EF1025, a Hypothetical Protein From Enterococcus faecalis, Interacts With DivIVA and Affects Cell Length and Cell Shape

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DivIVA plays multifaceted roles in Gram-positive organisms through its association with various cell division and non-cell division proteins. We report a novel DivIVA interacting protein in Enterococcus faecalis, named EF1025 (encoded by EF1025), which is conserved in Gram-positive bacteria. The interaction of EF1025 with DivIVA was confirmed by Bacterial Two-Hybrid, Glutathione S-Transferase pull-down, and co-immunoprecipitation assays. EF1025, which contains a DNA binding domain and two Cystathionine β-Synthase (CBS) domains, forms a decamer mediated by the two CBS domains. Viable cells were recovered after insertional inactivation or deletion of EF1025 only through complementation of EF1025 in trans. These cells were longer than the average length of E. faecalis cells and had distorted shapes. Overexpression of EF1025 also resulted in cell elongation. Immuno-staining revealed comparable localization patterns of EF1025 and DivIVA in the later stages of division in E. faecalis cells. In summary, EF1025 is a novel DivIVA interacting protein influencing cell length and morphology in E. faecalis.

Keywords: Gram-positive bacteria, Enterococcus faecalis, cell division, DivIVA, protein–protein interaction, Bacillus subtilis

INTRODUCTION

A key protein in Gram-positive bacteria is DivIVA which is implicated in cell division and other functions (Cha and Stewart, 1997; Ben-Yehuda et al., 2003; Fadda et al., 2003; Pinho and Errington, 2004; Ramirez-Arcos, 2005; Briley et al., 2011; Halbedel and Lewis, 2019). DivIVA self-interacts, oligomerizes and associates with a functionally different array of proteins in different Gram-positive bacteria (Halbedel and Lewis, 2019). In Bacillus subtilis (Bs), DivIVA functions as a mid-cell determinant by attracting the MinC/MinD protein complex to the cell poles, thereby preventing cell division at the polar region (Cha and Stewart, 1997; Edwards and Errington, 1997; Marston and Errington, 1999; Edwards et al., 2000; Karoui and Errington, 2001;
Harry and Lewis, 2003). DivIVA_{Bs} also associates with the DNA binding protein RacA, which acts as a bridge between the oriC region and the cell poles, anchoring the chromosome at the poles during sporulation (Ben-Yehuda et al., 2003). In addition, DivIVA_{Bs} interacts with Spo0J, participating in chromosome segregation during sporulation (Ben-Yehuda et al., 2003; Wu and Errington, 2003; Perry and Edwards, 2006); with ComN which is involved in competence development (dos Santos et al., 2012); and, with Maf, a regulator of cell shape and division (Butler et al., 1993). The interaction between Maf and DivIVA_{Bs} arrests cell division in competent cells (Briley et al., 2011).

DivIVA of Corynebacterium glutamicum interacts with RodA and ParB (Donovan et al., 2012; Sieger et al., 2013), which binds the origin of replication with ParA, resulting in chromosomal segregation (Mierzejewska and Jagura-Burdzy, 2012). DivIVA is involved in apical growth and control of cell polarity in Streptomyces coelicolor (Flärdh, 2010), by interacting with ParB to co-ordinate chromosomal segregation (Danczew et al., 2016).

DivIVA in Streptococcus pneumoniae interacts with several proteins implicated in divisive formation, including FtsZ, FtsA, ZapA, FtsK and FtsI, FtsB, FtsQ and FtsW (Fadda et al., 2007). These studies highlight the diverse functionality of DivIVA in Gram-positive organisms. There is no information regarding DivIVA-associating proteins in Enterococcus faecalis (Ef).

Enterococcus faecalis, an opportunistic, commensal, Gram-positive, ovococcal pathogen is recognized for its resistance to multiple antibiotics and for causing hospital-acquired infections (Murray, 1990; Cross and Jacobs, 1996; Hidron et al., 2008a,b; Sievert et al., 2013). Enterococcal infections are potentially fatal, causing neonatal and wound infections, endocarditis, meningitis, and urinary tract infections (Hidron et al., 2008a,b; Torelli et al., 2017). Due to its ability to form biofilms, catheter-related urinary tract infections with E. faecalis are difficult to treat (Mohamed and Huang, 2007). To formulate new therapeutic agents and targets for resisting antibiotic resistant E. faecalis infections, a greater understanding of enterococcal biology, physiology and genetics is required.

Enterococcus faecalis contains DivIVA (Ramirez-Arcos, 2005). This research describes a novel DivIVA-interacting protein, EF1025, which was annotated as a hypothetical protein in E. faecalis strain V583 (Paulsen et al., 2003). EF1025, which is conserved in most Gram-positive bacteria, contains a DNA binding domain at its N-terminus and two highly conserved Cystathionine β-Synthase (CBS) domains at the central and C-terminal regions. Bacterial Two-Hybrid (B2H), Glutathione S-Transferase (GST) pull-down, and Co-immunoprecipitation (Co-IP) assays were used to demonstrate interaction between EF1025 and DivIVA_{Ef}. EF1025 self-interacts and forms a decamer. It was not possible to obtain viable cells after the deletion or inserional inactivation of EF1025 without in trans expression of the gene. These rescued cells grew more slowly than wild type E. faecalis. Scanning electron microscopy (SEM) and atomic force microscopy (AFM) revealed cell elongation and aberrant cell shape in rescued cells. Cell elongation was also observed in SEM images when EF1025 was overexpressed in E. faecalis cells. Using an E. coli model, overexpression of EF1025 in E. coli PB103 resulted in filamentation. Immunofluorescence microscopy showed that EF1025 localized comparably to DivIVA_{Ef} localization during the later stages of cell division.

**MATERIALS AND METHODS**

**Strains, Plasmids and Growth Conditions**

Strains and plasmids used in this study are listed in Supplementary Tables S1, S2. E. coli XL1-Blue or DH5α were used as hosts for cloning. E. coli C41 (DE3) was used to overexpress cloned proteins, E. coli PB103 (de Boer et al., 1988) for heterologous overexpression of E. faecalis proteins, and E. coli R721 (Di Lallo et al., 2001, 2003) was used for the bacterial-two hybrid evaluations. E. coli strains were grown at 37°C in Luria-Bertani (LB) medium (Difco, Detroit, MI, United States) and antibiotics were included in the following concentrations as required: ampicillin (Amp) 100 µg/mL, kanamycin (Kan) 50 µg/mL and erythromycin (Ery) 125 µg/mL. E. faecalis JH2-2 (Jacob and Hobbs, 1974), the parental strain, was used for the preparation of genomic DNA. E. faecalis was cultured at 37°C without aeration in Brain Heart Infusion (BHI) broth (Difco, Detroit, MI, United States) and supplemented with appropriate antibiotics if required (Ramirez-Arcos, 2005; Rigden et al., 2008). Saccharomyces cerevisiae SFY526, used in yeast two-hybrid (Y2H) assays (Clontech Laboratories, Inc., Mountain View, CA, United States), was grown at 30°C for 2–4 days on yeast extract-peptone-dextrose-adenine medium (YPD) or on appropriate synthetic dropout media (Yeast Protocols Handbook, Clontech).

