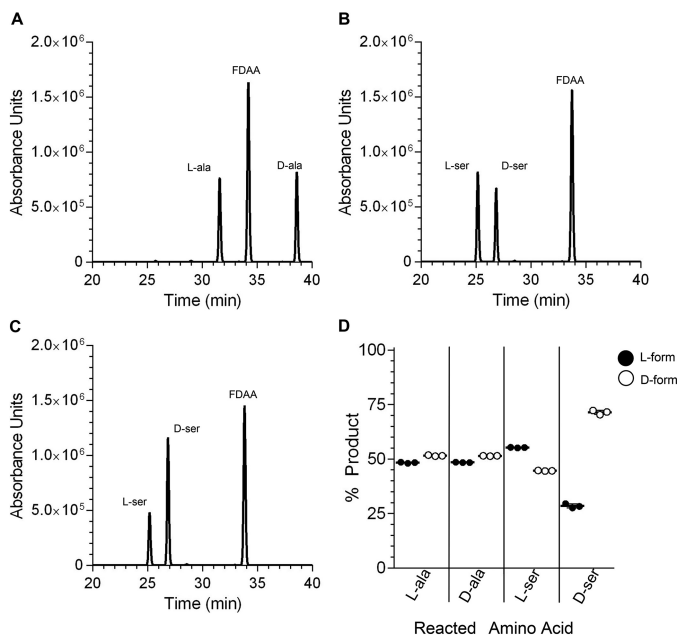


Figure 3. Alr2 interconverts L/D-alanine and serine. A–C, recombinantly expressed and purified Alr2 was incubated with L-alanine (A), L-serine (B), or D-serine (C) for 1 h. The reaction was labeled with FDAA and separated by HPLC. The HPLC retention times of each amino acid were determined using labeled amino acid standards. D, peak areas from each reaction were quantified and presented as percentages of product generated from a reaction involving either L-alanine or D-alanine, etc., and each data point is shown. Each bar represents the average peak area from three technical replicates. The error bars represent the standard deviation from the mean and in most cases are smaller than the bars.

by HPLC. As shown in Fig. 3A, when incubated with L-alanine, Alr2 converted L-alanine to an approximately equal amount of D-alanine (Fig. 3D); we use >98% pure D- or L-amino acid for these studies, and the opposite enantiomer is extremely low in these studies (supplemental Fig. S3). During the course of these experiments, we included L-serine as a would-be negative control for conversion. To our surprise, Alr2 converted L-serine to an approximately equal amount of D-serine (Fig. 3B). When tested with D-serine, Alr2 did not convert as much, and nearly 75% of the D-serine (Fig. 3C, quantified in Fig. 3D) remained in the D-form. Because Alr2 converted L- and D-serine, we tested every other soluble amino acid (data not shown). No other amino acid was converted by Alr2, indicating that Alr2 has specificity to the L- and D-forms of alanine and serine.

L- and D-serine are co-germinants for *C. difficile* spores

Because Alr2 interconverted L- and D-serine, we reasoned that *C. difficile* spores may respond to L-serine as a germinant. Thus, we determined the EC_{50} values for L- and D-serine during *C. difficile* spore germination, as described above (Table 2). Wild-type *C. difficile* UK1 displayed an EC_{50} for L-serine of 8.2 mM. This EC_{50} value decreased to 2.5 mM in the absence of *alr2*. Complementation of *C. difficile* RS07 led to a decrease in EC_{50} from the mutant levels to 4.9 mM but did not restore the EC_{50} to wild-type levels. Interestingly, *C. difficile* spores showed an EC_{50} for D-serine similar to that of L-serine. Also, similar to *C. difficile* RS07 and D-alanine, D-serine was a poor germinant in the absence of *alr2* (EC_{50} = 19.1 mM). Unfortunately, we were unable to complement the phenotype in response to

Table 2

ITC analysis of Alr2 and Alr2_{K39A} binding to L/D-alanine and serine

The averages and standard errors of the mean for at least three technical replicates are shown. The differences in binding between L-alanine and D-alanine or L-serine and D-serine are not statistically significant. The difference in the binding of Alr2 to L-alanine and L-serine or D-alanine and D-serine are significant ($p < 0.05$ and $p < 0.01$, respectively). CND, could not determine.

