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# Proline-Dependent Regulation of *Clostridium difficile* Stickland Metabolism

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***Clostridium difficile*, a proteolytic Gram-positive anaerobe, has emerged as a significant nosocomial pathogen. Stickland fermentation reactions are thought to be important for growth of *C. difficile* and appear to influence toxin production. In Stickland reactions, pairs of amino acids donate and accept electrons, generating ATP and reducing power in the process. Reduction of the electron acceptors proline and glycine requires the D-proline reductase (PR) and the glycine reductase (GR) enzyme complexes, respectively. Addition of proline in the medium increases the level of PR protein but decreases the level of GR. We report the identification of PrdR, a protein that activates transcription of the PR-encoding genes in the presence of proline and negatively regulates the GR-encoding genes. The results suggest that PrdR is a central metabolism regulator that controls preferential utilization of proline and glycine to produce energy via the Stickland reactions.**

*Clostridium difficile*, an anaerobic, Gram-positive, spore-forming bacterium, is an emerging nosocomial pathogen and the etiological agent of antibiotic-associated diarrhea and pseudomembranous colitis (1–3). In recent years, the severity and incidence of *C. difficile* infection have increased due to the emergence in North America and Europe of highly virulent NAP1/027 *C. difficile* strains (4, 5). Most pathogenic *C. difficile* strains produce two large cytotoxins (TcdA and TcdB) that are the primary virulence factors (6, 7).

Toxin production in *C. difficile* responds to environmental conditions, including the availability of specific nutrients, temperature changes, and alteration of the redox potential (8–10). In addition, the presence of a rapidly metabolizable carbon source (11, 12) or certain amino acids (13, 14) inhibits toxin gene expression. For instance, addition to the growth medium of a mixture of nine amino acids (cysteine, isoleucine, leucine, valine, methionine, threonine, tryptophan, glycine, and proline) strongly reduces toxin yield (12, 14). When cells are grown in rich medium, the toxin genes are transcribed only when the cells reach stationary phase (11, 15).

Some of the molecular mechanisms regulating *C. difficile* toxin gene expression have been elucidated (8, 11, 16–19). Transcription of the *tcdA* and *tcdB* genes depends on TcdR, an alternative sigma factor that directs RNA polymerase to the toxin gene promoters (20). Transcription of the *tcdR* gene is repressed during the rapid exponential growth phase by CodY, a global regulator of metabolism and of genes that allow adaptation to nutrient limitation (19, 21). CodY is active in cells with an excess of branched-chain amino acids (isoleucine, leucine, and valine) and GTP (22–24). When the cells reach stationary phase, the intracellular concentrations of these ligands decrease and CodY is less able to bind as a repressor, leading to derepression of *tcdR* transcription. How *C. difficile* responds to the levels of other amino acids to modulate toxin expression remains unclear.

The activity of TcdR is modulated by TcdC, an anti-sigma factor that destabilizes the TcdR–core RNA polymerase complex (25). TcdC seems to be most active in rapidly growing cells. Other sigma factors, such as SigD and SigH, are involved in toxin gene transcription by unknown mechanisms (8, 18).

Regulation of toxin production by the carbon source is mediated by CcpA, a global regulator of carbon metabolism in Gram-positive bacteria (16). CcpA is a direct repressor of the *tcdA* and *tcdB* genes. The roles of CodY and CcpA reflect a tight coupling between metabolism and virulence in *Clostridium* spp. and other pathogenic bacteria (26, 27).

In several nonpathogenic *Clostridium* species (e.g., *C. sporogenes* and *C. sticklandii*), amino acid metabolism by the Stickland reactions is a primary source of energy when bacteria are grown with amino acids as sole sources of carbon and nitrogen (28–31). Stickland reactions couple metabolism of pairs of amino acids in which one amino acid, acting as an electron donor, is oxidatively deaminated or decarboxylated and a second amino acid, acting as an electron acceptor, is reduced or reductively deaminated (Fig. 1). The most efficient electron donors are leucine, isoleucine, and alanine, and the most efficient acceptors are glycine, proline, and hydroxyproline (30, 32, 33). In *C. sticklandii*, the reduction of the Stickland acceptors glycine and proline is performed by two selenium-dependent reductases, glycine reductase (GR) and D-proline reductase (PR), respectively. GR catalyzes the reductive deamination of glycine to acetyl phosphate and ammonium, and PR reductively cleaves D-proline to 5-aminovalerate (34–37). Jackson et al. (38) showed that *C. difficile* growth requires selenium in a limiting basal medium with torula yeast extract, suggesting a key role of the Stickland reactions in *C. difficile* physiology (39). Based on sequence homology to *C. sticklandii*, genes encoding the *C. difficile* GR and PR subunits are clustered in two distinct genetic loci (*grd* and *prd*, respectively) on the chromosome of *C. difficile* strain 630 (38). The *grd* locus contains eight genes (Fig. 2). Two of the genes, *grdA* and *grdB*, likely encode the selenocysteine-containing subunits of GR (38). The seven genes of

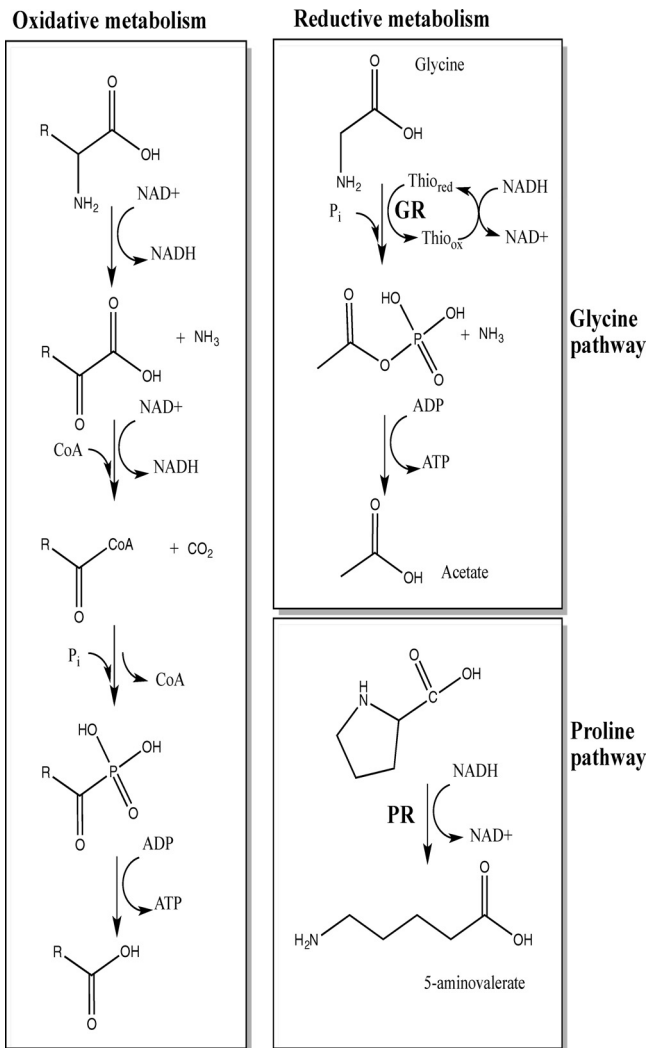
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**FIG 1** Overview of Stickland metabolism. Stickland reactions couple oxidation and reduction of amino acid pairs. D-Proline reductase catalyzes the reductive cleavage of the D-proline ring to yield  $\delta$ -aminovaleric acid. Glycine reductase catalyzes the reductive deamination of glycine to acetyl phosphate to generate ATP via substrate-level phosphorylation through acetate kinase.