**Bioinformatic Analysis**

DNA sequences interacting with DivIVA_{Ef}, identified after screening Y2H libraries of E. faecalis JH2-2 (Supplementary Methods) were blasted against the E. faecalis V583 genome (Paulsen et al., 2003) using NCBI BLAST1. A putative open reading frame, named EF1025 (GenBank accession number NC_004668), was identified from the E. faecalis V583 genome. The upstream sequence of EF1025 (∼ 480 bp) was analyzed for promoter prediction2 and the deduced amino acid sequence of EF1025 was ascertained using ProtParam3. Homologs of EF1025 were identified using BLASTp4 against the non-redundant protein sequences database. EF1025 was also analyzed by PROSITE (Sigrist et al., 2010)5 to identify functional domains. Transmembrane motifs in EF1025 were predicted using TMbase6 and potential coiled-coil structures were predicted using COILS7.

**EF1025-DivIVA_{Ef} Interactions in the Bacterial Two-Hybrid Assays (B2H)**

The B2H system of Di Lallo et al. (2001, 2003) was used to investigate interactions between DivIVA_{Ef} and EF1025 and its

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1 http://blast.ncbi.nlm.nih.gov/Blast.cgi
2 http://www.fruitfly.org/seq_tools/promoter.html
3 http://us.expasy.org/tools/protparam.html
4 https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins
5 https://prosite.expasy.org/
6 http://www.ch.embnet.org/cgi-bin/TMPRED_form.parser
7 http://www.ch.embnet.org/software/COILS_form.html
various domains. This particular assay involves a hybrid repressor which recognizes a chimeric operon. Potential interacting proteins are cloned at the two chimeric regions at the C-terminus of the hybrid repressor. The dimerization of the heterologous proteins permits reconstitution of the hybrid repressor which recognizes the chimeric operator and downregulates the activity of the downstream reporter gene, lacZ (Di Lallo et al., 2001). Modified B2H vectors pCl434-L and pClp22-L, containing a linker with multiple endonuclease restriction sites were used in B2H assays (Supplementary Table S2A) (Zou et al., 2017). EFl02S, EFl025-c (encoding A8A0-209 of EF102S) and divIVAEF were PCR-amplified from the E. faecalis JH2-2 using primers EF102S-F/R, EF1025C-F/R and CBdivIVA-F/R, respectively (Supplementary Table S3A) and cloned into the modified B2H vectors, resulting in plasmids pdivIVA22, pdivIVA34, pEF102S434, p22CBS1CBS2 and p434CBS1CBS2, respectively (Supplementary Table S2A). These plasmids were transformed into E. coli R721 alone or in combination (Di Lallo et al., 2001, 2003; Greco-Stewart et al., 2007). Freshly transformed single colonies were grown overnight in 4 mL LB medium supplemented with Amp 50 µg/mL and Kan 30 µg/mL. Cells were diluted 1:100 using fresh LB medium containing the same antibiotics and were incubated for ~1 h at 37°C, followed by the addition of 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Cells were further incubated to mid-log phase (OD600 ∼0.5) at 37°C, harvested, and tested for β-galactosidase activity, as previously described (Di Lallo et al., 2001). Each experiment was performed in triplicate and the average percentage β-galactosidase activity was calculated.

GST Pull-Down Assays

To create a GST-DivIVA_EF fusion, divIVAEF was PCR-amplified from genomic DNA from E. faecalis JH2-2 (see Supplementary Methods) using primers IVA-5/IVA-11 (Supplementary Table S3B) (Ramirez-Arcos, 2005). The amplicon was cloned into pGEX-2T, generating plasmid pGST-Div (Supplementary Table S2B). EF102S was PCR-amplified from E. faecalis JH2-2 DNA using primers EF102S-F/R (Supplementary Table S3B) and cloned into pETEF102S (Supplementary Table S2B). The two CBS domains, i.e. CBS1 and CBS2, of EF102S were PCR-amplified from E. faecalis JH2-2 DNA using primers EF102S-CF/R and cloned into pET30a(+), resulting in plasmid pETEF102S_CBS2 (Supplementary Tables S2B, S3B).

GST-DivIVAEF, 6 × His-EF102S, or 6 × His-EF102SCBS2 fusions were overexpressed in E. coli C41 (DE3) (Ramirez-Arcos, 2005). The GST-DivIVAEF fusion protein was purified using GST affinity beads (GST-Bind Kit, Novagen, United States). 6 × His-EF102S or 6 × His-EF102SCBS2 were purified from 200 mL log-phase growth of E. coli C41 by sonication in 5 mL interaction buffer (1B, 20 mM Tris/HCl pH 7.5, 10% glycerol, 50 mM KCl, 0.5 mM EDTA, 1% Triton X100, 1 mM DTT). The cell lysate was centrifuged and the supernatant (50 µL) was incubated with 20 µL GST-DivIVAEF bound beads, pre-equilibrated with 1B buffer, at 4°C for 2 h. Beads were washed with cold 1B buffer 3× and the retained protein was eluted using a 40 µL 1 × SDS loading buffer and heating at 95°C for 10 min. Eluted protein was separated by SDS-PAGE, followed by Western blot analysis using anti-6 × His monoclonal antibody (Biorad, United States). The same protocol was used to study the DivIVA_EF and EF102S-CBS2 interaction. Purified GST protein was used as a control and was produced in E. coli C41 (DE3) from plasmid pGEX2T.

Production of Anti-EF102S Polyclonal Antibody

6 × His-EF102S was overexpressed in E. coli C41DE3 from plasmid pETEF102S (Supplementary Table S2B) and was purified as described previously (Ramirez-Arcos, 2005). Female New Zealand White rabbits were injected with ~30 µg/mL purified 6 × His-EF102S in Freund’s adjuvant (Sigma; v/v = 1:1) at the Animal Core Facility of the Vaccine and Infectious Diseases Organization (University of Saskatchewan) with a booster dose on day 21 after the initial injection. Polyclonal IgG antibody was purified by affinity purification of antisera using Protein-A sepharose beads (Pharmacia Biocience; Ramirez-Arcos, 2005). Antibody specificity was tested by western blotting assay using an E. faecalis JH2-2 whole cell protein extract which was prepared by sonicating 50 mL of cell culture and resuspending the cells in 2.5 mL of Tris buffer (Supplementary Figure S1). Previously prepared anti-DivIVA_EF (Ramirez-Arcos, 2005) was used as a positive control.