	Alr2 _{WT}		Alr2 _{K39A}	
	K_D	ΔH	K_D	ΔH
L-Alanine	9.2 ± 0.7	-7 ± 0.7	64 ± 6	-1.1 ± 0.1
D-Alanine	9.2 ± 0.4	-7.8 ± 0.7	46 ± 9	-1.7 ± 0.1
L-Serine	14 ± 1	-16 ± 1	CND	CND
D-Serine	17 ± 1	-21 ± 0.2	CND	CND

D-serine, for unknown reasons. These results suggest that both L- and D-serine are co-germinants for *C. difficile* spores.

Determining the affinity of Alr2 and Alr2_{K39A} to L/D alanine and serine

Previously, kinetic analysis of L- to D-alanine conversion (and vice versa) was used to determine the affinities of Alr2 to the alanine isoforms (35). Because we found that Alr2 can convert L- and D-serine, we tested the affinity of Alr2 to alanine and serine by isothermal titration calorimetry (ITC) (Fig. 4). As shown in Fig. 4A, when L-alanine was injected into the ITC cell, we observed exothermic binding between Alr2 and L-alanine, which could be saturated. We found that Alr2 had similar affinities for L-alanine (9.2 mM) and D-alanine (9.2 mM). The affinities for L-serine (14 mM) and D-serine (17 mM) were weaker (Table 2). Because the ITC method injects ligand into the ITC cell, Alr2 converted the injected ligand to its isomer (supplemental Fig. S4).

Because of the conversion of the L- to the D-form (and vice versa) in the ITC cell, the affinity of Alr2 for each of the tested amino acids could be an average of the affinities for tested and converted amino acids. For this reason, we engineered a mutation that disrupts the binding of the co-factor that is essential for catalysis (pyridoxal 5'-phosphate). The Alr2_{K39A} mutant was confirmed to be enzymatically dead by HPLC analysis of Alr2_{K39A} reactions with L- and D-alanine (data not shown). When the binding of Alr2_{K39A} to L- or D-alanine was tested by ITC, we observed significantly less signal (Fig. 4B) compared with that of wild-type Alr2 (Fig. 4A). However, we observed a signal that could be saturated (Fig. 4B). From this data, we determined that Alr2_{K39A} had an affinity of 65 μM for L-alanine and 50 μM for D-alanine (140 times that of wild-type for L-alanine and 184 times that of wild-type for D-alanine). Unfortunately, we could not determine the affinity of Alr_{K39A} and L-serine or D-serine, and the signal-to-noise ratio was much too great to yield usable data.

Discussion

Spore germination is most often triggered by L-alanine (5). In the model spore-forming organism, *B. subtilis*, L-alanine interacts with the GerAA-AB-AC germinant receptor (7). This interaction leads to the release of CaDPA from the spore core and subsequent cortex degradation. The GerA germinant receptor is conserved across most spore-forming bacteria (36). In *Bacillus anthracis*, and other organisms, L-alanine is a ger-

