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Glutamate Racemase Mutants of *Bacillus anthracis*

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ABSTRACT

D-Glutamate is an essential component of bacterial peptidoglycan and a building block of the poly-γ-D-glutamic acid (PDGA) capsule of *Bacillus anthracis*, the causative agent of anthrax. Earlier work suggested that two glutamate racemases, encoded by *racE1* and *racE2*, are each essential for growth of *B. anthracis*, supplying D-glutamic acid for the synthesis of peptidoglycan and PDGA capsule. Earlier work could not explain, however, why two enzymes that catalyze the same reaction may be needed for bacterial growth. Here, we report that deletion of *racE1* or *racE2* did not prevent growth of *B. anthracis* Sterne ($pXO1^+$ $pXO2^-$), the noncapsulating vaccine strain, or of *B. anthracis* Ames ($pXO1^+$ $pXO2^+$), a fully virulent, capsulating isolate. While mutants with deletions in *racE1* and *racE2* were not viable, *racE2* deletion delayed vegetative growth of *B. anthracis* following spore germination and caused aberrant cell shapes, phenotypes that were partially restored by exogenous D-glutamate. Deletion of *racE1* or *racE2* from *B. anthracis* Ames did not affect the production or stereochemical composition of the PDGA capsule. A model is presented whereby *B. anthracis*, similar to *Bacillus subtilis*, utilizes two functionally redundant racemase enzymes to synthesize D-glutamic acid for peptidoglycan synthesis.

IMPORTANCE

Glutamate racemases, enzymes that convert L-glutamate to D-glutamate, are targeted for antibiotic development. Glutamate racemase inhibitors may be useful for the treatment of bacterial infections such as anthrax, where the causative agent, *B. anthracis*, requires D-glutamate for the synthesis of peptidoglycan and poly-γ-D-glutamic acid (PDGA) capsule. Here we show that *B. anthracis* possesses two glutamate racemase genes that can be deleted without abolishing either bacterial growth or PDGA synthesis. These data indicate that drug candidates must inhibit both glutamate racemases, RacE1 and RacE2, in order to block *B. anthracis* growth and achieve therapeutic efficacy.

D-Glutamate is a component of peptidoglycan, a structure essential for the physical integrity and the growth of bacteria (1, 2). Earlier work sought to target bacterial D-glutamate synthesis for antibiotic development; however, broad-spectrum inhibitors of D-glutamate synthesis have not yet been reported (3–7). Two distinct enzymatic routes have been identified for bacterial D-glutamate biosynthesis. Glutamate racemase produces D-glutamate by the reversible stereoinversion of L-glutamate (4, 8). D-Amino-acid transaminase catalyzes the pyridoxal phosphate (PLP)-mediated, reversible transamination of D-alanine and α-ketoglutarate, generating D-glutamate and pyruvate (9–11). Some bacteria, including *Escherichia coli* and *Mycobacterium tuberculosis*, employ glutamate racemase as the sole generator of D-glutamate, and the structural gene for this enzyme, *murI*, is essential for growth (12, 13). Other bacteria, for example, *Bacillus* spp. and *Staphylococcus* spp., express two different glutamate racemases and/or D-amino-acid transaminase (*dat*), which, at least in principle, may contribute to the synthesis of D-glutamate (14, 15).

Bacillus subtilis requires D-glutamate for the synthesis of peptidoglycan and of the poly-γ-glutamic acid (γ-PGA) capsule, which is composed of both D- and L-glutamate stereoisomers (16). Three genes of *B. subtilis* encode enzymes that could contribute to D-glutamate synthesis: *racE* (glutamate racemase), *yrpC* (glutamate racemase), and *yheM* (D-amino-acid transaminase) (17). RacE was initially proposed to provide D-glutamate for γ-PGA synthesis (18), whereas YrpC was thought to synthesize D-glutamate for peptidoglycan synthesis (19). However, when grown in minimal (chemically defined) medium, *B. subtilis* does not require *racE*, *yrpC*, or *yheM* for peptidoglycan and γ-PGA synthesis (17, 20). In rich medium (Luria broth), *racE* expression is essential for *B. subtilis* growth and γ-PGA synthesis, as *yrpC* is not expressed under this condition (17). Thus, D-glutamate synthesis in *B. subtilis* appears to require two glutamate racemases with different expression patterns. The D-amino-acid transaminase activity of *Bacillus* and other species has been proposed to prevent racemase-mediated D-glutamate overflow and toxicity (9, 21).

Bacillus anthracis, the causative agent of anthrax, elaborates a poly-γ-D-glutamic acid (PDGA) capsule that blocks phagocytic clearance of its vegetative forms replicating in mammalian hosts (22, 23). The *capBCADE* operon on the pXO2 virulence plasmid contains the genes for the assembly and transport of PDGA (24). The PDGA capsule is essential for *B. anthracis* virulence but dispensable for vegetative growth in laboratory medium (25, 26). The *B. anthracis* genome encodes two glutamate racemases, RacE1 and RacE2, that are closely related to *B. subtilis* RacE but not to YrpC (17). Further, *B. anthracis* harbors two genes (BAS2100 and BAS5082) whose products can be predicted to function as D-amino-acid transaminases. Shatalin and Neyfakh reported that

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TABLE 1 Oligonucleotides used in this study

Primer	Nucleotide sequence[a]
P292	AAAGAATTCGCTATTACAAATACACTAGGGTG
P293	AAACCATGGCACCCGTTTTCTCATTCATCATC
P294	AAACCCGGGATAAAACTAACGACTTCAATACG
P295	TTTCCATGGTAGAATCCATATAAGTGAACTCC
P390	TTTGTCGACAGAAGCAGTTTAAGTGCGC
P391	TTTGGATCCTCAGGAGTTCACTTATATGGATTC
P397	TTAGAGCTCCAACTTCTACGTTAAACGAACC
P398	ACAGCTAGCCATATACTTCATCCCCTCCTC
P399	ATCGCTAGCTAGGCTTTATTAAAGTACTTTTTGTTC
P400	GTCCCCGGGAATGAAGGATCTACAACAAGCGC
P401	AAAGTCGACGTTATTGACGCTAGAAGATTG
P402	AAAGGATCCAACTAACCTAATTTATGG