the putative *prd* operon (Fig. 2) include *prdF*, predicted to encode a D-proline racemase, and *prdB*, predicted to encode the selenium-containing subunit of PR. It is interesting to note that *proP*, downstream of the *grd* operon, is predicted to encode a proline amino-

peptidase, suggesting a link between metabolism of glycine and that of proline (38). Moreover, Jackson et al. (38) found that proline and glycine specifically stimulate production of the selenoenzymes PR and GR, respectively. Interestingly, addition to the medium of glycine and proline in combination with other amino acids has been shown to decrease toxin yield (14). Therefore, a detailed understanding of the amino acid metabolism pathways and their regulation may give important insights into *C. difficile* pathogenesis.

In this study, we investigated the transcriptional regulation of the *C. difficile* *grd* and *prd* gene clusters. We found that addition of proline to the growth medium increases the expression of the *prd* operon and decreases the expression of the *grd* operon. By constructing *prd* and *grd* null mutant strains, we were able to demonstrate the interdependent regulation of the two operons. That is, expression of a functional PR decreased transcription of the *grd* operon. Furthermore, we identified PrdR, the product of a gene located upstream of the *prd* operon, as the mediator of proline-dependent activation of the *prd* operon and proline-dependent repression of the *grd* operon.

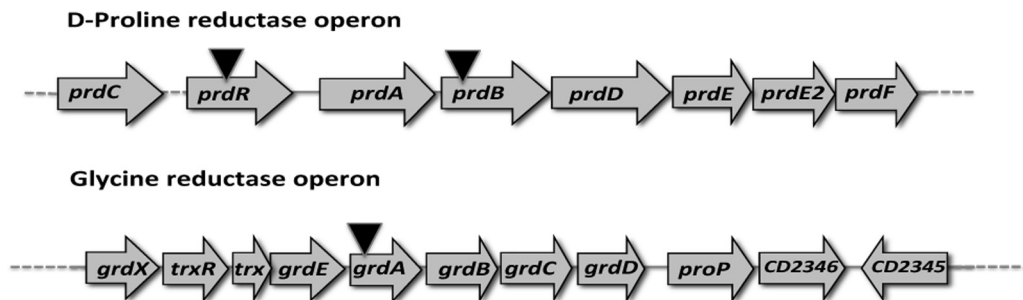
## MATERIALS AND METHODS

**Strains and growth conditions.** The bacterial strains and plasmids used in this study are listed in Table 1. *C. difficile* strains were grown routinely in TY medium (11), supplemented with 250  $\mu$ g/ml D-cycloserine, 40  $\mu$ g/ml kanamycin, 20  $\mu$ g/ml thiamphenicol, or 5  $\mu$ g/ml erythromycin as needed. *C. difficile* strains were maintained at 37°C in an anaerobic chamber (Coy Laboratory Products) with an atmosphere of 10% H<sub>2</sub>, 5% CO<sub>2</sub>, and 85% N<sub>2</sub>. *Escherichia coli* strains were grown at 37°C in L medium supplemented with 20  $\mu$ g/ml chloramphenicol or 100  $\mu$ g/ml ampicillin, as needed.

Growth behavior analyses of *C. difficile* strains were performed in TY medium, with or without supplementation as indicated below, in a microtiter plate format. Briefly, mid-exponential-phase cultures adjusted to an optical density at 600 nm (OD<sub>600</sub>) of 0.1 were diluted (1:10) in 200  $\mu$ l TY medium supplemented with proline (30 mM) or glycine (30 mM) in wells of a 96-well microtiter plate (Costar). Cultures were grown anaerobically at 37°C. At 10-min intervals over a 12-h period, the plates were agitated for 2 min before the OD<sub>600</sub> was recorded using a plate reader (Bio-Tek). Data from two biological replicates, each with six technical replicates, were used to construct the growth curve for each strain.

**Strain and plasmid construction.** Oligonucleotides used in this study are listed in Table 2. *C. difficile* strain 630 DNA, GenBank accession number AM180355 (41), was used as a template for PCR amplification. Sequencing of cloned DNA fragments was performed by the Tufts University Core Facility using an ABI 3130XL DNA sequencer.

Null mutations in *prdB* and *prdR* were created in several steps. First, plasmids pBL33 and pBL67 were constructed by retargeting of the *erm*



**FIG 2** Organization of the D-proline reductase and glycine reductase gene clusters. Arrows indicate the locations of group II intron insertions in various mutant strains. The coordinates refer to the positions of the gene clusters in the genome of *C. difficile* strain 630.

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristics	Reference or source
<b>Strains</b>		
<i>E. coli</i>		
HB101	F <sup>-</sup> <i>supE44 hsdS20</i> (r <sub>B</sub> <sup>-</sup> m <sub>B</sub> <sup>-</sup> ) <i>recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1</i>	B. Dupuy
DH5α	<i>fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17</i>	New England Biolabs, USA
<i>C. difficile</i>		
JIR8094	Em <sup>s</sup> derivative of strain 630	19
LB-CD4	JIR8094 <i>prdB::ermB</i>	This study
LB-CD12	JIR8094 <i>grdA::ermB</i>	This study
LB-CD8	JIR8094 <i>prdR::ermB</i>	This study
LB-CD13	JIR8094 pBL58	This study
LB-CD14	LB-CD8 pBL93	This study
<b>Plasmids</b>		
pBL33	pCE240 targeted to <i>prdB</i>	This study
pBL64	pCR2.1-intron template part A	This study
pBL65	pCR2.1-intron template part B	This study
pBL67	pCE240 targeted to <i>prdR</i>	This study
pBL68	pMC123 without HindIII and BsrGI sites	This study
pBL71	pMC123 containing groupII intron targeted to <i>prdR</i> from pBL67	
pBL91	pBL100 targeted to <i>grdA</i>	This study
pBL93	pBL68 containing <i>prdR</i>	This study
pBL100	pBL68 containing un-targeted groupII intron–TargeTron vector	This study
pCE240	Derivative of pJIR750ai (Sigma-Aldrich), Tm <sup>r</sup>	42
pCR2.1		Invitrogen
pMC123	Amp <sup>r</sup> Tc <sup>r</sup>	44
pRK24	Tra <sup>+</sup> Mob <sup>+</sup> Amp <sup>r</sup> Tc <sup>r</sup>	45