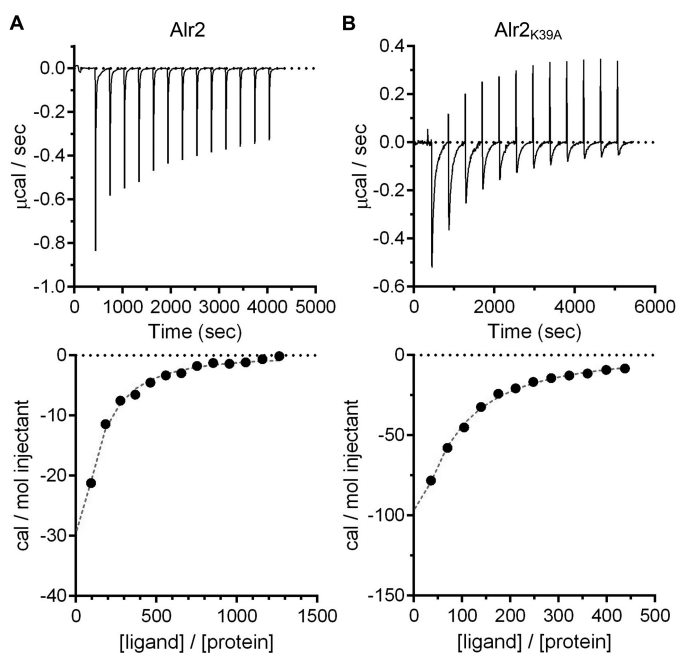


Figure 4. Binding of Alr2_{WT} and Alr2_{K39A} to L-alanine. Recombinantly expressed and purified Alr2_{WT} (A) or Alr2_{K39A} (B) was tested for their ability to bind to L-alanine by ITC. The thermograms are shown in the top panels. A binding isotherm was generated from the total heat following each ligand injection and is plotted in the bottom panels. The data are representative of at least three technical experiments. The summarized data can be found in Table 2.

minant whose action is dependent on the presence of the GerA germinant receptor. Importantly, *C. difficile* does not encode orthologues of the *gerA* germinant receptor but germinates through a novel spore germination pathway (5, 12, 13, 15, 17).

The bile acid germinant receptor, CspC, activates germination through an unknown mechanism (12). Subsequently, the CspB protease activates pro-SleC by cleaving the N terminus from the protein, generating active cortex hydrolase (14). SleC then degrades the cortex resulting in osmotic changes that are perceived at the inner spore membrane (15, 17). This change in osmotic pressure at the inner spore membrane is relieved by the SpoVAC mechanosensing protein and results in CaDPA release from the core (17). Although bile acids are necessary to trigger germination, they are not sufficient. An amino acid signal is required to function as a co-germinant (10).

The most effective amino acid co-germinant is glycine (10). However, other amino acids can function as co-germinants (5). Previously, Howerton *et al.* (11) found that L-alanine could function as a co-germinant with TA for *C. difficile* spores. In their study, they found that D-alanine did not inhibit L-alanine-mediated spore germination, unlike what is observed in other spore-forming bacteria. Importantly, the authors found that D-alanine did not function as an activator of spore germination at 30 °C (11). Herein, we found that D-alanine can stimulate spore germination in combination with TA at 37 °C. Moreover, in the same study, L-serine was nearly inactive as a co-germinant (L-serine generated ~5% germination when compared with glycine); D-serine was not tested (11). However, we found that both L-serine and D-serine can function as co-germinants for *C. difficile* spores at 37 °C. Our data suggest that tempera-

ture is an important consideration when determining activators and inhibitors of *C. difficile* spore germination.

Surprisingly, *C. difficile* Alr2 converted L-serine to D-serine (and vice versa). The affinities of Alr2 to L-serine and D-serine were similar, but the difference was not statistically significant. When tested for the ability to convert L-serine to D-serine, we observed that Alr2 generated nearly 50% D-serine, suggesting that Alr2 efficiently converts L-serine to D-serine. When Alr2 was given with D-serine as a substrate, Alr2 only generated ~25% L-serine from 100% D-serine. Because the binding affinities of Alr2 and L- or D-serine are similar, this could indicate that the rate of conversion of D-serine to L-serine is much slower than the reverse reaction. Because of the conversion of L/D-alanine and serine during ITC, we tested the binding of Alr2_{K39A} to these amino acids. Although the K_D for Alr2_{K39A} and L- and D-serine could not be determined because of a low signal-to-noise ratio, we found the K_D for L- and D-alanine to be much lower than the K_D for the wild-type protein (Table 2). This suggests that the pyridoxyl 5'-phosphate (PLP) co-factor may destabilize the interaction of Alr2 with its substrate, potentially as part of the catalytic mechanism.