[a] Primer sequences encompassing restriction enzyme cleavage sites are underlined.

each glutamate racemase gene, *racE1* or *racE2*, cannot be deleted from *B. anthracis* ΔANR (pXO1$^-$ pXO2$^-$) (27) unless laboratory media are supplemented with D-glutamate and proposed that RacE1 and RacE2 are each required for peptidoglycan synthesis and growth (28). However, biochemical studies showed that RacE1 and RacE both catalyze the reversible stereoisomerization of L-glutamate and D-glutamate with similar steady-state kinetic properties (29). A contribution of *racE1* and *racE2* to *B. anthracis* PDGA synthesis had heretofore not been investigated.

Here we show that *racE1* and *racE2* are not essential for growth of *B. anthracis* Sterne (pXO1$^+$ pXO2$^-$) and *B. anthracis* Ames (pXO1$^+$ pXO2$^+$), even in the absence of exogenous D-glutamate. The *racE1* deletion did not affect growth rates, whereas *racE2* deletion delayed the vegetative growth of *B. anthracis* following spore germination, producing aberrant cell shapes that could be only partially suppressed with exogenous D-glutamate. Deletion of *racE1* or *racE2* did not affect the synthesis or stereochemical composition of *B. anthracis* PDGA capsule, and mutants with a deletion of both genes, *racE1* and *racE2*, were not viable.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *B. anthracis* Sterne 34F2 and its mutants were grown in brain heart infusion (BHI) broth. *E. coli* strain K1077 was cultured in Luria-Bertani (LB) broth at 30°C. Kanamycin (Kan) was added at a concentration of 20 μg ml^{-1} for *B. anthracis* to achieve plasmid or mutant allele selection and at 50 μg ml^{-1} for *E. coli*. Spectinomycin and chloramphenicol were added at concentrations of 200 and 5 μg ml^{-1}, respectively. Media were supplemented with 2 mM D-glutamic acid during *racE2* mutant generation. For exogenous complementation, either 2 or 5 mM D-glutamic acid was used. *B. anthracis* was sporulated in modG medium as previously described (30). Spore preparations were heat treated to kill vegetative bacilli and then washed and stored in water. Spores were germinated by inoculation into BHI broth at 10^6 CFU ml^{-1} and grown at 37°C. For capsule production, *B. anthracis* Ames and its variants were grown on nutrient broth-yeast extract (NBY) agar supplemented with 0.8% sodium bicarbonate and 10% heat-inactivated horse serum at 37°C in 5% CO_2 (31).

***B. anthracis* mutants and plasmids.** Deletion mutants were generated with the temperature-sensitive replication plasmid pLM4 (32). PCR products derived from the primer pairs P292/P293 and P294/P295 were inserted into pLM4 via EcoRI, NcoI, and XmaI restriction, thereby generating pSY149, which carries a mutant *racE2* allele (Table 1). Linearized plasmid pSY149 was ligated with the *aad9* gene via the NcoI restriction site, resulting in pSY174. Similarly, primer pairs P397/P398 and P399/P400 were used to generate pSY180 carrying the *racE1* allele. Plasmids were electroporated into *B. anthracis*, and transformants were grown, screened, and verified for allelic replacement as described previously (33). To place the *racE2* allele on the chromosome, *B. anthracis* Sterne with pSY174 was used for allelic exchange; the mutant allele was transduced into *B. anthracis* strain Sterne or Ames by using bacteriophage CP-51 as described previously (34, 35). For complementation studies, pSY179 (pRacE2) and pSY181 (pRacE1) were constructed in pWWW412 (36) (referred to here as "vector") by using primer pairs P390/P391 and P401/P402, respectively. The glutamate racemase genes of pRacE1 and pRacE2 are expressed via the constitutive *hprK* promoter (36). Two additional alleles with a *bursa aurealis* insertion in the *racE1* gene of *B. anthracis* Sterne were used in this study (Fig. 1A) (37). The variant with the *bursa aurealis* insertion at the 5′ end of *racE1* was used to generate the *racE1*^{Ames} mutant allele in *B. anthracis* Ames by transduction with the CP-51 bacteriophage (34, 35).

Microscopy. Digital micrographs of bacterial samples that had been fixed with 4% formalin were captured with an Olympus IX81 microscope equipped with a 100× objective. Phase-contrast images of samples and FM4-64 or boron-dipyrromethene (BODIPY)–vancomycin-stained fluorescence images were captured with a charge-coupled-device camera.

Capsule purification. Capsule from *B. anthracis* Ames or its variants was prepared as described previously, with some modifications (26). Briefly, cells grown on capsule-inducing medium were boiled in 250 mM Tris-HCl (pH 7.5)–4% SDS at 100°C for 30 min. Cells were discarded by sedimentation, and the supernatant was precipitated with 4 volumes of ethanol. The pellet was treated with Benzonase (25 U ml^{-1}) for 2 h at 37°C, followed by proteinase K treatment for 4 h. Any insoluble materials were removed by centrifugation for 10 min at 10,000 ×*g*, and the supernatant was dialyzed against water.