gene-containing group II intron from pCE240 (42), using for *prdB* the primers oLB64, oLB65, oLB66, and the EBS Universal primer and for *prdR* the primers oLB102, oLB103, oLB104, and the EBS Universal primer as outlined in the TargeTron user manual (Sigma-Aldrich), followed by initial cloning of the retargeted fragment in pCE240 digested with BsrGI/HindIII. Plasmid pCE240 is a derivative of pJIR750ai (Sigma-Aldrich) and is similar to pMTL007 (43). The retargeted group II intron from pBL67 was then extracted by digestion with SfoI and SphI and cloned between the SphI and SnaBI sites of pMC123 (44), resulting in pBL71. pBL33 (targeted for *prdB*) and pBL71 (targeted for *prdR*) were then introduced by transformation into *E. coli* strain HB101(pRK24). pRK24 is a derivative of the broad-host-range plasmid RP4, which mobilizes IncP *oriT* plasmids. The resulting strains were then mated with *C. difficile* strain JIR8094, resulting in transfer of pBL33 and pBL71 by conjugation, as previously described (45), except that transconjugants were selected on BHIS plates (45) supplemented with D-cycloserine, kanamycin, and thiamphenicol. Inactivation of *prdB* and *prdR* was selected for by screening transconjugants for erythromycin resistance, resulting in strains LB-CD4 and LB-CD8, respectively. These strains were also thiamphenicol sensitive, indicating that they no longer carried the replicating plasmid. Insertional disruption of *prdB* or *prdR* was confirmed using primer pairs oLB78/oLB79 and oLB120/oLB121, which anneal outside the region of insertion in the *prdB* and *prdR* genes, respectively.

To interrupt *grdA* and to make future mutant construction in *C. difficile* easier, we created a new set of plasmids for retargeting of the group II intron. We amplified two portions of the intron by PCR using pBL71 as the template and primers oLB102/EBS Universal and oLB103/oLB104. Amplicons were TA cloned in the pCR2.1 vector (Invitrogen), resulting in pBL64 and pBL65. A mixture (1:1) of pBL64 and pBL65 was used as the template to generate the retargeted intron fragment by mutagenic PCR.

We generated a *grdA* targeted intron fragment using primers oLB99, oLB100, oLB101, and EBS Universal and a pBL64-pBL65 mixed template. Following HindIII/BsrGI digestion, the PCR fragment was cloned in HindIII/BsrGI-digested pBL100 (see below). The resulting plasmid, pBL91,

was introduced into *E. coli* strain HB101(pRK24), and the obtained strain was subsequently mated with *C. difficile* JIR8094 as described above. Insertional disruption was verified by PCR using primers oLB118 and oLB119.

To construct pBL100, the BsrGI and HindIII sites of pMC123 (44) were first removed in multiple steps by PCR-directed mutagenesis and blunting of digested DNA, creating pBL68. Then, pBL68 was digested by EcoRI and blunted using Klenow fragment (New England BioLabs, USA) and subsequently digested by SphI. The intron portion of pCE240 was extracted by SphI/SfoI digestion and cloned in digested pBL68, yielding pBL100.

To complement the *prdR* disruption, a 2,119-bp fragment containing the *prdR* gene and its upstream region was amplified using primers oLB187 and oLB188 and cloned between the EcoRI and HindIII sites of pBL58, generating pBL93. This plasmid was introduced into LB-CD8 as previously described (45), resulting in LB-CD14.

**Southern hybridization.** *C. difficile* genomic DNA was digested with HindIII and subjected to agarose gel electrophoresis (0.8%). Digested DNA was then transferred to a nylon membrane (pore size = 0.45 μm) in 20× saline-sodium citrate (SSC; 1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) according to the method of Southern (46). The membrane was prehybridized for 3 h at 42°C in 50% formamide, 5× SSC, 2× Denhardt's solution, and 100 mg denatured salmon sperm DNA per ml. Overnight hybridization was carried out in the same solution at 42°C with an intron-specific, <sup>32</sup>P-labeled PCR fragment generated using oLB277 and oLB278 as primers. The membrane was washed for 30 min in 1× SSC–0.1% SDS and for 30 min in 0.1× SSC–0.1% SDS at room temperature. Radioactive bands were visualized by PhosphorImager analysis (Molecular Dynamics).

**qPCR analysis.** Cultures of *C. difficile* grown in TY medium were harvested at OD<sub>600s</sub> of 0.4 to 0.7 (mid-exponential phase) or 1.2 to 1.4 (stationary phase), and DNA-free RNA was prepared as previously described (19, 44). RNA was quantitated by absorbance (A<sub>260</sub> and A<sub>260</sub>/A<sub>280</sub> ratio) using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific). Primers for quantitative reverse transcription-

TABLE 2 Primers used in this study

Primer name	Sequence
EBS Universal	CGAAATTAGAACTTGCGTTCAGTAAAC
oLB64	AAAAGCTTTTGCAACCCACGTCGATCGTGAATTCTGCAGCTAAGTGCGCCAGATAGGGTG
oLB65	CAGATTGTACAAATGTGGTGATAACAGATAAGTCAGCTAATTTAACTTACCTTTCTTTGT
oLB66	CGCAAGTTTCTAATTTTCGGTTAAGTTCGATAGAGGAAAGTGCT
oLB67	AAAAGCTTTTGCAACCCACGTCGATCGTGAAGACCCAAGTAAAGTGCGCCAGATAGGGTG
oLB78	TCCTCCAGTTGGACTCCTGTAAC
oLB79	ATTGGAAC TAATGGGCAACTG
oLB99	AAAAGCTTTTGCAACCCACGTCGATCGTGAACAGTTACTGTTTGTGCGCCAGATAGGGTG
oLB100	CAGATTGTACAAATGTGGTGATAACAGATAAGTCCTGTTTCGTAACCTTACCTTTCTTTGT
oLB101	CGCAAGTTTCTAATTTTCGGTTAAGTTCGATAGAGGAAAGTGCT
oLB102	AAAAGCTTTTGCAACCCACGTCGATCGTGAATGTATCATTAGTGCGCCAGATAGGGTG
oLB103	CAGATTGTACAAATGTGGTGATAACAGATAAGTCCATTTACTTAACTTACCTTTCTTTGT
oLB104	CGCAAGTTTCTAATTTTCGGTTTACATTCGATAGAGGAAAGTGCT
oLB118	ATAATAGGTGACCGTGATGG
oLB119	TTCTTCTTTATACCAGACATTTT
oLB120	ATAGGGATCTTCTGTTCTTAG
oLB121	TCCTATGTCTTCTTTCTCTCTCT
oLB122	CTAGCTGCTCCTATGTCTCACATC
oLB123	CCAGTCTCTCCTGGATCAACTA
oLB131	GTATGGATAGGTGGAGAAGTCA
oLB132	CTCTTCTCTAGTAGCTGTAATGC
oLB261	CTATAGACTCTCATAACAGCAGGTG
oLB262	CATTATGTCTCTTGGCTCTAAC
oLB265	GGGAGAGGGTATTACACATACT
oLB266	GGAGTTCATATCTCCAAATACC
oLB170	GGTCAAGTACTAGGAGCTAAGT
oLB171	CTACTTCTTCTTAGCCTCTCTCTG
oLB176	CCCTGGTATCATGTCTAAAGTTG
oLB177	GAGTATACTTAGCTCCTTCTCCAG
oLB187	CCCGAATTCTGGATACAGGCATTCATTAATTGT
oLB188	CCCAAGCTTAACACATCTATATACACAACAC
oLB221	GAACTCTAATCGCAGAACCAAC
oLB222	CATTCTACTACTCCAGCTTCT
oLB277	GTAGGAGAACCTATGGGAAC
oLB278	TCCTTACCATTAAAGCACAC