Co-immunoprecipitation (Co-IP)

An overnight culture of E. faecalis JH2-2 was diluted 1:100 in BHI broth and incubated for 16–20 h at 37°C without aeration. Two hundred mL were centrifuged at 10,000 rpm for 10 min and the pellet was re-suspended in 5 mL Co-IP buffer (25 mM HEPES pH7.9, 100 mM NaCl, 5% glycerol, 0.5 mM EDTA, 0.1% Triton X100, 1 mM DTT and 0.5 mM PMSF). The suspension was sonicated, on ice, 3×, for 30 s each, with an interval of 20 s. The cell lysate was centrifuged under the same conditions (above) and the supernatant was collected for Co-IP assays.

Protein-A Sepharose beads (Pharmacia Inc., Canada) were cross-linked with 20 µg of either anti-DivIVA_EF or anti-EF102S polyclonal antibody in 200 µL PBS as follows: antibody was incubated with 50 µL Protein-A Sepharose beads at room temperature (RT) for 1 h. Beads were washed with PBS once and then washed twice with 0.2 M sodium borate (pH 9.0). Dimethylpimelimidate (Sigma) was added to the beads to a final concentration of 20 mM and incubated for 30 min at RT to allow cross-linking. The reaction was stopped by adding 0.2 M ethanolamine (final concentration 20 mM) pH8.0 (Sigma) and incubating at RT for 2 h. Beads were then washed with PBS and stored at 4°C for later use. Prior to Co-IP, 20 µL antibody-bound beads were incubated with 10 mg/mL BSA overnight at 4°C to block non-specific binding sites. Beads were then equilibrated with Co-IP buffer and subsequently incubated with 200 µL of E. faecalis JH2-2 cell extract for 2 h at 4°C. After removing the supernatant, beads were washed with Co-IP buffer 3× for 10 min each. Proteins retained on the beads were eluted in 80 µL 1 × SDS loading buffer, separated on 12% SDS-PAGE, and transferred onto a nitrocellulose membrane for Western blot assay. Blots were probed with either anti-DivIVA_EF or anti-EF102S polyclonal
antibody. Beads alone or beads cross-linked with anti-MinC<sub>β</sub> polyclonal antibody (Ramirez-Arcos et al., 2001) were used as negative controls.

**EF1025 Self-Interaction**

To determine whether EF1025 self-interacts, and to map the sites responsible for self-interaction, the predicted functional domains of EF1025 were cloned, in different combinations, into Y2H vectors as follows: EF1025CBS1 (AA80-204) carrying CBS1 and CBS2 domains, NCBS1-EF1025 (AA6-137) containing the N-terminus HTH domain and CBS1 domain, CBS2-EF1025 (AA144-204) containing the CBS2 domain, and N-EF1025 (AA6-50) containing the N-terminus HTH domain. *E. faecalis* JH2-2 DNA was used as a template for PCR amplification of these fragments. Primers for the amplification of various fragments are described in Supplementary Table S3C. These amplicons were cloned into the vectors pGAD424 and pGBT7 resulting in plasmids pGADEF1025CBS12, pGBDEBF1025CBS12, pGADEF1025NCBS1, pGBDEBF1025NCBS1, pGADEF1025CBS2, pGBDEBF1025CBS2, pGADEF1025-N, and pGBDEBF1025-N, respectively (Supplementary Table S2C). Each plasmid construct was co-transformed with a plasmid expressing full-length EF1025 (e.g. pGADEF1025 or pGBDEBF1025) into *S. cerevisiae* FY5256. Transformation efficiencies were calculated by plating 50 µL of diluted transformants on separate plates followed by counting the number of colonies produced. Transformants were selected on complete synthetic medium lacking leucine and tryptophan (SD-leu-trp) (Clontech). Transformation efficiencies were calculated by plating 50 µL of diluted transformants on separate plates followed by counting the number of colonies produced. After 3–4 days of incubation at 30°C, using a colony lift assay (Clontech), cells were screened for blue color development in the presence of 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal, Sigma-Aldrich; St. Louis, MS) to study the self-interaction ability of EF1025. Positive clones were further subcultured in SD-leu-trp broth and a spectrophotometric assay for β-galactosidase activity, using the substrate o-nitrophenyl-β-D-galactopyranoside was performed (Ramirez-Arcos, 2005).

SEC-MALS, the combination of Size Exclusion Chromatography with Multi-Angle Light Scattering analysis (Wyatt Technology, United States), was used to determine the oligomerization state of EF1025. Using His-bind resin (Novagen, Canada), 1mg of purified 6 × Hi-Fi-EF1025 was loaded onto a Superdex 200 column (Biorad) equilibrated with a buffer comprising 50 mM Tris base, 400 mM NaCl, pH 7.4. A single peak, corresponding to EF1025 eluted by SEC, was detected by the MALS detector to estimate molar mass.

**Overexpression of EF1025 in *E. faecalis* JH2-2**

To overexpress EF1025 in *E. faecalis* JH2-2, EF1025 was cloned into pMSP3545 (Supplementary Table S2). pMSP3545 was first modified by introducing an Amp-encoding gene that was PCR amplified from pCDNA3.1(+) using primer pairs AmpF/R (Supplementary Table S3D), into pMSP3545 creating pMSP3545A (Supplementary Table S2D). Linkers LinkA/B (Supplementary Table S3D), which contained restriction sites BamHI and Ncol, were ligated to the Amp gene amplicon prior to ligation in pMSP3545. pMSP3545A was electroporated into electrocompetent *E. faecalis* JH2-2 cells using previously described methods (Ramirez-Arcos, 2005) and colonies were selected on BHI supplemented with Ery (125 µg/mL), creating *E. faecalis* MK0, *E. faecalis* JH2-2 and *E. faecalis* MK0 served as controls for all electroporation experiments. EF1025 and 80 bp upstream, which included the predicted promoter sequence, was PCR amplified using primers EF1025npF/R, and the amplicon was digested with Ncol and XbaI, purified and subcloned into pMSP3545A, digested with the same enzymes, creating pMSPEF1025A (Supplementary Table S2D). pMSPEF1025A was transformed into *E. coli* DH5α and transformants were selected for Amp resistance. Clones were confirmed for the presence of EF1025 using restriction digestion and PCR amplification with primers EF1025npF/R. pMSPEF1025A was electroporated into electrocompetent *E. faecalis* JH2-2 cells creating *E. faecalis* MK23 (Supplementary Table S1) using previous methods (Ramirez-Arcos, 2005). To ascertain whether EF1025 was expressed from its native promoter in pMSPEF1025A, pMSPEF1025-flag was created by fusing a flag-tag encoding sequence which was PCR amplified from pcDNA3.1(+) using primers flagF/R (Supplementary Table S3D). The amplicon was ligated in pMSPEF1025A downstream of EF1025 and electroporated into electrocompetent *E. faecalis* JH2-2 cells to create *E. faecalis* MK24 (Supplementary Table S1). EF1025 expression from pMSPEF1025-flag in *E. faecalis* MK24 was evaluated using an anti-flag monoclonal antibody (GenScript, United States) by Western blot analysis. Whole cell extracts of both *E. faecalis* JH2-2, *E. faecalis* MK23 and *E. faecalis* MK24 were prepared for these blots. In a separate Western blot, anti-EF1025 antibody was used to compare EF1025 expression levels in the same strains.