D- and L-glutamate content of capsular material. Purified capsular polymers were hydrolyzed in 6 M HCl at 105°C for 16 h and dried under vacuum at 60°C. Sediments were dissolved in a buffer with 25% triethylamine and 25% methanol. As controls, 50 mM D- and L-glutamate and poly-L-glutamate (Sigma-Aldrich) were treated as described above. Samples were labeled with 1% FDAA (1-fluoro-2-3-dinitrophenyl-5-L-alanine amide) in acetone, followed by the addition of 120 mM sodium bicarbonate and incubation at 40°C for 1 h. Hydrolyzed labeled glutamate was separated on a Hypersil octadecylsilane column (4.6 by 250 mm; particle size, 3 μm; Thermo Electron Co.) and eluted over a period of 60 min with a linear gradient of 5 to 60% acetonitrile in 1% methanol–10 mM ammonium formate, pH 5.2. The flow rate (1 ml min^{-1}) and column temperature (35°C) were constant during the course of the experiments. Peaks were detected by recording the absorbance at absorbance at 340 nm (A_{340}).

RESULTS

***B. anthracis* Sterne *racE1* and *racE2* mutants.** The genome of *B. anthracis* harbors two glutamate racemase genes, *racE1* (BAS0806) and *racE2* (BAS4379), whose deduced amino acid sequences are 53 and 60% identical to that of *B. subtilis* RacE (17). Of note, genes neighboring *B. anthracis racE2* are similar to those flanking *B. subtilis racE* (Fig. 1A) (17). Earlier work generated *bursa aurealis* transposon mutants of *B. anthracis* Sterne and determined the locations of some insertional lesions by DNA sequencing (37). This approach identified two insertions in *racE1*, suggesting that its glutamate racemase product is not essential for *B. anthracis* growth (Fig. 1A). Using allelic replacement, we deleted the *racE1* gene in *B. anthracis* Sterne to generate strain SYO1 (Δ*racE1*, Fig. 1BC). Because earlier studies proposed that *racE2* may be essential for the growth of anthrax bacilli (28), we constructed pRacE2, a glutamate racemase expression plasmid, which was transformed to generate *B. anthracis* Sterne (pRacE2). Using allelic replacement, chromosomal *racE2* was replaced with the spectinomycin resistance gene (*aad9*) and transduced via bacteriophage CP-51 into *B. anthracis* Sterne, generating strain SYO2 (Δ*racE2*::*aad9*).

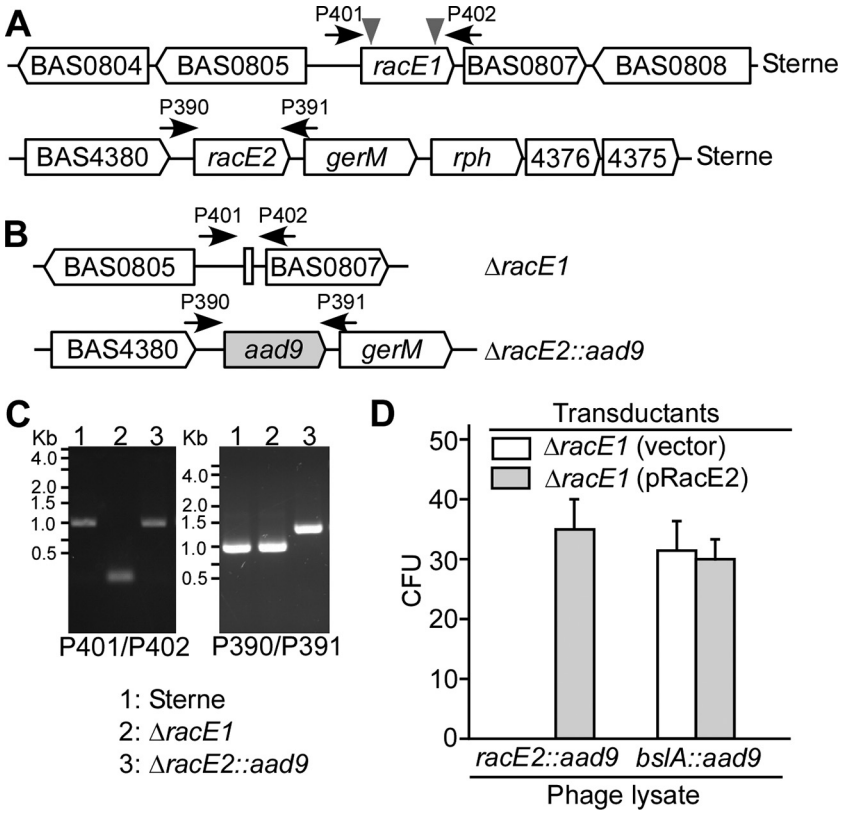

FIG 1 Two glutamate racemases in *B. anthracis*. (A) Organization of glutamate racemase genes *racE1* and *racE2* on the chromosome of *B. anthracis* Sterne. Arrowheads denote *bursa aurealis* insertion sites. (B) Disruption of glutamate racemase genes by allelic replacement. (C) Confirmation of *racE1* and *racE2* gene disruption by PCR amplification of the deleted locus with flanking primers. (D) Efficiency of transduction of the *racE2* allele into the *B. anthracis* Δ*racE1* mutant strain carrying plasmid pRacE2 or the vector (cloning plasmid) control. Spectinomycin-resistant colonies were enumerated after incubation of plates at 30°C for 24 to 30 h. Insertion of the correct *racE2*::*aad9* allele was verified by DNA sequencing. Phage lysate of *bslA*::*aad9* was used as a control. Data from three independent experiments are averaged, and the standard error of the mean is presented.

CP-51 bacteriophage lysates were derived from *B. anthracis* strains SYO2 (Δ*racE2*::*aad9*) and JWK1 (*bslA*::*aad9*), where the spectinomycin resistance gene replaces *bslA*, a gene encoding an S-layer-associated protein (38). CP-51 transduction of Δ*racE2*::*aad9* and *bslA*::*aad9* alleles was successful when *B. anthracis* SYO1 carrying the *racE2*-complementing plasmid (Δ*racE1*/pRacE2) was used as the recipient (Fig. 1D). However, only transduction of *bslA*::*aad9*, but not that of the *racE2*::*aad9* allele, was successful when *B. anthracis* SYO1 carrying the vector control alone (Δ*racE1*/vector) was used as the recipient. Similarly, on agar plates with 2 mM D-glutamate, phage transductions of *bslA*::*aad9* but not of the *racE2*::*aad9* allele were successful with *B. anthracis* SYO1. Thus, *B. anthracis* Sterne does not require *racE1* or *racE2* for growth, whereas mutants lacking both *racE1* and *racE2* appear not to be viable.