PCR (qPCR) were designed using the online PrimerQuest tool from Integrated DNA Technologies (<http://www.idtdna.com/Scitools/Applications/Primerquest>), and amplification efficiencies for each primer set were determined prior to use. To control for chromosomal DNA contamination, mock cDNA synthesis reaction mixtures containing no reverse transcriptase were used as negative controls in subsequent amplifications. cDNA samples were diluted 4-fold and used as the templates for qPCR of *rpoC* (primers oLB122/oLB123), *prdA* (primers oLB170/oLB171), *prdC* (primers oLB221/oLB222), *prdD* (primers oLB265/oLB266), *prdF* (primers oLB261/oLB262), *grdE* (primers oLB176/oLB177), and *tcdA* (primers oLB131/oLB132) using Roche SYBR green I PCR mix and a Roche LightCycler 480 II thermocycler. Reactions were performed in a final volume of 20  $\mu$ l using 4  $\mu$ l diluted cDNA and 1  $\mu$ M each primer. Reactions were performed in triplicate using cDNA extracted from each of a minimum of three biological replicates, and results are presented as the means and standard deviations of the data obtained. Amplification included 45 cycles of the following steps: 10 s at 95°C, 10 s at 53°C, and 15 s at 72°C. Results were calculated using the comparative cycle threshold method (47), in which the amount of target mRNA is normalized to that of an internal control transcript (*rpoC*).

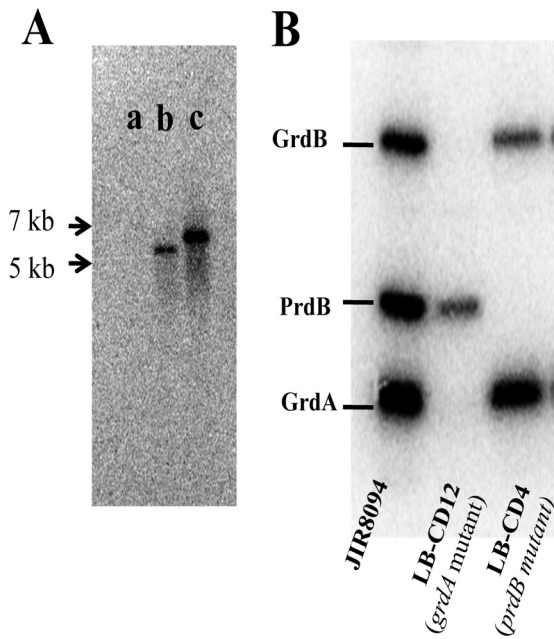
**Proline assay.** Overnight cultures in TY medium were diluted in TY medium to an OD<sub>600</sub> of 0.05 and incubated anaerobically at 37°C. Cells were harvested at mid-exponential growth phase (OD<sub>600</sub> of 0.4 to 0.7) by centrifugation (10 min at 4,000 rpm) and resuspended in 3% (wt/vol) 5-sulfosalicylic acid. Silica-glass beads (0.1 mm) were added, and cells

were disrupted using a Mini-BeadBeater (BioSpec Products). Silica beads and cell debris were removed by centrifugation. The proline concentration was determined by a published procedure (48), and the results were expressed relative to those for the wild-type strain.

**<sup>75</sup>Se labeling experiment.** *C. difficile* strains were cultivated in TY medium with or without sodium selenite (50 nM, 4  $\mu$ Ci of <sup>75</sup>Se, University of Missouri Research Reactor), and cell extracts were obtained as previously described (38). The protein concentration was determined by the Bradford assay (49) using bovine serum albumin as a standard. Selenoproteins in cell extracts were identified after electrophoresis in 15% polyacrylamide gels containing SDS and visualization of radioisotope bands by PhosphorImager analysis (Molecular Dynamics), after overlaying a Coomassie blue-stained gel to determine the approximate molecular weights of the radioactive protein bands.

## RESULTS

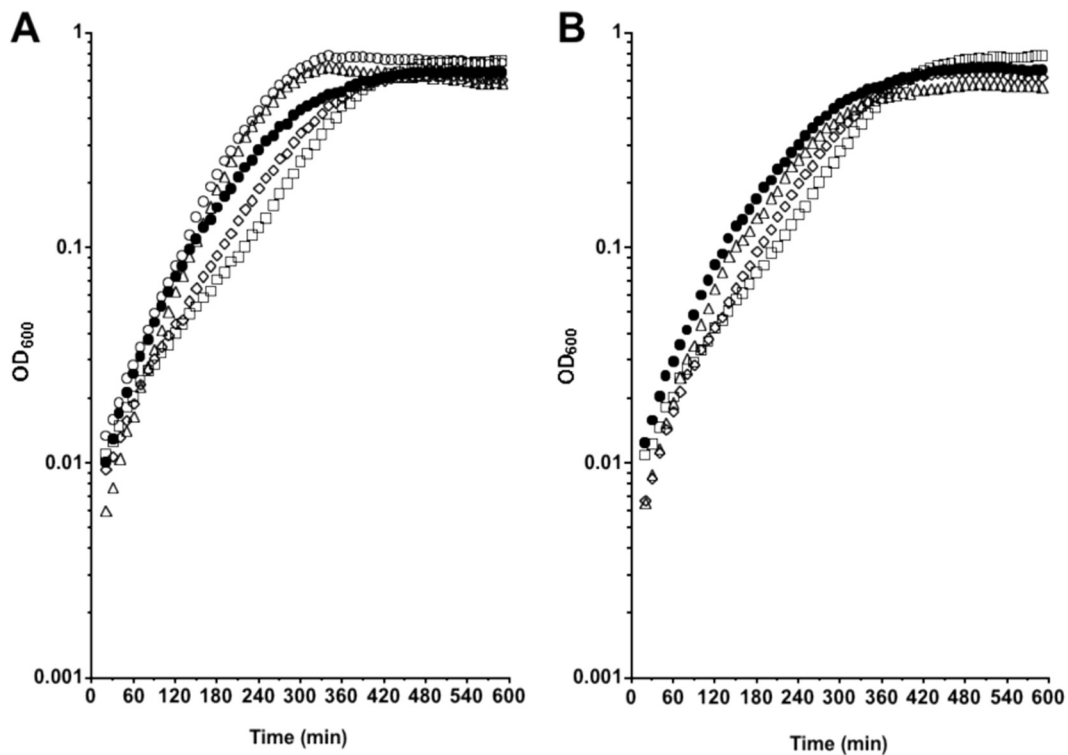
**Stickland-type metabolism of proline is required for optimal growth of *C. difficile*.** In many *Clostridium* spp., including *C. difficile*, Stickland reactions are thought to be important for growth, at least under certain conditions (38, 50). In order to confirm the role of Stickland reactions in growth of *C. difficile*, we introduced insertion mutations into the *prdB* and *grdA* genes (Fig. 2). Insertional disruptions were verified by PCR (see Materials and Methods), and a Southern blot confirmed that each mutant strain had a single intron insertion (Fig. 3A). The resulting strains, LB-