**Complementation of EF1025 Deletions and Insertional Mutants in *E. faecalis* JH2-2**

Clones of insertionally inactivated or deleted EF1025 in *E. faecalis* JH2-2 could not be recovered unless EF1025 was expressed in trans. Therefore, *E. faecalis* JH2-2 was co-transformed both with plasmids expressing EF1025 (i.e. either pMSPEF1025-pro or pMSPEF1025A) and plasmid constructs designed to insertionally inactive (i.e. p3ERM EF1025::Kan) or delete (i.e. p3ERMΔEF1025::Cat) EF1025.

To create p3ERM EF1025::Kan, first the N-terminal sequence of EF1025 (AA1-55) was PCR-amplified from *E. faecalis* JH2-2 using primers CBSDFP/CBS55R-Hind (Supplementary Table S3D). The amplicon was ligated and ligated to predigested pUC18, resulting in pUCEF1025-N (Supplementary Table S2D). Then, a kanamycin cassette (Kan<sup>β</sup>) was PCR-amplified from pTVC-lac (Supplementary Table S2D; Poyart and Trieu-Cuot, 1997) with primers KanF/R (Supplementary Table S3D), and the amplicon was inserted into pUCEF1025-N at its HindIII/SmaI sites, producing plasmid pUCEF1025-N-Kan.
(Supplementary Table S2D). The C-terminal sequence of EF1025 (AA56-209) was PCR-amplified from E. faecalis JH2-2 with primers CBB55F-Smal/EF1025-BamHI (Supplementary Table S3D) and the amplicon was inserted into pUCEF1025-N-Kan creating the plasmid pUCEF1025::Kan (Supplementary Table S2D). Finally, pUCEF1025::Kan was digested with EcoRI and BamHI, yielding a fragment containing EF1025-N, KanR and EF1025-C. This fragment was ligated into p3ERM-H, creating the suicide vector p3ERMEF1025::Kan (Supplementary Table S2D; Ramirez-Arcos, 2005). This plasmid was electroporated into E. faecalis JH2-2 (Ramirez-Arcos, 2005) with selection attempted using BHI agar containing Kan 500 µg/mL and incubation at 37°C for 2–3 days. Transformants were never obtained after multiple attempts, so p3ERMEF1025::Kan was co-electroporated with the shuttle plasmid pMSPEF1025-Pro that expresses wild type EF1025 in trans from its native promoter (Supplementary Table S2D) into E. faecalis JH2-2 to create E. faecalis MJ26 (Supplementary Table S3C; Ramirez-Arcos, 2005). Transformants were selected on BHI supplemented with Ery (125 µg/mL) and Kan (500 µg/mL). For each electroporation experiment, we used E. faecalis JH2-2 and MK0 as controls for growth on BHI supplemented with erythromycin. E. faecalis JH2-2 failed to grow in the presence of erythromycin while E. faecalis MK0 grew well. To confirm that transformants contained both an insertionally inactivated chromosomal EF1025 as well as EF1025 expressed in trans from pMSPEF1025-pro in E. faecalis MJ26, primers mutF/Kan-R, KanF/KanR, EF1025-Pro/KanR and KanF/CBSDP were used to amplify chromosomal and plasmid fragments, followed by DNA sequencing of all amplified fragments for confirmation of the insertion (Supplementary Table S3D).

To ensure that phenotypes observed in E. faecalis MJ26 were not caused by polar effects of the insertional mutagenesis of EF1025 on the downstream gene EF1026, qPCR was performed to study the expression of both genes (Supplementary Methods).

A second strategy to inactivate EF1025 in E. faecalis JH2-2 involved the non-polar deletion of chromosomal EF1025 (LeDeaux et al., 1997) by introduction of the suicide plasmid pERMÆEF1025-Cat. Partial overlapping flanking primers ppdkF/R-BamHI (Supplementary Table S3D) were used to amplify 500 bp upstream (includes the native promoter of EF1025) of the start codon of EF1025 and 500 bp downstream of the stop codon of EF1025 using primers 1026F/R-EcoRI (Supplementary Table S3D) of E. faecalis JH2-2 DNA. A chloramphenicol cassette was amplified from pLEmo (NEB) using primers CatF/R (Supplementary Table S3D). The three fragments were combined by overlap PCR amplification (Hussain and Chong, 2016), creating a fragment that contained the chloramphenicol cassette flanked by the 500 bp upstream fragment and 500 bp downstream fragment. The resultant fragment was purified, digested and ligated into p3ERM-H, creating the suicide vector p3ERMÆEF1025-Cat (Supplementary Table S2D). As no transformants were recovered after electroporation of p3ERMÆEF1025-Cat into E. faecalis JH2-2, this plasmid along with pMSPEF1025A (Supplementary Table S2D) were co-electroporated into E. faecalis JH2-2 (Shepard and Gilmore, 1995) creating E. faecalis MK12. Transformants were selected on BHI agar plates containing Chl 5 µg/mL and Ery 125 µg/mL, incubated at 37°C for 2–3 days. The deletion of EF1025 in E. faecalis MK12 was confirmed by PCR-amplification using primers ppdkF/EF26b-R, mutF/EF26b-R, ppdkF/EF1025npR, EF1025npF/1026R, CatF/1026R and CatF/R (Supplementary Tables S3C,D) followed by DNA sequencing of these amplified fragments (data not shown).

Enterococcus faecalis JH2-2 did not grow at this concentration of chloramphenicol. As a positive control, p3ERMÆEF1025::Cat was electroporated into E. coli DH5α and transformants were selected on LB agar plates containing Chl 33 µg/mL after incubation for 24 h at 37°C.