Growth defects of the *B. anthracis racE2* mutant. *B. anthracis* strain Sterne, the Δ*racE1* and Δ*racE2*::*aad9* mutants, and the Δ*racE2*::*aad9*/pRacE2 complemented strain were propagated on BHI agar and incubated at 23, 30, 37, or 42°C, and colony formation was assessed as a measure of vegetative growth. When incubated at 30 or 37°C, the Δ*racE2*::*aad9* variant formed smaller colonies than *B. anthracis* strain Sterne and the Δ*racE1* mutant (Fig. 2A). This growth defect was not observed at 23 or 42°C, and the wild-type colony size was restored in the Δ*racE2*::*aad9*/pRacE2 complemented strain incubated at 30 or 37°C. Similar complementation was observed when the Δ*racE2*::*aad9* variant was transformed with the plasmid expressing the RacE1 glutamate racemase (Fig. 2B, Δ*racE2*::*aad9*/pRacE1 strain). Further, when propagated on LB agar, the *B. anthracis* Δ*racE2*::*aad9* variant formed colonies similar in size to those of the isogenic parent, strain Sterne (Fig. 2). These data suggest that loss of endogenous *racE2* diminishes *B. anthracis* growth at physiological temperature (30 to 37°C) on rich medium and that this phenotype can be complemented by plasmid-borne expression of either of the two glutamate racemases, RacE1 or RacE2.

The *racE2* mutation causes growth phase-dependent defects in *B. anthracis* survival. To detect germination or vegetative growth defects, spores of *B. anthracis* (1×10^6 CFU) were inoculated into BHI broth and incubated at 37°C and growth was assessed by measuring A_{600}. Compared to *B. anthracis* Sterne or SYO1 (Δ*racE1*), SYO2 (Δ*racE2*::*aad9*) commenced growth after an 8-h delay; however, the strain eventually replicated at a rate similar to that of the other two strains (Fig. 3A). The delay in the commencement of *B. anthracis* SYO2 growth was eliminated by plasmid-borne expression of glutamate racemase (pRacE2) (Fig. 3A, Δ*racE2*::*aad9*/pRacE2). Addition of 2 or 5 mM D-glutamate partially suppressed the delay in growth commencement of *B. anthracis* SYO2 (Fig. 3B). Plating of culture aliquots on agar and enumeration of colonies revealed a 20-fold drop in the viable CFU count of *B. anthracis* SYO2 at 6 h after spore dilution and germi-

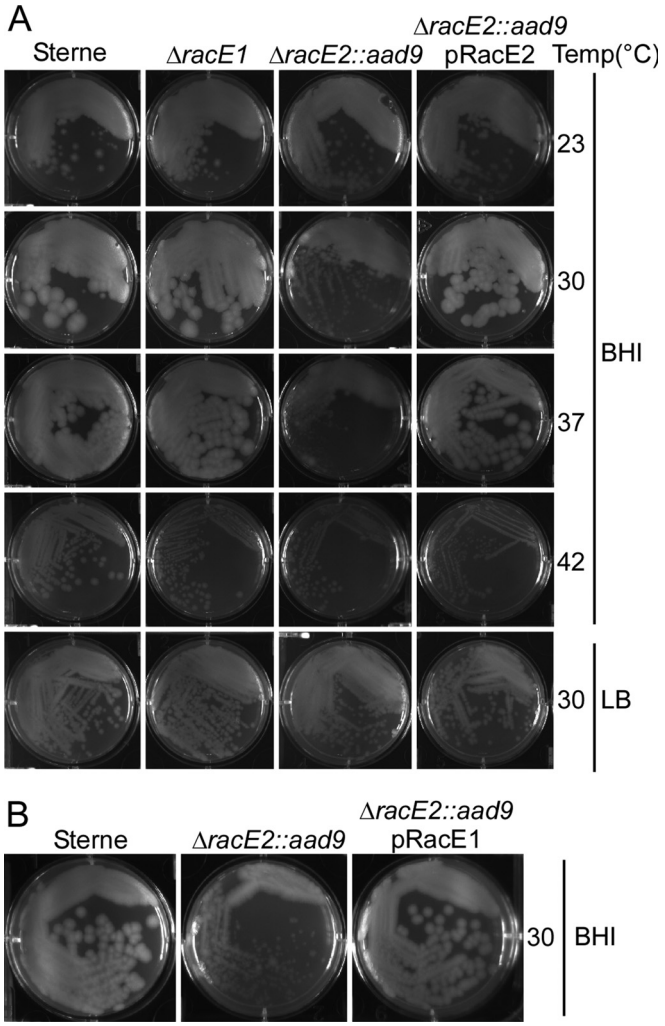

FIG 2 Colony size phenotypes of the *B. anthracis* Sterne glutamate racemase mutants. (A) *B. anthracis* Sterne and the Δ*racE1*, Δ*racE2*::*aad9*, and complemented Δ*racE2*::*aad9*/pRacE2 variants were spread on BHI agar and incubated at 23, 30, 37, and 42°C or on LB agar and incubated at 30°C. The diminished colony size of the *racE2* mutant at 30 and 37°C was complemented by plasmid-borne *racE2* (pRacE2). (B) The diminished colony size of the *racE2* mutant was also complemented by plasmid-borne *racE1* (pRacE1).