**FIG 3** Mutant characterization. (A) Southern blot analysis of HindIII-digested genomic DNA from wild-type (JIR8094) (a), *prdB* mutant (LB-CD4) (b), and *grdA* mutant (LB-CD12) (c) strains with an intron probe. (B) Radiolabeling ( $^{75}\text{Se}$ ) of *C. difficile* wild-type and mutant strains. Wild-type (JIR8094), *grdA* mutant (LB-CD12), and *prdB* mutant (LB-CD4) strains were grown in TY medium with  $^{75}\text{Se}$  (4  $\mu\text{Ci}$ ) for 24 h, harvested, and lysed by sonication. The extracts were subjected to SDS-15% polyacrylamide gel electrophoresis.

CD4 and LB-CD12, respectively, were tested for the ability to produce the selenoenzymes PR and GR by labeling of the proteins with  $^{75}\text{Se}$ . Figure 3B shows that three major radioactive bands corresponding to the PrdB subunit of PR and the GrdA and GrdB subunits of GR were detected in extracts of wild-type cells. No band corresponding to the PrdB subunit of PR was detected in strain LB-CD4 (*prdB* mutant), and no bands corresponding to the GrdA and GrdB subunits of GR were detected in the *grdA* null mutant. The lack of the GrdB band in the *grdA* null mutant suggests that the *grdA* mutation is polar (*grdB* is downstream of *grdA* [Fig. 2]). These results indicate that LB-CD4 and LB-CD12 are defective in producing the cysteine-containing subunits of the selenoenzyme complexes PR and GR, respectively.

We then assessed the growth behavior of the mutant strains in TY medium supplemented with 30 mM proline (Fig. 4A) or 30 mM glycine (Fig. 4B). The growth rate of the mutant strains was similar to that of the wild-type (JIR8094) strain in TY medium alone (Table 3) and in TY medium supplemented with glycine (Fig. 4B and Table 3). In contrast, addition of L-proline to the medium increased the growth rate of the wild-type and the *grdA* mutant strains but had no effect on the doubling time or the growth behavior of the *prdB* mutant (LB-CD4) (Fig. 4A and Table 3). It should be noted, however, that at low cell densities, the growth rates of all strains in all media were similar, suggesting that the bacteria do not begin to utilize proline until some other constituents of TY medium have been consumed. In addition, the growth of the mutant strains in defined medium (14) supplemented with proline was appraised. Surprisingly, mutants were viable and did not exhibit a severe growth defect compared to the



**FIG 4** Growth of *C. difficile* wild-type and mutant strains supplemented with L-proline or glycine. *C. difficile* wild-type (JIR8094 [○]), LB-CD4 (*prdB::ermB* [◇]), and LB-CD12 (*grdA::ermB* [△]) strains were grown in TY medium alone or supplemented with 30 mM L-proline (A) or 30 mM glycine (B). The *C. difficile* wild-type (JIR8094 [●]) was grown in TY medium alone. Cultures were grown anaerobically in a microtiter plate format at 37°C, and OD<sub>600</sub> measurements were taken every 10 min using a plate reader (Bio-Tek).

**TABLE 3** Doubling times of wild-type and mutant strains in TY medium with proline and glycine<sup>a</sup>

Strain	Genotype	Doubling time (min) in:		
		TY medium alone	TY medium + proline	TY medium + glycine
JIR8094	Wild type	55.41 ± 2.00	41.51 ± 0.18	56.51 ± 2.72
LB-CD4	<i>prdB::erm</i>	57.43 ± 2.43	55.81 ± 0.68	54.43 ± 2.57
LB-CD12	<i>grdA::erm</i>	57.44 ± 3.00	39.11 ± 0.57	58.67 ± 1.57
LB-CD8	<i>prdR::erm</i>	63.83 ± 6.99	70.41 ± 4.13	64.15 ± 4.63

<sup>a</sup> Strains were grown in TY medium alone or supplemented with 30 mM L-proline or 30 mM glycine. Doubling time was calculated during a 60-min period starting at 120 min of growth; values are means ± standard deviations.

wild type (data not shown), suggesting that other metabolic pathways bypass the lack of a functional PR or GR. Therefore, the proline reductase pathway is needed for optimal growth of *C. difficile* in proline-supplemented medium, but the glycine reduction pathway does not play a significant role in the growth of *C. difficile* under the conditions tested.

**Proline activates transcription of the *prd* operon and inhibits transcription of the *grd* operon.** It has been shown previously for *C. difficile* that addition of glycine to the growth medium increases the level of GR and that addition of proline to the growth medium both increases PR levels and decreases GR levels (38). To see if these effects could be explained by regulation at the transcriptional level, we used qPCR to compare the relative amounts of *prdA*, *prdD*, *prfF*, and *grdE* mRNAs in the mutant and wild-type strains. As shown in Fig. 5A, the level of *prdA* mRNA increased about 10-fold when wild-type cells were grown in the presence of 30 mM L-proline. Similarly, *prdD* and *prfF* transcripts were about 10-fold more abundant in the presence of exogenous L-proline (Fig. 5C and D). The levels of *grdE* mRNA increased about 3-fold in wild-type cells grown in the presence of 30 mM glycine (Fig. 5B). Interestingly, we found that *grdE* mRNA in the wild-type strain was reduced about 80-fold when proline was added to the medium (Fig. 5B), consistent with the reduced amount of GR protein found in cells grown with proline (18). These data demonstrate that the *prd* and *grd* gene clusters are induced at the transcriptional level in response to proline and glycine, respectively, and that proline plays a role in the transcription of the *grd* genes.

**PrdR is required for proline-dependent activation of the *prd* pathway.** Jackson et al. (38) reported the presence, immediately upstream of the *C. difficile* *prd* operon, of a 1,761-bp open reading frame, named *prdR*, that encodes a putative activator of sigma-54-dependent transcription. A previous study revealed that *prdR* homologs are found upstream of the proline reductase gene cluster in most of the species that possess such genes (51). BLAST analysis indicated that *prdR* is also present upstream of a *prd* gene cluster in all sequenced *C. difficile* genomes (data not shown). To test whether PrdR plays a role in the proline-dependent regulation of the Stickland reaction genes, we created an insertion mutation in *prdR*, using TargeTron technology (see Materials and Methods). Disruption of the *prdR* gene was verified by PCR (see Materials and Methods). Using qPCR, we measured the relative amounts of *prdA*, *prdD*, *prfF*, and *grdE* mRNAs in the wild-type and *prdR* mutant (LB-CD8) strains. Even in the absence of exogenous proline, the amount of *prdA* transcript in the *prdR* mutant was reduced about 2-fold compared to that in the wild type (Fig. 5A). Moreover, when the cells were grown in the presence of proline,

*prdA* mRNA levels were ~35-fold lower in the *prdR* mutant than in wild-type cells (Fig. 5A). Similarly, *prdD* and *prfF* transcripts were less abundant in the *prdR* mutant than in the wild-type strain grown in TY medium containing L-proline (Fig. 5C and D) or in TY medium alone. Interestingly, *grdE* levels were increased about 3-fold in the *prdR* mutant compared to those in the wild type and were not affected by the presence of proline in the medium (Fig. 5B), suggesting that PrdR participates in proline-dependent negative regulation of the *grd* operon.