Microscopy

A SU8010 Cold Field Emission Ultra-High-Resolution scanning electron microscope (WCVM, University of Saskatchewan, Saskatoon, SK, Canada) was used to image E. faecalis strains JH2-2, MK0, MK12, MJ26, MK23, MK24 (Supplementary Table S1). Strains were cultured in BHI medium with or without appropriate antibiotics, without agitation, at 37°C, either overnight (~20 h) or to stationary phase. Cells were fixed on poly-L-lysine coverslips, dehydrated in ethanol, critical point dried, sputter coated with gold and imaged (Ramirez-Arcos et al., 2001). Length measurements were performed across the poles of the diplococcal bacteria and the percentage of elongated cells was calculated by measuring the lengths of 110–250 cells.

A Hitachi HT7700 High Contrast High-Resolution Digital Transmission Electron Microscope (WCVM, University of Saskatchewan, Saskatoon, Saskatchewan) was used to image E. faecalis strains JH2-2 and MJ26 prepared as previously described (Ramirez-Arcos, 2005).

Immuno-Fluorescence Microscopy of E. faecalis JH2-2

To visualize DivIVA\textsubscript{Ef} and EF1025 localization, E. faecalis JH2-2 cells in exponential phase were collected and fixed using a procedure modified from Harry and Lewis (2003). One mL of cell culture was harvested and the resuspended pellet was fixed with 1 mL fixation buffer (2.5% paraformaldehyde, 0.03% glutaraldehyde in 30 mM sodium phosphate buffer pH 7.5) for 30 min, at RT, then for 2 h at 4°C. Cells were washed 3\times with 1 \times PBS and resuspended in 200 \µL GTE (50 mM glucose, 20 mM Tris-HCl pH 7.5, 10 mM EDTA) to which a freshly prepared lysozyme solution (2 mg/mL) was added. This volume was transferred to and fixed on poly-L-lysine coated coverslips. Cells attached to the coverslips were blocked with BSA-PBST (3% bovine serum albumin [wt/vol] and 0.2% Triton X-100 [vol/vol] in PBS) for 2 h at RT. Cells were then incubated with either anti-DivIVA\textsubscript{Ef} (1:200) or anti-EF1025 (1:100) in BSA-PBST for 3 h at RT. After washing with PBST, cells were incubated with a fluorescence-labeled secondary antibody (1:500 dilutions in BSA-PBST, goat anti-rabbit Alexa Fluor 488, Invitrogen) for 45 min. Images were acquired using U-M655 and U-M665 filters and processed using InVitro 3 and ImagePro 6.0 software (Media Cybernetics). Each experiment was performed...
4× using 2 independent cell cultures, and about 300 cells were counted for each immuno-staining. Cells were also stained with DAPI (Thermo Fisher, CA) and were mounted and observed under a 100X oil immersion objective using an Olympus BX61 microscope with standard filters. DAPI-stained cells were divided into five cell division stages. Stage 1 was defined as a single cell with a central condensed chromosome. Stage 2 cells contained a segregating chromosome as the cell started to divide. Stages 3 and 4 defined by the presence of two newly replicated cells with segregated chromosomes. As the cell completed one round of cell division, Stage 5 comprised of two daughter cells with condensed DNA in the center. *E. faecalis* MWMR16 cells, containing point mutations in the coiled coil region of DivIV A*Ef*, were used as negative control (Rigden et al., 2008).

**RESULTS**

**Identification and *in silico* Analysis of a Novel DivIVA*Ef* Interacting Protein in *E. faecalis***

To identify DivIVA*Ef* interacting proteins from *E. faecalis*, a Y2H system was used to screen an *E. faecalis* genomic DNA library using DivIVA*Ef* as the bait protein (data not shown). Positive clones were sequenced and bioinformatic analysis indicated a sequence corresponding to the C-terminus of the hypothetical protein EF1025 (GenBank accession # NP_814759) of the *E. faecalis* V583 genome; EF1025 spans nucleotide positions 983760-984389 (*Figure 1A*). *In silico* analysis of EF1025 indicated that a ribosome binding site (GGAGG) is located at nucleotide position (nt) –6 to –10, and a putative promoter at position nt –36 to –87. EF1025 has a transcriptional orientation (*Figure 1B*) similar to the downstream gene EF1026, a hypothetical protein with a kinase phosphoprotein phosphatase (PPPase) domain. A predicated terminator sequence is located downstream of EF1026. The upstream gene, EF1024, is transcribed in the opposite orientation of EF1025 and EF1026 and encodes a putative pyruvate phosphate dikinase (PPDK) domain (*Figure 1B*).

EF1025 comprises 209 amino acids (AA), with a molecular weight of ~23 kDa and a theoretical isoelectric point of 6.75. Domain prediction studies (*Figure 1B*) showed that EF1025 contains an N-terminal Helix-turn-Helix (HTH) DNA binding domain (AA 6-50), and two CBS domains (i.e. CBS1, AA 80-137 and CBS2, AA 144-204). The CBS1 domain is in the central
region of EF1025 and CBS2 is located at the C-terminus. EF1025 does not contain any transmembrane motifs (suggesting that it is a cytosolic protein), nor does it contain coiled-coil regions.

The EF1025 protein sequence was used as a query in BLASTp against 10000 targeted sequences in the non-redundant (nr) protein sequences database (last accessed May 2019). EF1025 was identified as belonging to the CBS pair superfamily and is conserved predominantly in Gram-positive bacteria, primarily in Firmicutes. As with EF1025, Gram-positive homologs contain an N-terminal HTH domain and two CBS domains located centrally and at the C-terminal. In *B. subtilis*, the EF1025 homolog is named CcpN and is involved in the gluconeogenic pathway (Servant et al., 2005).

**EF1025 Oligomerizes and Self-Interacts**

To determine whether EF1025 self-interacts, fragments comprising different combinations of domains of EF1025 were cloned into Y2H vectors and initially tested for interactions using the colony lift assay (data not shown), followed by a quantitative assay for β-galactosidase activity. The quantitative assay indicated that EF1025 strongly self-interacts (Figure 2). Furthermore, the EF1025CBS12, containing the CBS1 and CBS2 domains, strongly interacted with EF1025. Fragments containing the N-terminus HTH domain and the central CBS1 domain (i.e. EF1025NCBS1) and fragments EF1025CBS2 (contains CBS2 domain) and EF1025-N (i.e. N-terminus HTH domain) showed no interaction with EF1025.

6×His-EF1025 was found to be a decamer, with an estimated molecular mass of 222 kDa, using a combination of Size Exclusion Chromatography (SEC) with Multi-Angle Light Scattering (MALS) analysis (Supplementary Figure S2). Reduced disulfide linkages, achieved by adding 1 mM Dithiothreitol, did not change the overall molecular weight of 6×His-EF1025.