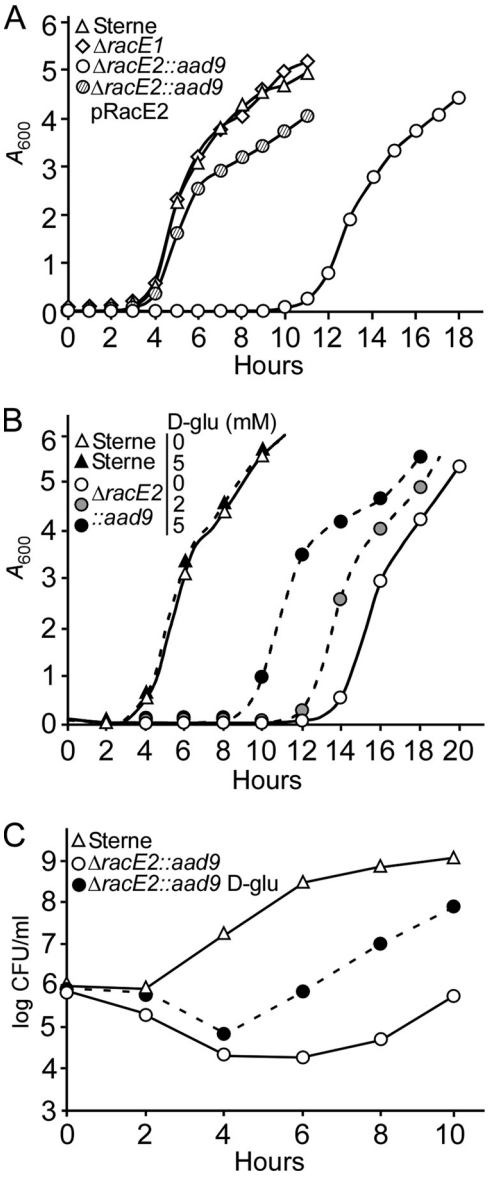

FIG 3 Growth phenotypes of *B. anthracis* Sterne glutamate racemase mutants. (A) Spores (1×10^6/ml) derived from wild-type Sterne and the Δ*racE1*, Δ*racE2*::*aad9*, and complemented Δ*racE2*::*aad9*/pRacE2 variants were inoculated into BHI broth, and vegetative growth was monitored as increased A_{600}. (B) Where indicated, BHI broth was supplemented with 2 or 5 mM D-glutamate (C) Viability of germinated bacilli was quantified by dilution and enumeration of CFU on LB agar at timed intervals following the dilution of spores in BHI broth. Data are representative of two independent experiments.

nation, indicating that large numbers of germinated *racE2* mutant spores did not survive (Fig. 3C). Nevertheless, the decline in the CFU count was followed by expansive growth of the surviving *racE2* mutant vegetative forms. The survival defect of newly germinated *B. anthracis* SYO2 vegetative forms was only partially restored by the addition of 5 mM D-glutamate to the growth medium (Fig. 3C).

The *racE2* mutation causes growth phase-dependent defects in *B. anthracis* cell shape. *B. anthracis* spores (1×10^6 CFU) were inoculated into BHI broth and incubated at 37°C for germination and growth, and culture aliquots were fixed with formaldehyde at timed intervals. Germinated bacilli were stained with the membrane dye FM4-64 and with BODIPY-vancomycin, which binds to newly formed peptidoglycan and its precursors in vegetative forms but not spores of *B. anthracis* (32). Two hours after dilution in BHI broth, *B. anthracis* Sterne formed elongated chains of vegetative forms with cell wall septation (Fig. 4). In contrast, for the first 5 h following spore dilution and germination, the SYO2 (Δ*racE2*::*aad9*) mutant formed small round or oval cells without detectable cell division events (Fig. 4). After 3 to 5 h of incubation, *racE2* mutant cells had expanded in size but did not generate vancomycin-stained septal peptidoglycan (Fig. 5). After 10 h of incubation, the *racE2* mutant formed short chains of vegetative cells with regularly spaced septal (vancomycin-stained) peptidoglycan (Fig. 5).

***B. anthracis* Ames *racE1* and *racE2* mutants.** Bacteriophage CP-51 was used to transduce the *bursa aurealis racE1*::*aad9* (37) and racE2::*aad9* (this study) alleles into *B. anthracis* Ames

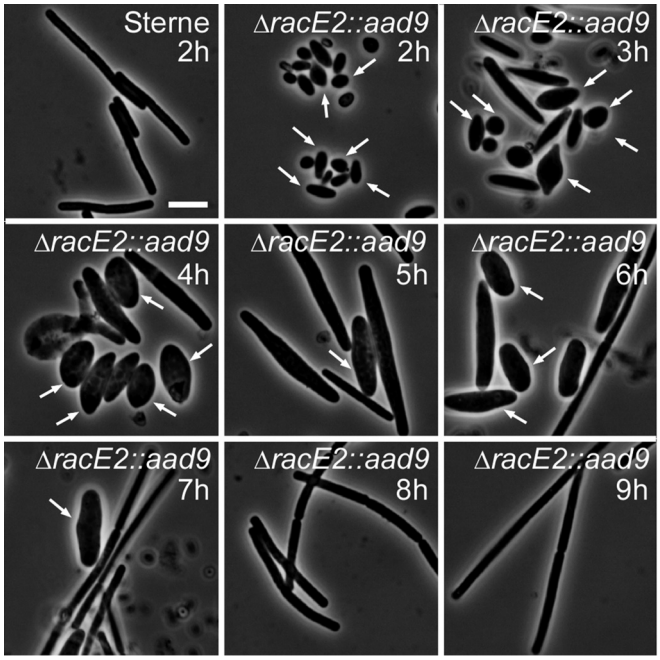

FIG 4 Cell shape abnormalities of germinated *racE2* mutants. Spores (1×10^6/ml) derived from wild-type and Δ*racE2*::*aad9* mutant *B. anthracis* Sterne were inoculated into BHI broth. Aliquots were withdrawn at timed intervals, fixed with formalin, and analyzed by phase-contrast microscopy. Scale bar, 5 μm. Arrows identify round and oval cells.