Upstream of *prdR*, we found two genes, CD3246 and *prdC*. In *C. sticklandii*, *prdC* encodes a selenoprotein required for electron transfer by the PR complex (51). Despite the lack of a selenium signature in *C. difficile* PrdC, we hypothesized that PrdC might be part of the PR complex and thus might be regulated by PrdR. To test this hypothesis, we measured by qPCR the amount of *prdC* mRNA in the wild type and *prdR* mutant. We observed that *prdC* levels increased about 10-fold when wild-type cells were grown in the presence of L-proline (Fig. 5E). In the *prdR* mutant, *prdC* levels were 2-fold lower than in the wild type grown in TY medium alone and 30-fold less abundant than in the wild type grown in TY medium supplemented with L-proline (Fig. 5E), suggesting that PrdR is responsible for the proline-dependent activation of *prdC* expression.

To confirm that proline-dependent regulation was due to the disruption of *prdR*, we complemented strain LB-CD8 (*prdR* mutant) in *trans* with a plasmid carrying the *prdR* coding sequence and 260 bp of upstream sequence. The resulting strain, LB-CD14, was tested for its ability to restore *prdA* mRNA to wild-type levels. The empty vector, pBL58, was introduced into the wild-type strain to control for plasmid presence. The strain containing pBL58 (LB-CD13) and the wild-type strain exhibited comparable levels of *prdA* mRNA (data not shown). As shown in Fig. 5A, *prdA* levels in LB-CD14 were similar to or slightly higher than those in the wild-type strain grown under the same conditions, i.e., with or without added L-proline. Hence, we conclude that *prdR* is required for proline-dependent activation of the *prd* operon.

Given the importance of PrdR in the expression of the Stickland reaction genes and the role of PR in growth (see above), we assessed the effect of a *prdR* mutation on growth in TY medium with and without supplementation with L-proline or glycine. Results shown in Fig. 3 and Table 3 indicate that the *prdR* mutant exhibited a slightly lower growth rate than the wild type under all conditions tested. This result shows that PrdR is needed for maximal growth of *C. difficile*.

**Effects of disruption of *prdB* and *grdA* on expression of Stickland metabolism genes.** Because the presence of the Stickland acceptors proline and glycine affects expression of the Stickland metabolism genes, we hypothesized that the PR and GR enzymes control their own expression by modulating the intracellular pools of proline. To test this hypothesis, we first measured the intracellular concentration of free proline in strains LB-CD4, LB-CD8, and LB-CD12. As expected, free proline in LB-CD4 (*prdB*) was about 5-fold more abundant than in the wild-type control. No changes were observed in strain LB-CD12 (*grdA*) compared to the wild type, and intermediate levels were observed in strain LB-CD8 (*prdR*) (Fig. 6). These data show that intracellular proline concentrations depend on the activity of PR but not on the activity of GR.

Therefore, we tested if expression of Stickland metabolism genes was altered in the absence of PR. The level of *prdA* mRNA in strain LB-CD4 (*prdB*) was much higher than in wild-type cells