Alanine racemases play important roles during the growth of Gram-negative and Gram-positive bacteria (20). These racemases are used to convert L-alanine to the D-alanine that is used to synthesize stem peptides during cell wall synthesis (20). As such, these proteins can be targeted by antibiotic treatment to prevent cell wall synthesis. D-Cycloserine is a commonly used antibiotic that targets alanine racemase, indicating that the racemization of alanine is important for cellular physiology (35). Alr2 can be inactivated by D-cycloserine, but *C. difficile* is naturally resistant to the antibiotic action of D-cycloserine, and D-cycloserine is included in medium that is selective for *C. difficile* growth (35, 37). This suggests that the Alr2 that is incorporated into the *C. difficile* spores is unlikely to be used during cell wall synthesis. *C. difficile* encodes other amino acid racemases. For example, CDR20291_2507 is annotated as a putative alanine racemase. However, there are several other putative amino acid racemases, including a putative VanTG orthologue (serine/alanine racemase). It is likely that one of these other racemases is insensitive, or has reduced sensitivity, to D-cycloserine. We tested the effects of pre-exposure of D-cycloserine to *C. difficile* spores as a means to inactivate Alr2 to prevent the conversion of amino acids in the *C. difficile* spore. Unfortunately, D-cycloserine functioned as a weak co-germinant (data not shown). Because of the background spore germination generated by D-cycloserine, we could not inactivate Alr2 chemically.

Autoinhibition of spore germination is important for the development of a spore and ensuring that the spore germinates under appropriate conditions (21, 24). Alanine racemase is important for autoinhibition by preventing premature germination of the developing spore within the mother cell or under less-than-suitable environmental conditions. We did not observe a difference between spores produced by the wild-type UK1 strain and spores produced by the *alr2* mutant strain. Because *C. difficile* spore germination is activated in response to bile acids and an amino acid co-germinant, it is not surprising that the *alr2* mutation did not affect spore formation. The

After screening for tetracycline-sensitive and thiamphenicol-resistant colonies (transposon-negative, plasmid-positive), the colonies were confirmed to have the desired plasmid using PCR. To isolate a TargeTron insertion into *alr2*, the above isolates were spread onto BHIS plates containing 20 $\mu\text{g}/\text{ml}$ lincomycin and incubated for 24–36 h to obtain colonies. Lincomycin-resistant colonies were isolated and tested for insertion of TargeTron by PCR using the primers 5' *alr* and 3' *alr* (Table 3). An isolate with the desired group II intron insertion was identified, and this strain was renamed *C. difficile* RS07.

Preparation of spores

All spores were purified from BHIS agar medium as described previously (8, 10, 12). Briefly, both wild-type *C. difficile* UK1 and *C. difficile* RS07 were grown on BHIS agar medium, and *C. difficile* RS07 pRS89 was grown on BHIS agar medium supplemented with 5 $\mu\text{g}/\text{ml}$ thiamphenicol. Strains were grown for 4–5 days before isolating the growth and suspending in 1 ml of sterile water/plate. The suspension was stored overnight at 4 °C to promote release of the spores from the mother cells. The suspensions were then washed five times with sterile water to remove cell debris. The resulting spores were purified using a bed of 60% sucrose by centrifuging at $4,000 \times g$ for 30 min. After centrifugation, the sucrose was removed, and the spores at the bottom of the tube were isolated and washed five times with sterile water to remove the sucrose. The spores were >99% pure and heat-activated at 65 °C for 30 min before use.

Spore germination

Spore germination was analyzed at 25 or 37 °C using both a DPA release assay and an optical density assay both. All assays were carried out in 100 μl of total volume using buffer containing a final concentration of 50 mM HEPES, 100 mM NaCl (pH 7.5), 10 mM TA, and varying concentrations of amino acids. The DPA release assay was performed as described previously using a final spore density of 0.5 optical density with 30 mM of amino acids and 250 μM of TbCl_3 in a 96-well plate reader at low PMT settings (excitation at 270, emission at 420 nm) (17). The optical density-based germination assay was also carried out using a plate reader at 595 nm with final 0.5 optical density spores and 30 mM of amino acids.

For the calculation of EC_{50} , the DPA release assay was used at 37 °C with increasing concentrations of amino acids (0–200 mM and a final optical density at 600 nm of 0.3 spores). The rates of germination were determined using the slopes of the linear portions of the germination plots. The data are reported as the averages from three independent experiments with one standard deviation from the mean.