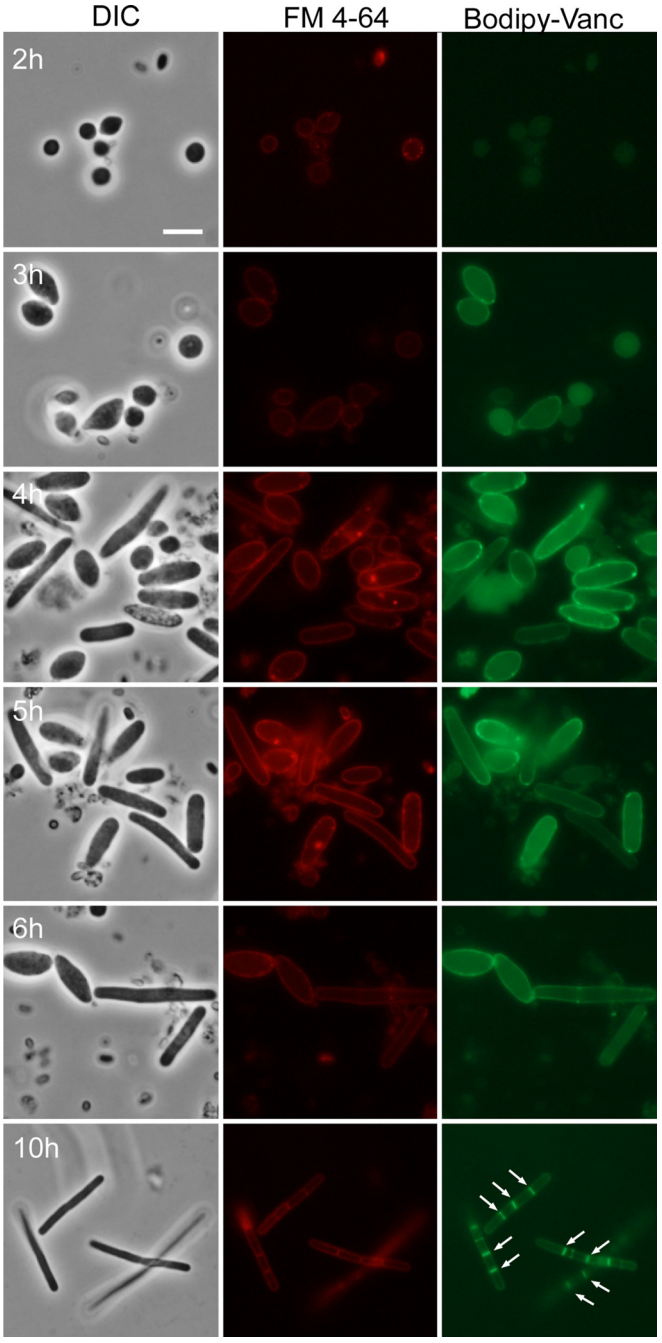

FIG 5 Cell division defect of germinated *racE2* mutants. Spores (1×10^6/ml) derived from *B. anthracis* and the Δ*racE2*::*aad9* mutant were inoculated into BHI broth. Aliquots were withdrawn at timed intervals, fixed with formalin, and stained with BODIPY-vancomycin to identify cell wall septa (arrows) and with FM4-64 to reveal membranes and then viewed by fluorescence microscopy. Scale bar, 5 μm.

(pXO1$^+$ pXO2$^+$), the fully virulent, capsulating American anthrax isolate (39). Transductants were plated on BHI agar and capsule-inducing NBY agar (Fig. 6A and B). Unlike its parent, *B. anthracis* Ames, the *racE1*^{Ames} variant did not display growth defects on BHI broth and capsule-inducing medium (Fig. 6A and B). The *racE2*^{Ames} mutant formed small colonies on BHI broth and small, glossy colonies on NBY capsulating medium (Fig. 6A and B). When colony material was stained with India ink and viewed by light microscopy, *B. anthracis* Ames excluded India ink from the bacterial surface, a phenotype known to be associated with PDGA capsule production (26). The *racE1*^{Ames} mutant formed encapsulated chains of vegetative bacilli, similar to wild-type Ames (Fig. 6B). The *racE2*^{Ames} mutant produced similar amounts of capsular material, albeit that the chain lengths of its vegetative forms were expanded compared to those of wild-type Ames and the *racE1*^{Ames} variant (Fig. 6B).

When analyzed for growth in BHI broth and in capsule-inducing broth culture, the *racE1*^{Ames} mutant replicated at the same rate as its parent, *B. anthracis* Ames. Similar to its growth phenotype in *B. anthracis* Sterne, disruption of *racE2* delayed expansion of *B. anthracis* Ames vegetative forms by 6 h in BHI broth and by 8 h in NBY capsulating medium (Fig. 6C and D). This growth defect was only partially suppressed by the addition of 5 mM D-glutamate.