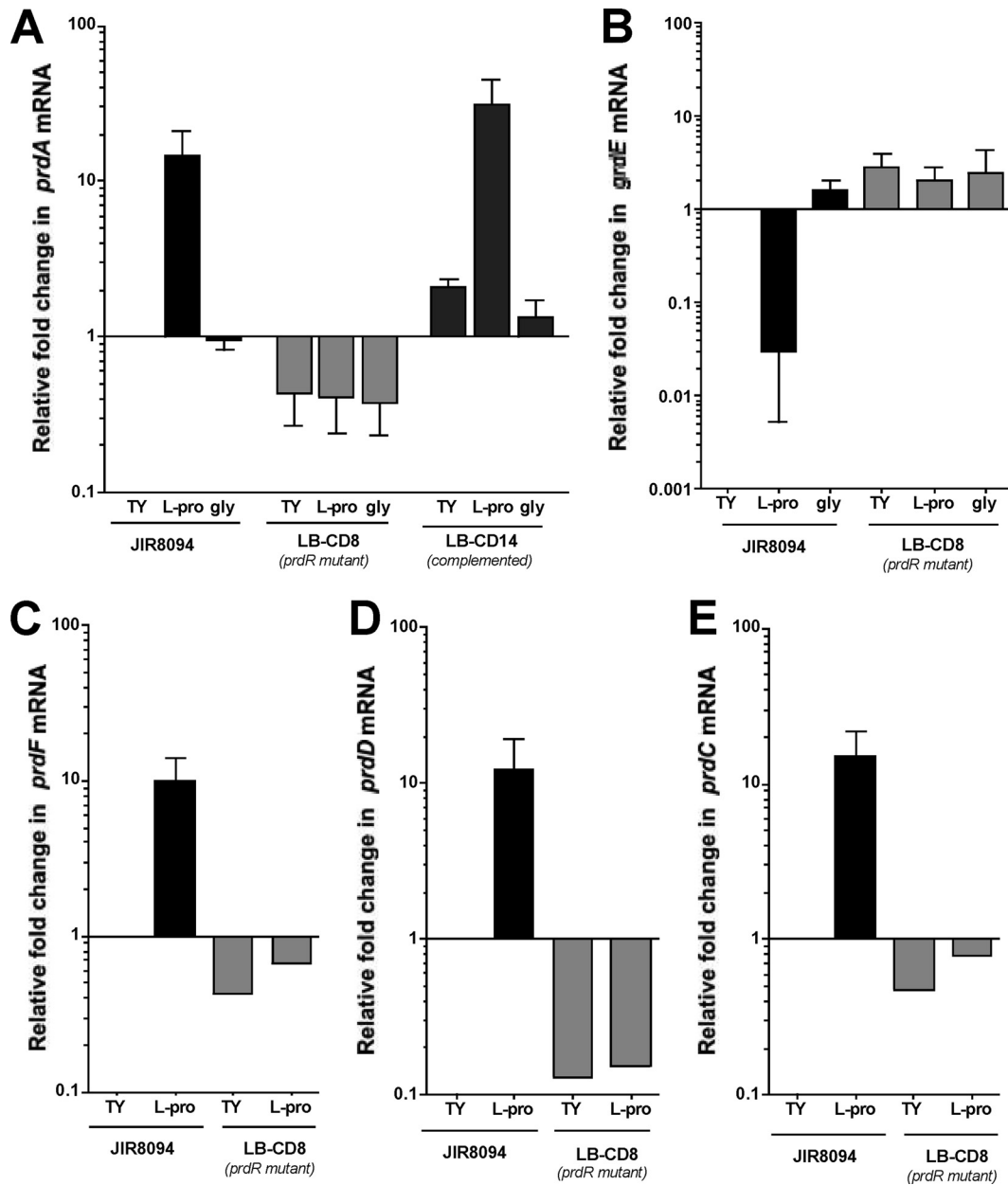


FIG 5 Expression of the *prd* and *grd* genes of wild-type and *prdR* mutant strains in the presence of L-proline or glycine. *C. difficile* wild-type (JIR8094), LB-CD8 (*prdR::ermB*), and LB-CD14 (*prdR::ermB, prdR<sup>+</sup>*) strains were grown to mid-exponential phase in TY medium alone or supplemented with 30 mM L-proline or 30 mM glycine. RNA was harvested, cDNA was synthesized, and qPCR was performed using gene-specific primers for *prdA* (A), *grdE* (B), *prdF* (C), *prdD* (D), and *prdC* (E). Results were normalized to an internal control gene (*rpoC*) and are presented as the ratio of each transcript level to that of wild-type cells grown in TY medium. The means and standard deviations of at least three biological replicates, each assayed in triplicate, are shown, with the exception of LB-CD8 samples in panels C, D, and E, for which data were obtained from a single biological replicate performed in triplicate.

(Fig. 7A), consistent with the idea that blocking metabolism of proline causes it to accumulate to a level that induces *prdA* transcription. Furthermore, whereas the addition of proline to wild-type cells led to a dramatic increase in *prdA* transcription, there was no significant proline-dependent increase in *prdA* expression in *prdB* mutant cells (Fig. 7A), implying that *prdA* transcription had reached its maximum level in strain LB-CD4 without added proline.

Surprisingly, *grdE* transcripts in strain LB-CD4 were more abundant (~10-fold) than in wild-type cells (Fig. 7B) and ~3-fold

more abundant than in the *prdR* mutant (Fig. 5B and 7B). Because PrdR is present in LB-CD4 and the high intracellular concentration of proline in LB-CD4 does not repress *grdE* expression, we conclude that PrdR does not directly mediate the inhibition of the *grd* operon by proline. Instead, our results suggest that the activity of PR plays a more direct role than does PrdR in negatively controlling expression of the glycine reductase pathway.

In a similar manner, we tested whether GR plays a role in regulating expression of the *prd* and *grd* genes. No changes in *prdA* mRNA levels were seen in strain LB-CD12 (*grdA*) relative to those



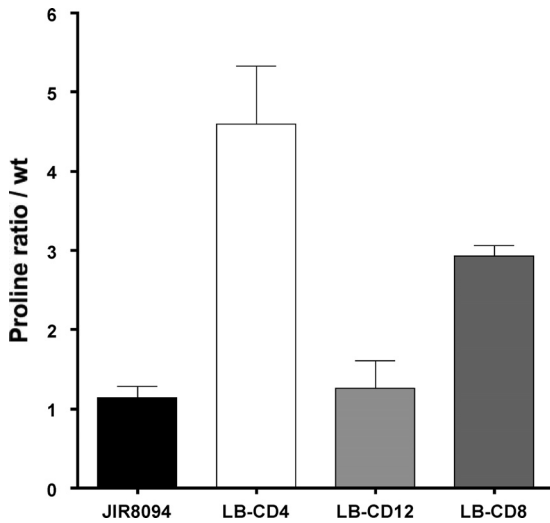


FIG 6 Analysis of proline content in *C. difficile* cells. Extracts from *C. difficile* wild-type (JIR8094), LB-CD4 (*prdA::ermB*), LB-CD12 (*grdA::ermB*), and LB-CD8 (*prdB::ermB*) strains harvested after growth to mid-exponential phase in TY medium were assayed for free proline using a published procedure (48), and the results were expressed relative to those for the wild-type strain. Results represent the means and standard deviations of three biological replicates.

in wild-type cells when the cells were grown in the presence of proline (Fig. 7A). However, we noticed a small increase (~3-fold) in the amount of *prdA* transcript in the *grdA* mutant grown with or without glycine, indicating that GR plays a minor role in the repression of PR. In contrast, *grdE* levels in LB-CD12 were ~10-fold higher than in wild-type cells and ~12-fold more abundant in glycine-supplemented medium (Fig. 7B). Furthermore, induction of *grdE* expression in the presence of glycine was notably higher in the *grdA* mutant than in the wild-type strain (Fig. 7B). These results indicate that the *grd* gene cluster is activated by glycine and

repressed directly or indirectly by GR or by another protein or metabolite product of the *grd* gene cluster.

**Effects of disruption of *prdB* and *prdB* on expression of a *C. difficile* toxin gene.** The presence of certain amino acids, including proline, inhibits toxin production (13, 14). To test whether the effect of proline on toxin production is mediated by the Stickland reactions, we measured the relative amounts of *tcdA* transcript in wild-type, LB-CD4, and LB-CD8 strains in the early stationary phase of growth. We observed that *tcdA* mRNA levels decreased about 2.5-fold ( $P$  value < 0.05) when wild-type cells were grown in the presence of exogenous proline (Fig. 8). Similarly, *tcdA* levels in LB-CD4 grown with or without proline were ~2.5-fold lower than in the wild-type cells (Fig. 8). On the other hand, we observed similar (or slightly higher) levels of *tcdA* transcripts in strain LB-CD8 compared to those in the wild type in cells grown without proline supplementation. However, the relative amount of *tcdA* mRNA in LB-CD8 was ~4-fold higher than in the wild type grown in the presence of exogenous proline, suggesting that PrdR represses *tcdA* expression. Thus, this result indicates that proline negatively affects toxin expression at the transcriptional level and shows that PrdR mediates this repression either directly or indirectly.

## DISCUSSION

Stickland metabolism has been shown to be an important source of energy for nontoxicogenic *Clostridium* spp. in media containing amino acids as sole carbon and nitrogen sources. For *C. difficile*, both glycine and L-proline are required for optimal growth in defined media (50, 52, 53). In addition, Jackson et al. (38) suggested that because of the selenium requirement of *C. difficile* for growth in limiting basal medium, the Stickland selenium-containing reductases (PR and GR) play a key role in the physiology of the bacteria. In the current work, we show that even in rich complex medium where bacteria can find and utilize a multitude of carbon and nitrogen sources, the proline-dependent expression of the PR is used for optimal growth of *C. difficile* (Fig. 4). However,