**EF1025 Interacts With DivIVA<sub>EF</sub> in vitro and in vivo**

A B2H system was used to confirm preliminary Y2H results, showing the interaction of EF1025 with DivIVA<sub>EF</sub>. In this assay, less than 50% residual β-galactosidase activity is indicative of a positive interaction (Di Lallo et al., 2001; Zou et al., 2017). *E. coli* R721 cells showed a baseline residual β-galactosidase activity of 100%. *E. coli* R721 transformed, with one of pdivIVA22, pdivIVA434, pEF1025434, p434CBS1CBS2, or p22CBS1CBS2, showed residual β-galactosidase activities of 78, 82, 55, 66, and 77%, respectively, and served as negative controls. The positive control (*E. coli* R721 cells containing plasmids pdivIVA22 and pdivIVA434), which demonstrated the self-interaction of DivIVA<sub>EF</sub> (Ramirez-Arcos, 2005), displayed 36% residual β-galactosidase activity. Our results indicate an interaction between DivIVA<sub>EF</sub> and EF1025 (Figure 3; pdivIVA434 and p22EF1025 together) with a residual β-galactosidase activity of 21%. The two CBS domains together (i.e. p22CBS1CBS2 or
FIGURE 3 | EF1025 interacts with DivIVA<sub>Ef</sub> in B2H assay. The β-galactosidase activity was expressed in percentage Miller Units (y-axis). The x-axis shows the combination of B2H plasmids used in the experiment. Average values were obtained from three independent assays in triplicates. Values of less than 50% indicate a positive interaction. The error bars represent 1 standard deviation.

p434CBS1CBS2) also interacted with DivIVA<sub>Ef</sub> (pdivIVA434 or pdivIVA22) with 14% residual β-galactosidase activity.

The interaction between EF1025 and DivIVA<sub>Ef</sub> was also ascertained using a GST-pull down assay. A Western blot using anti-EF1025 antibody revealed that GST-DivIVA<sub>Ef</sub> was pulled down by 6×His-EF1025 (Figure 4A, Lane 3) or 6×His-EF1025CBS12 (Supplementary Figure S3, Lane 3). GST did not interact with 6×His-EF1025 (Figure 4A, Lane 2) or 6×His-EF1025-C (Supplementary Figure S3, Lane 2).

The interaction between EF1025 and DivIVA<sub>Ef</sub> was also determined using a Co-IP assay. EF1025 co-precipitated with DivIVA<sub>Ef</sub> using anti-DivIVA<sub>Ef</sub> antibody (Figure 4B, Lane 2), and DivIVA<sub>Ef</sub> co-precipitated with EF1025 with anti-EF1025 antibody (Figure 4C, Lane 2). As a negative control, anti-MinC<sub>Ng</sub> (MinC from N. gonorrhoeae) antiserum failed to precipitate EF1025 or DivIVA<sub>Ef</sub> (Figures 4B,C Lane 4).

**In trans Complementation of Inactivated or Deleted EF1025**

Two strategies were used to inactivate or delete EF1025 in *E. faecalis* JH2-2. First, we attempted to insert a Kan<sup>R</sup> cassette at position nt151 (AA50 and Supplementary Figure S4A) of EF1025 using p3ERM<sub>EF1025</sub>:Kan. No transformants were recovered after several attempts. The second strategy, in which an EF1025 deletion mutant would be created by in frame replacement of EF1025 (p3ERM EF1025:Cat) with a Cat<sup>R</sup> cassette in *E. faecalis* JH2-2 also failed to produce transformant colonies.

Expression of EF1025 was rescued by co-transformation with plasmid combinations p3ERM<sub>EF1025</sub>:Kan and pMSPEF1025-pro, and p3ERMAEF1025:Cat and pMSPEF1025A. These rescue strategies were successful, creating transformant strains *E. faecalis* MJ26 and MK12, respectively (Supplementary Figure S4B).

Taken together, the data suggest that EF1025 may be an essential gene. *E. faecalis* MJ26 and MK12 grew more slowly than *E. faecalis* JH2-2 (Supplementary Figure S5).

The expression EF1026 in *E. faecalis* MJ26 was determined by RT-PCR in order to ascertain that the apparently lethal effects of the Kan<sup>R</sup> insertion in EF1025 was not due to polar effects on this gene. Amplified DNA fragments corresponding to the various regions of EF1026 indicated that the gene was transcribed (Supplementary Figure S6). Expression levels (i.e. ΔC<sub>T</sub> values) for EF1026 in *E. faecalis* JH2-2 (i.e. 16.88 ± 0.13) and *E. faecalis* MJ26 (i.e. 16.79 ± 0.04) were equal.

The phenotypes of *E. faecalis* MJ26 and MK12 differed from wild type *E. faecalis* JH2-2. SEM of *E. faecalis* JH2-2 showed cells with symmetrical division at the mid-cell with characteristic ovococcal cell morphology (Figures 5A, 6A). *E. faecalis* MJ26 and *E. faecalis* MK12 cells formed elongated cells with distorted cell shapes (Figures 5B,C) which were aggregated, failed to segregate (Figure 5B) and had multiple division sites within a single elongated cell (Figure 5C). Compared to the length of the wild type *E. faecalis* JH2-2 cells (1.16 ± 0.14 μm, n = 141), 47% of *E. faecalis* MJ26 (1.63 ± 0.29 μm, n = 174) and 49% of *E. faecalis* MK12 (1.74 ± 0.27 μm, n = 127) cells were significantly (p < 0.05)
Overexpression of EF1025 in E. faecalis and E. coli Induces Cell Elongation

Enterococcus faecalis MK23 was created in which EF1025 is expressed from its native promoter both from the chromosome and from pMSPEF1025A. In order to ensure that EF1025 could be expressed from its native promoter in trans, E. faecalis MK24 was constructed (contains pMSPEF1025-flag) and the protein detected in whole cell extract by Western blot using a monoclonal anti-flag antibody (Supplementary Figure S8A, Lane 3). Expression of EF1025-flag was not detected in E. faecalis JH2-2 or MK23 cell extracts (Supplementary Figure S8A, Lanes 1 and 2). This confirmed expression of an extrachromosomal copy of EF1025 in E. faecalis MK24 when electroporated with pMSPEF1025-flag. This shows that E. faecalis MK23 also carries enhanced expression levels of EF1025 due to the presence of an extrachromosomal copy of EF1025. When anti-EF1025 antibody was used to identify the expression levels of EF1025, overexpression of EF1025 in E. faecalis MK23 and E. faecalis MK24 was observed as determined by densitometric quantification of band intensities, as compared to its expression in E. faecalis JH2-2 (Supplementary Figures S8B,C).