Glutamate composition of capsule from *racE1* and *racE2* mutants. To analyze the stereochemical composition of glutamate in capsular material, wild-type *B. anthracis* Ames and the *racE1*^{Ames} and *racE2*^{Ames} mutant strains were grown under capsulating conditions on NBY agar. Bacteria were washed in buffer, boiled in hot SDS, and centrifuged, and capsular material was purified from the culture supernatant. Capsular polymers were hydrolyzed in acid and labeled with Marfey's reagent (FDAA [1-fluoro-2,4-dinitrophenyl-5-L-alanine amide]), which forms an adduct with the primary amine of glutamic acid, enabling separation of derivatized D- and L-glutamate by reverse-phase high-performance liquid chromatography (rpHPLC). For calibration, FDAA-derivatized L- and D-glutamate eluted at 18 and 21 min during rpHPLC (Fig. 7). As a control, acid hydrolysis and FDAA derivatization of poly-L-glutamate generated a large absorbance

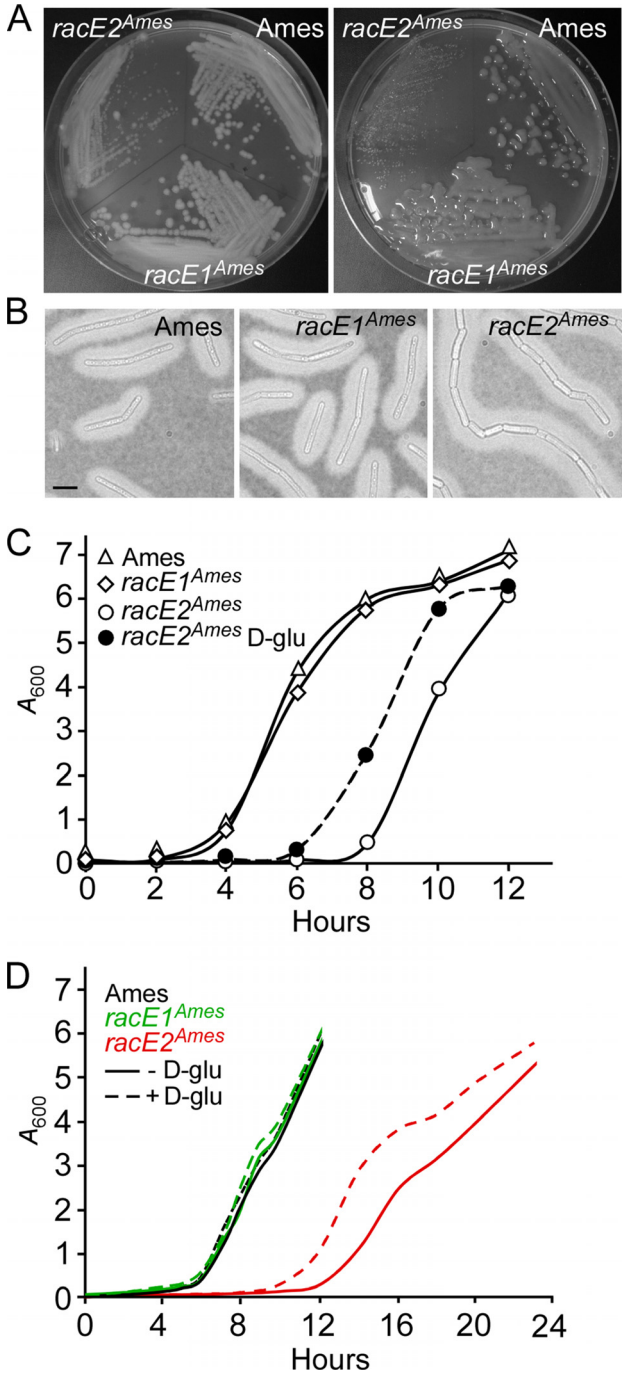

FIG 6 Capsule production and growth phenotypes of *B. anthracis* Ames glutamate racemase mutants. (A) The *racE1*::*aad9* and *racE2*::*aad9* alleles were transduced into *B. anthracis* Ames, yielding the *racE1*^{Ames} and *racE1*^{Ames} mutant isolates, which were spread along with wild-type strain Ames on BHI agar (left) or NYB agar with horse serum and incubated at 37°C with 5% CO_2 (right, capsule-inducing condition). (B) PDGA capsule production of colony material was analyzed by India ink staining and phase-contrast microscopy. Scale bar, 10 μm. (C) Spores (5×10^6/ml) of *B. anthracis* Ames and its *racE1*^{Ames} and *racE2*^{Ames} mutants were inoculated into BHI broth (with or without 5 mM D-glutamate), and vegetative growth was monitored as increased A_{600}. (D) Spores (5×10^6/ml) of *B. anthracis* Ames and its *racE1*^{Ames} and *racE2*^{Ames} mutants were inoculated into NBY broth supplemented with 0.8% sodium bicarbonate and 10% heat-inactivated horse serum (with or without 5 mM D-glutamate), and vegetative growth was monitored as increased A_{600}.

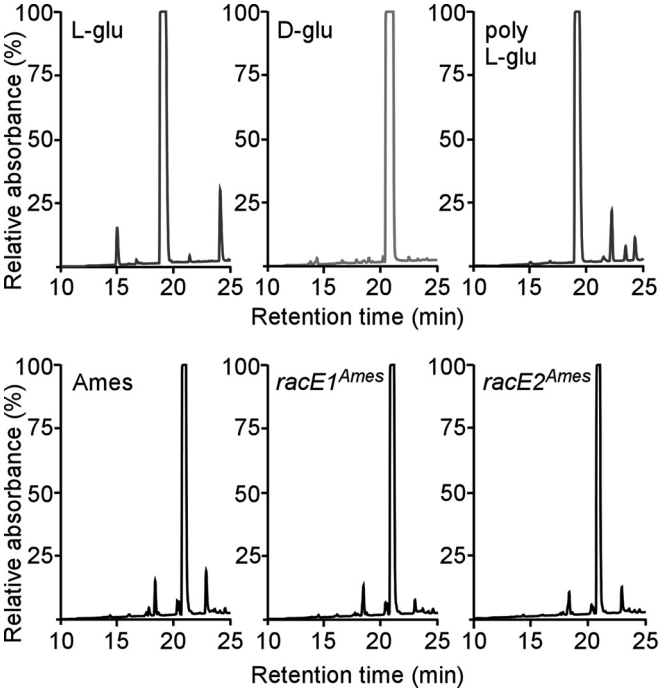

FIG 7 Composition of the polyglutamate capsule in *B. anthracis* Ames glutamate racemase mutants. Purified capsule polymers from *B. anthracis* Ames and its *racE1*^{Ames} or *racE2*^{Ames} mutants were hydrolyzed in hydrochloric acid, labeled with 1% FDAA, and separated by rpHPLC. L-Glutamic acid, D-glutamic acid, and poly-L-glutamic acid were used as controls.

peak at 18 min during rpHPLC (Fig. 7). In contrast, acid hydrolysis and FDAA derivatization of capsular material from *B. anthracis* Ames resulted in a large absorbance peak at 21 min (Fig. 7). Acid hydrolysis and FDAA derivatization of capsular material from the mutant strains also generated absorbance peaks at 21 min; the rpHPLC elution spectra of glutamate from the capsule of *racE1*^{Ames} and *racE2*^{Ames} mutants could not be distinguished from the spectrum of *B. anthracis* Ames (Fig. 7).