Purification of Alr2 and Alr_{K39A}

pJS164 and pRS100 were transformed into *E. coli* BL21(DE3) *slyD::kan*. To express and purify Alr2_{6His}, the cells were grown at 37 °C to an $A_{600} = 0.8$ in $2 \times \text{TY}$ medium supplemented with ampicillin before inducing for 4 h at 37 °C in the presence of 500 μM isopropyl β -D-thiogalactopyranoside. The cells were pelleted at $6,000 \times g$ for 15 min in a Sorvall centrifuge (Beckman Coulter). The pellets were suspended in lysis buffer (LIB) containing 50 mM

Tris-HCl (pH 7.5), 250 mM NaCl, 1 mM PLP, 1 mM tris-(2-carboxyethyl) phosphine hydrochloride, 15 mM imidazole and protease inhibitor (1 mM PMSF) before freezing at -80 °C.

The cell pellets were thawed and suspended in LIB (25 ml/liter of cell pellet). Lysozyme and DNase were added, and the cell suspension was incubated on ice for 30 min. The cells were lysed using sonication. The sonicated extract was clarified by centrifugation at $15,000 \times g$ for 30 min at 4 °C. Alr2_{6his} was purified from the extract by nickel-affinity chromatography. Beads were washed once in wash buffer LIB containing 30 mM imidazole. The recombinant protein was eluted from the beads using the wash buffer with 500 mM imidazole. The eluted protein was dialyzed in LIB for 3 h and further purified by gel filtration chromatography (Akta Pure, GE Healthcare) on a Sephadex G200 size-exclusion column. Protein was injected and separated at 1 ml/min using LIB. Protein was detected at 280 nm and the eluted protein was collected (supplemental Fig. S3).

Enzymatic assays and HPLC separation of stereoisomers

L- to D-alanine and L- to D-serine conversion (and vice versa) was initiated by adding 500 ng of purified Alr2 to 50 mM amino acid in 50 mM Tris-HCl (pH 7.5), 250 mM NaCl, 1 mM PLP, and 1 mM tris-(2-carboxyethyl) phosphine hydrochloride (total reaction volume was 100 μl (155 nM)). The reactions were incubated at 37 °C for 1 h.

To assay for the presence of L- and D-forms of the tested amino acids, 200 μl of 1% FDAA (Marfey's reagent) in acetone and 40 μl of 1 M sodium bicarbonate was added to the reaction conditions above and incubated for 1 h at 40 °C (41). After the 1-h incubation, 20 μl of 2 M HCl was added to stop the reaction. The reacted material was separated by reverse-phase HPLC (Prominence system; Shimadzu) using a Thermo Scientific Synchronis C18 column. 3 μl of the reacted material was injected and separated at a 0.65 ml/min flow rate of buffer A (50 mM triethylammonium phosphate, pH 3.5, in 25% methanol) and a gradient of 0% buffer B (50 mM triethylammonium phosphate, pH 3.5, in 75% methanol) to 100% buffer B in 40 min before returning to 0% buffer B for another 30 min (70 min total time). Elution of the FDAA and FDAA-labeled compounds was detected by absorbance at 340 nm, and the peak area was quantified.

Isothermal titration calorimetry

All ITC experiments were done using a MicroCal iTC200 System (Malvern) at a constant temperature of 25 °C. The ITC base buffer was 50 mM Tris-HCl (pH 7.5), 250 mM NaCl, and 1 mM PLP. In the ITC chamber, wild-type Alr2 was used at a concentration of 32–45 μM in the ITC base buffer, whereas Alr2_{K39A} was at 100 μM . In the syringe, D- and L-alanine ligands were dissolved in ITC base buffer at 200 mM for wild-type protein or 20 mM for the Alr2_{K39A} experiments, and D- and L-serine were made to 200 mM. All solutions were filtered or centrifuged at $18,000 \times g$ for 5 min prior to use. Thermograms were processed and fit using a single-ligand model (with n fixed to unity), using Origin software, to obtain K (association constant) and ΔH (enthalpy). The dissociation constant (K_D) was calculated from the relationship $K_D = 1/K$.

Influence of *C. difficile* *alr2* on spore germination

Author contributions—R. S. and J. A. S. designed the study and wrote the manuscript. R. S. and S. W. L. performed the experiments. S. W. L. provided comments regarding the paper. All authors analyzed the results and approved the final version of the manuscript.

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