SEM analysis showed a statistically significant (p < 0.05) increase in cell length (1.37 ± 0.21 μm, n = 202; Figures 7B,C) in E. faecalis MK23 as compared to wild type E. faecalis JH2-2 cells (1.16 ± 0.14 μm, n = 141; Figures 7A,C).

Seventy per cent of cells (63/89) overexpressing EF1025 in E. coli PB103 (i.e. E. coli MK23) were filamentous (Supplementary Figure S9B) as compared to none of the control cells, i.e. E. coli cells with pUC18 and cells overexpressing prgXeff, a transcriptional regulator encoding gene (Supplementary Figures S9A,C) (Christie and Dunny, 1986; Bae et al., 2000) in the same vector.

EF1025 Localizes at the Septum and Cell Poles in E. faecalis

Immunofluorescence studies of E. faecalis JH2-2 cells, using anti-DivIVA_Ef or anti-EF1025 polyclonal antibody, determined the localization patterns of DivIVA_Ef and EF1025 during cell division. Cell division entailed 5 stages (273 cells counted for DivIVA_Ef and 281 for EF1025 localization). During Stage 1, as the cell started to divide and the chromosome started to segregate, DivIVA_Ef (20.5%, 56/273 cells) localized at the poles and along the length of the cell. In this stage, EF1025 (23.1%, 65/273 cells) was dispersed along the inner membrane (Figure 8, Stage 1). In Stage 2, EF1025 (14.9%, 42/281) localized along the length of the cell in contrast with DivIVA_Ef (36.7%, 100/273) that remained localized at the poles and the midcell (Figure 8, Stage 2).
Stage 2). At Stage 3, EF1025 (36%, 104/281 cells) and DivIVA_{Ef} (16.1%, 44/273) localized similarly, i.e. to the cell poles and midcell. In Stage 4, as the cells progressed toward completion of cell division, EF1025 (13.2%, 37/281) and DivIVA_{Ef} (16.8%, 46/273) localized as disks and bands along the cell length and septum. With one completed round of cell division (i.e. Stage 5), EF1025 (11.7%, 33/281 cells) was redistributed along the inner membrane before another round of cell division, while DivIVA_{Ef} (9.9%, 27/73) once again localized as dots at the cell poles of the newly formed daughter cells (Figure 8, Stage 5), like Stage 1 cells. The coiled-coil region of DivIVA_{Ef} facilitates oligomerization and is essential for its biological functioning (Rigden et al., 2008). E. faecalis MWMR16 which contains point mutations in the coiled-coil region of DivIVA_{Ef} (Rigden et al., 2008) exhibited loss of DivIVA_{Ef} localization at the cell poles and midcell position (Supplementary Figure S10). The signal was observed to be dispersed all along the membrane and distinct stages of cell division were absent.

DISCUSSION

In the present study, we investigated a novel DivIVA_{Ef} interacting protein, EF1025, from E. faecalis. EF1025 belongs to the CBS pair superfamily and is conserved in Firmicutes including Bacillus, Streptococcus, Clostridium, Paenibacillus, Staphylococcus, Lactobacillus, Streptomyces and Listeria. Surprisingly, EF1025 homologs in the Firmicutes S. pneumoniae, S. pyogenes and L. lactis did not belong to the CBS pair superfamily as they contained an N-terminal HTH domain, but no CBS domains and their sequence similarities ranged from 40 to 44%. We also determined that EF1025 homologs, with uncharacterized functions and different combinations of CBS and HTH domains, may be present in species of the Proteobacteria and Euryarcheota such as Vibrio, Campylobacter, Burkholderia, Acinetobacter, Fusobacterium, Methanosarcina, and Methanoculleus. Proteins containing CBS domains are present in organisms ranging from archaea to humans and were originally identified in
Methanococcus jannaschii as sequence motifs of approximately 60 amino acids (Bateman, 1997). Although several crystallographic studies have been carried out on CBS domains from bacteria, their precise function remains unexplained (Baykov et al., 2011). It has been postulated that CBS domains may act as allosteric “internal inhibitors” of the functional domains of proteins (Aravind and Koonin, 1999; Biemans-Oldehinkel et al., 2006). Proteins with CBS domains can form dimers through interaction of these domains. For example, TM0935 of Thermotoga maritima self-interacts through its two CBS domains forming a dimer (Miller et al., 2004). An Mg^{2+} transporter from E. faecalis, MgtE, also contains two CBS domains but the precise function of these domains remains unelucidated (Ragumani et al., 2010). Our experiments show the importance of the two CBS domains in EF1025 self-interaction. The absence of one CBS domain resulted in the loss of EF1025 self-interaction.

DivIVA, a topological factor in Gram-positive bacteria, interacts with a variety of proteins in various bacteria (Muchová et al., 2002; Halbedel and Lewis, 2019). The range of DivIVA interacting partners changes from one genus to another (Kaval and Halbedel, 2012). In Listeria monocytogenes (Lm), DivIVA<sub>Lm</sub>, performs a variety of functions through its interaction with different proteins (i.e. MinCD and SecA2), including precise positioning of the septum at midcell, assistance in the secretion of autolysins, and enabling swarming motility (Kaval et al., 2014, 2017). In Streptococcus suis (Ss) serotype 2, Ser/Thr kinases (STK) directly phosphorylate DivIVA<sub>Ss</sub> thereby affecting cell growth and division (Nováková et al., 2010). DivIVA from S. aureus (Sa) associates with various divisome proteins (FtsZ<sub>Sa</sub>, FtsA<sub>Sa</sub>, EzrA<sub>Sa</sub>, DivIC<sub>Sa</sub>, DivIB<sub>Sa</sub>, PBP1<sub>Sa</sub> and PBP2<sub>Sa</sub>) to ensure cell division and chromosome segregation (Bottomley et al., 2017). The molecular chaperone, DnaK, interacts and stabilizes DivIVA<sub>Sa</sub> in S. aureus (Bukau and Walker, 1989; Bottomley et al., 2017). Bottomley et al. (2017) also reported an indirect function of DivIVA<sub>Sa</sub> in chromosomal segregation by its interaction with the chromosome segregation protein, SMC (Bottomley et al., 2017). In Mycobacterium smegmatis (Ms) and M. tuberculosis (Mt), the DivIVA homolog is Wag31 (Nguyen et al., 2007; Kang et al., 2008; Meniche et al., 2014). Wag31<sub>Mt</sub> interacts with the penicillin-binding protein, PBP3 (Mukherjee et al., 2009) as well as ParB (Donovan et al., 2012).
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FIGURE 7 | EF1025 overexpression in E. faecalis JH2-2 cells causes cell elongation. Scanning electron micrographs showing (A) E. faecalis JH2-2 lancet-shaped cells; (B) E. faecalis MK23 cells harboring pMSPEF1025A, showing elongated cell morphology; 3 µm bar scale at the bottom right corner of each image establishes the comparison in cell length for E. faecalis JH2-2 and MK23; and (C) Comparison of cell lengths of E. faecalis strains: JH2-2 (n = 141), MK23 (n = 202) and MK24 (n = 226) where “n” represents a number of cells counted for each sample; two-tail p-value from t-test for each group set has been indicated in the graph. All data was introduced to t-test and ANOVA analysis with a p-value < 0.05. The error bars represent 1 standard deviation.