DISCUSSION

D isomers of two amino acids, alanine and glutamic acid, are key elements of the cell wall of bacteria and essential for the assembly of peptidoglycan (12, 40). Synthesis of D isomers is dependent on racemases, and these enzymes have been studied as potential drug targets with the goal of identifying new antibiotics (3, 41, 42). Alanine racemase requires PLP as a coenzyme, which contributes to catalysis by forming the intermediate D-alanyl-PLP aldimine (43, 44). Cycloserine (4-amino-3-isoxazolidinone), a drug that is approved as a second-line therapeutic against multidrug-resistant *M. tuberculosis* (45), is an inhibitor of alanine racemase and D-Ala–D-Ala ligase, which contribute to lipid II precursor synthesis (46). Cycloserine has limited efficacy against other bacteria, particularly against microbes that express two different alanine racemase enzymes (47). Cycloserine also has considerable side effects due to the inhibition of PLP-dependent racemases in the human nervous system (45).

Glutamate racemase does not require PLP for catalysis and instead employs cysteine thiolates to deprotonate its L-glutamate substrate and generate the D isomer (48, 49). Considerable sequence variation has been reported for glutamate racemases from

different bacteria, which may explain differences in allosteric activation and kinetic regulation of these enzymes (7). Efforts to identify inhibitors of glutamate racemases have thus far been successful for pyrazolopyrimidinediones, which block *Helicobacter pylori* MurI racemase activity, presumably by binding to a regulatory site of the enzyme (3, 7). Nevertheless, the activity spectrum of these compounds is very narrow and they cannot be used for treatment of infectious diseases caused by drug-resistant bacteria (3). Similar to alanine racemase inhibitors, development of drugs with broad-spectrum activity against glutamate racemase must address the sequence variation of this enzyme family and the potentially confounding effect that several microbes express two different glutamate racemases.

B. anthracis, a Gram-positive, spore-forming firmicute, is the causative agent of anthrax, a zoonotic disease that is rapidly fatal unless treated early with antibiotics (50). Because of the ease with which infectious spores are generated, the agent is disseminated, and drug-resistant strains are isolated, *B. anthracis* is considered a weapon of mass destruction (51). Glutamate racemase has been identified as a target for the development of antibiotics that can combat drug-resistant *B. anthracis* (4). This concept was supported by findings of Shatalin and Neyfakh, who reported that each of the two glutamate racemase genes of *B. anthracis*, *racE1* and *racE2*, is essential for vegetative growth (28). Here we demonstrate that this model is not correct. Each gene, *racE1* or *racE2*, can be deleted from the chromosome of *B. anthracis* Sterne, a noncapsulating vaccine strain lacking the pXO2 virulence plasmid, and from *B. anthracis* Ames, a fully virulent, capsulating isolate. We do not know the reasons for the discrepancy between the findings of the two studies because Shatalin and Neyfakh did not include data on *racE1* and *racE2* mutants in their publication on a plasmid system for allelic replacement (28). It seems unlikely that these differences are caused by strain differences; Shatalin and Neyfakh used *B. anthracis* ΔANR, a plasmidless variant of *B. anthracis* Ames (pXO1 pXO2), which was examined in our study.

Unlike *racE1* mutants, *B. anthracis* lacking *racE2* displays delayed growth and even cell death following germination. *racE2* mutant cells had a round or oval shape, similar to the spore outgrowth phenotype of *B. subtilis pbpA* mutants, which cannot adequately support cell wall elongation because of loss of transpeptidase activity but are not defective in the formation of septal peptidoglycan (52, 53). *racE2* mutant cells that did survive eventually replicated with kinetics similar to those of wild-type *B. anthracis*. Taken together, these data suggest that RacE2 is a key enzyme for newly germinated spores, supplying D-glutamate for peptidoglycan synthesis. These observations are in agreement with gene expression studies showing that *racE2* is preferentially expressed during early vegetative growth (54). At a later stage of vegetative growth, both RacE1 and RacE2 are independently capable of providing *B. anthracis* with D-glutamate for peptidoglycan and PDGA capsule synthesis. Of note, *B. anthracis* mutants lacking *racE1* and *racE2* appear nonviable, suggesting that D-amino-acid transaminases are not sufficient to provide anthrax bacilli with D-glutamate, as has been reported for *B. subtilis* (17).

Homology modeling of RacE1 and RacE2 revealed potential differences within the active-site pocket that might affect the design of inhibitory pharmacophores (55). Earlier work discovered a natural product, dipicolinic acid, that acts as an allosteric inhibitor of *B. anthracis* RacE1 and RacE2 (55). The dipicolinic acid binding pocket is distinct from that of pyrazolopyrimidinedione on *H. pylori* MurI (55). Dipicolinic acid occurs at high concentrations in the *B. anthracis* spore and has been implicated as a key factor in the regulation of this organism's life cycle (56). The inhibitory attributes of dipicolinic acid for glutamate racemases may explain the requirement of *racE2* for vegetative growth of *B. anthracis* immediately following the germination of spores.

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