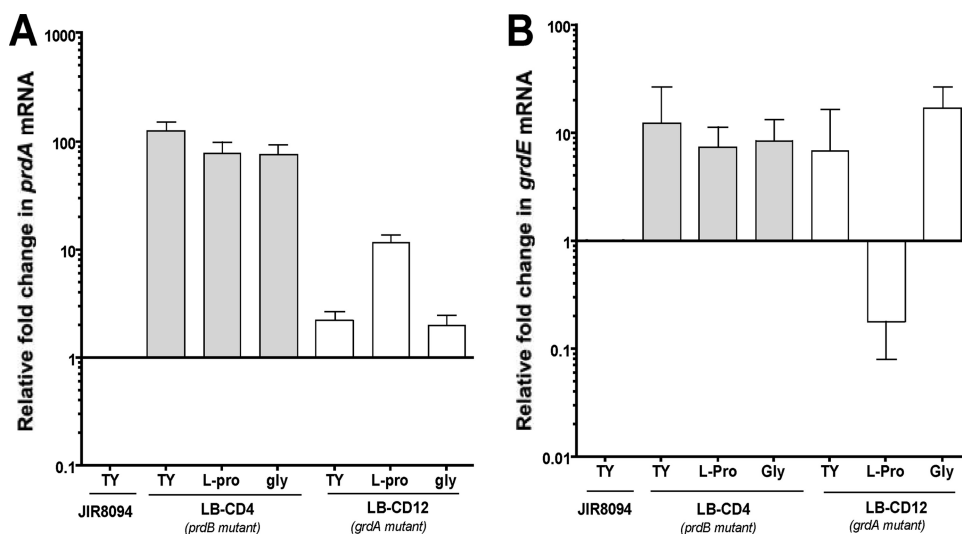
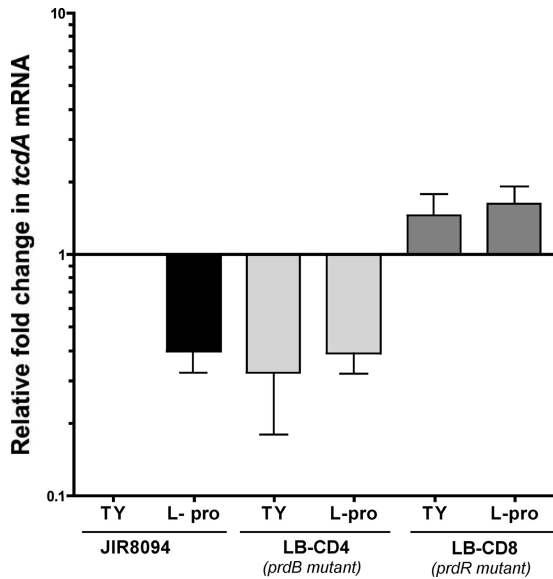


FIG 7 Expression of the *prdA* and *grdE* genes of wild-type and mutant strains in the presence of L-proline or glycine. *C. difficile* wild-type (JIR8094), LB-CD4 (*prdB::ermB*), and LB-CD12 (*grdA::ermB*) strains were grown to mid-exponential phase in TY medium alone or supplemented with 30 mM L-proline or 30 mM glycine. RNA was harvested, cDNA was synthesized, and qPCR was performed using gene-specific primers for *prdA* (A) and *grdE* (B). Results were normalized to an internal control gene (*rpoC*) and are presented as the ratio of each transcript level to that of wild-type cells grown in BHIS. The means and standard deviations of at least three biological replicates, each assayed in triplicate, are shown.



**FIG 8** Expression of the *tcdA* gene of wild-type and mutant strains in the presence of L-proline. *C. difficile* wild-type (JIR8094), LB-CD4 (*prdB::ermB*), and LB-CD8 (*prdR::ermB*) strains were grown for 7 h to stationary phase in TY medium alone or supplemented with 30 mM L-proline. RNA was harvested, cDNA was synthesized, and qPCR was performed using gene-specific primers for *tcdA*. Results were normalized to an internal control gene (*rpoC*) and are presented as the ratio of each transcript level to that of wild-type cells grown in TY medium. The means and standard deviations of biological replicates are shown.

the lack of GR does not affect growth in rich medium. Hence, the proline pathway of Stickland metabolism might be an important source of energy in rich medium but the glycine pathway is not.

It has been shown previously for *C. difficile* that (i) proline stimulates production of PR and inhibits production of GR (38) and (ii) glycine stimulates production of GR. In addition, an earlier report (54) indicated that supplementation of the medium with L-proline decreases GR activity in *C. sporogenes* and suggested that the inhibition occurs at the level of GR synthesis. We show here that addition of proline to the medium increases the expression of the *prd* genes and decreases the expression of *grdE* (Fig. 5A and B). Furthermore, we identified PrdR, an apparent sigma-54-dependent activator encoded by a gene located upstream of the *prd* operon (Fig. 2), as the mediator of proline-dependent activation of the *prd* gene cluster and *prdC* and proline-dependent repression of the *grd* operon (Fig. 5). We also found that glycine activates expression of *grdE* and that a functional PR is required for proline-dependent repression of *grdE*. (The *prdB* mutation in fact resulted in increased expression of *grdE* [Fig. 5B and Fig. 7].) Given that the *prdB* mutant accumulates proline and that proline seems to activate PrdR, we surmise that the proline-dependent repression of *grdE* expression is not directly mediated by PrdR.

If it is not activation of PrdR that explains repression of *grdE* in proline-containing medium, what could be responsible? First, the enzymatic activity of PR could produce an inhibitor of *grdE* expression or consume an activator of *grdE*. Second, the *prdB* mutation is likely to have a polar effect on expression of downstream genes, including *prdF*, which encodes proline racemase. If D-proline, the substrate of PR, rather than L-proline regulates *grdE* expression, the absence of *prdF* expression in a *prdB* mutant would limit inhibition of *grdE* expression. Alternatively, 5-aminovalerate,

the end product of D-proline reduction (Fig. 1), might be a signal for *grdE* repression. However, addition of either 5-aminovalerate or D-proline to *prdB* mutant cells failed to restore repression of *grdE* (data not shown), suggesting that neither D-proline nor 5-aminovalerate, assuming it is imported, is the mediator of *grdE* repression.

Recent work showed that CodY, a global regulator in Gram-positive bacteria that modulates gene expression in response to the availability of branched-chain amino acids (isoleucine, leucine, and valine) and GTP (23), represses toxin expression in *C. difficile* by binding to the promoter region of *tcdR*, which encodes a sigma factor needed for transcription of the toxin genes (19). CodY proteins also control virulence gene expression in other Gram-positive pathogens, such as *Staphylococcus aureus* (40, 55, 56), *Streptococcus pneumoniae* (57), and *Listeria monocytogenes* (58). Interestingly, isoleucine and leucine are among the most efficient Stickland donors (59). Therefore, it is tantalizing to think that Stickland metabolism might play an indirect role in toxin gene expression by reducing CodY activity by draining the pools of isoleucine, leucine, and valine. On the other hand, the effect of proline on toxin production (12, 14) might be at least partly independent of CodY. Consistent with this idea, our results show that toxin expression is reduced in the *prdB* null mutant, which accumulates proline, and is slightly increased in the *prdR* mutant compared to the wild type (Fig. 8). Whether PrdR acts directly on toxin gene expression remains to be tested.

In conclusion, our results and those of others (38, 54) show that the Stickland metabolism genes of *C. difficile* are regulated in response to the availability of proline and glycine and that these genes are needed for optimal growth in defined and complex media. With the mutants in hand, we will be able to assess the role of Stickland metabolism in animal models of *C. difficile* infection.

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