and Wag31Ms interacts with ParA (Donovan et al., 2012; Ginda et al., 2013). DivIVA from E. faecalis is essential for cell viability and growth, proper cell division and chromosome segregation (Ramirez-Arcos, 2005). Rigden et al. (2008) showed that the oligomerization of DivIVA_Ef is mediated by two centrally located coiled coils that are important for its proper biological functioning (Rigden et al., 2008). E. faecalis DivIVA_Ef mutant, E. faecalis MWMR16, contains a disrupted coiled coil region and failed to interact with EF1025 in a B2H assay due to the loss of a functional coiled-coil region in DivIVA_Ef (Rigden et al., 2008; Hedlin, 2009). Our research addressed the essentiality, localization and function of EF1025 during cell division.

Immunostaining showed that EF1025 localized in a pattern comparable to DivIVA_Ef in E. faecalis. Previously, Fadda et al. (2007) showed DivIVA localization to the mid-cell septa and poles in S. pneumoniae using similar methods (Fadda et al., 2007). EF1025 localized laterally along the cell length in Stages 1 and 2 and a pattern comparable to DivIVA_Ef in Stages 3, 4, and 5 of cell division. This localization progression may assist proper cell segregation required for cell division during the later stages of cell division when these two proteins interact. GpsB, an essential protein which determines the ellipsoidal shape in S. pneumoniae, localized in a similar but not identical manner to FtsZ, and is implicated in determining cell shape by septal ring closure (Land et al., 2013). There
is a possibility that the localization of EF1025 (a cytosolic protein) to the lateral cell regions could be facilitated by DivIVA\textsubscript{Ef} association. Different domains of DivIVA\textsubscript{Bs} have been reported to interact with different partners that are membrane proteins as well as cytosolic proteins (Perry and Edwards, 2006; Bramkamp et al., 2008; Patrick and Kearns, 2008; Briley et al., 2011; dos Santos et al., 2012; Baarle et al., 2013; Halbedel et al., 2014; Schumacher, 2017; Halbedel and Lewis, 2019). Membrane localization of cytosolic proteins enhances the interaction abilities of interacting partners during processes such as cell division which involves multi-protein complex formation (Yogurtcu and Johnson, 2018).

We postulate that EF1025 may be an essential gene since, during our attempts to delete or insertionally inactivate the gene, we were never able to recover viable cells. When these strains were complemented with EF1025 (i.e. \textit{E. faecalis} MJ26 and MK12) they grew more slowly, with a longer log phase as compared to the \textit{E. faecalis} JH2-2. The most likely explanation is that the rescue plasmids (i.e. pMSPEF1025-pro and pMSPEF1025A) failed to provide full complementation, which also led to altered cell shape and length. In \textit{S. pneumoniae}, depletion of GpsB, caused cessation of growth and substantial cell elongation (Chastanet and Carballido-Lopez, 2012; Land et al., 2013). Based on the localization pattern of EF1025 and the elongated and aberrant phenotypes exhibited by \textit{E. faecalis} MK12 cells, and the similarity of their localization patterns, we postulate that EF1025 could be one of the members of the septal machinery in \textit{E. faecalis}, which has an unstudied GpsB homolog.

An interesting EF1025 homolog (41% identity) in \textit{B. subtilis}, named CcpN (control catabolite protein of gluconeogenic genes), has two CBS domains and an HTH domain (Servant et al., 2005). CcpN plays a negative regulatory role in the transcription of the gluconeogenic genes \textit{gapB} (one of the GAPDH-encoding genes) and \textit{pckA} (encodes PEP carboxykinase), which are required in carbon catabolite repression pathways (Licht et al., 2005; Servant et al., 2005; Tännler et al., 2008; Licht and Brantl, 2009). Transcription regulation by CcpN has been attributed to its HTH domain which binds to the conserved upstream promoter regions of \textit{gapB} and \textit{pckA} (Licht et al., 2005; Servant et al., 2005; Tännler et al., 2008; Licht and Brantl, 2009). We detected strong interactions between CcpN and DivIVA\textsubscript{Bs} by B2H and GST-pull down assay (paper in preparation). We observed that \textit{gapB} from \textit{B. subtilis} shared 48% homology with \textit{type I} \textit{gap} from \textit{E. faecalis} while \textit{pckA} from \textit{B. subtilis} and \textit{E. faecalis} showed 20% homology. \textit{E. faecalis} was observed to have \textit{type I} and \textit{type II} \textit{gap} as two homologs of \textit{gapB}. Our preliminary sequence searches indicate that the conserved upstream promoter sequences from \textit{B. subtilis} are absent in \textit{E. faecalis} for \textit{type I} \textit{gapB} and \textit{pckA} (unpublished data). This suggests that even though CcpN and EF1025 belong to the same superfamily, they possibly regulate the expression of different genes. CcpN is not an essential gene in contrast to EF1025 (Servant et al., 2005; Tännler et al., 2008), which may result from each protein regulating different genes.

In conclusion, this research presents the first evidence of a DivIVA\textsubscript{Ef} interacting protein, EF1025, in \textit{E. faecalis} that affects cell viability, cell length and shape. Using immunofluorescence, we showed that the localization patterns of EF1025 and DivIVA\textsubscript{Ef} during the later stages of cell division in \textit{E. faecalis} were similar. Our inability to insertionally inactivate or delete EF1025 without in \textit{trans} complementation of the gene indicates that gene is important for viability. Different microscopy methods showed cell elongation, aggregation and impaired cell division in complemented cells with a deleted or inactivated chromosomal gene.

**DATA AVAILABILITY STATEMENT**

The datasets generated for this study are available on request to the corresponding author.

**AUTHOR CONTRIBUTIONS**

KS designed and completed majority of the experiments, analyzed the results, and drafted the manuscript. ML performed and analyzed Y2H and other experiments. TS and TD designed and analyzed AFM experiments and edited the manuscript. J-AD directed the project and its implementation, analyzed the data, edited the manuscript drafts and approved the final submission in collaboration with all authors.
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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.00083/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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