Mutation of a *degS* Homologue in *Enterobacter cloacae* Decreases Colonization and Biological Control of Damping-Off on Cucumber

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ABSTRACT

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We have been using mutagenesis to determine how biocontrol bacteria such as *Enterobacter cloacae* 501R3 deal with complex nutritional environments found in association with plants. *E. cloacae* C10, a mutant of 501R3 with a transposon insertion in *degS*, was diminished in growth on synthetic cucumber root exudate (SRE), colonization of cucumber seed and roots, and control of damping-off of cucumber caused by *Pythium ultimum*. DegS, a periplasmic serine protease in the closely related bacterium *Escherichia coli* K12, is required for the RpoE-mediated stress response. C10 containing wild-type *degS* from 501R3 or

from *E. coli* K12 on pBeloBAC11 was significantly increased in growth on SRE, colonization of cucumber roots, and control of *P. ultimum* relative to C10 containing pBeloBAC11 alone. C10 and 501R3 were similar in sensitivity to acidic conditions, plant-derived phenolic compounds, oxidative stress caused by hydrogen peroxide, desiccation, and high osmoticum; stress conditions potentially associated with plants. This study demonstrates a role for *degS* in the spermosphere and rhizosphere during colonization and disease control by *Enterobacter cloacae*. This study implicates, for the first time, the involvement of DegS and, by extension, the RpoE-mediated stress response, in reducing stress on *E. cloacae* resulting from the complex nutritional environments in the spermosphere and rhizosphere.

Additional keywords: environmental stress, extracytoplasmic stress.

Plant-associated bacteria can perform a variety of beneficial functions, including promotion of plant growth, biological control of plant diseases, and inactivation of toxic pollutants (17,18, 38,41). Unfortunately, there has been limited commercialization of these beneficial, plant-associated bacteria despite considerable research interest (51); inconsistent performance by these beneficial microorganisms being a major obstacle to commercialization (15). This inconsistent performance has been attributed to highly varied soil microenvironments, where a large number of abiotic and biotic factors can negatively influence their behavior (21,74,76,77). Soil abiotic factors such as texture, pH, concentration of micronutrients, and water-holding capacity can cause stress and have been shown to influence the persistence and performance of these bacteria (8,21–23,49). Plants are highly important biotic factors having tremendous influence on environmental parameters such as pH, redox potential, ionic strength, water potential, C:N ratio, and the concentration of nutrients in soil (26,30). The concentration and fluctuation in availability of factors such as nutrients (e.g., C, N, and P), oxygen, and water in the rhizosphere can impose stress on beneficial bacteria and are thought to be capable of negatively impacting their performance (76,77).

Bacteria have evolved mechanisms for dealing with environmental stresses which, in many cases, involve the alternative σ

factors RpoE (σ^E), RpoH (σ^H), and RpoS (σ^S). Cpx and Bae two-component stress response regulator systems, Rcs phosphorelays, and the phage shock response have also been shown to be involved in *Escherichia coli* (40,55,69,71). The RpoE pathway senses damage to polypeptides in the cell envelope (periplasm and outer membrane) and upregulates expression of genes encoding factors that combat this damage such as proteases and the periplasmic folding factors disulfide bond catalysts, peptidyl-prolyl *cis/trans* isomerases, and chaperones (24,71). In addition, a number of genes involved in biosynthesis of phospholipid, fatty acid, LPS, and cell envelope proteins are transcribed under the direction of RpoE (19,57,58). The RpoE pathway has been shown to be activated by heat, ethanol, hyperosmotic shock, sodium dodecyl sulfate, EDTA, and crystal violet (10,14). RpoE in *Pseudomonas* spp. (AlgU and AlgT) has been shown to be important for tolerance to oxidative and desiccation stress (33,73).

DegS, a periplasmic serine protease, is pivotal in the activation of the RpoE pathway by serving as a sensor of extracytoplasmic stress (75,80). DegS is a trimer of identical subunits, each containing a membrane anchor, a trypsin-like protease domain, and a PDZ domain (75). In *E. coli*, binding of misfolded outer membrane proteins to the PDZ domain of DegS activates proteolytic activity by this enzyme, initiating the proteolytic cascade that ultimately results in the RpoE-mediated stress response (75,80). Under nonstress conditions, RpoE is sequestered by the membrane-spanning anti- σ factor RseA. Under conditions of extracytoplasmic stress, RseA is degraded through intramembrane proteolysis by DegS, followed by RseP (YaeL) and ClpXP. RpoE is then released into the cytoplasm, where it directs core RNA polymerase to stress-related genes in the RpoE regulon (3,25,71).

Enterobacter cloacae is an enteric, plant-beneficial bacterium that controls damping-off caused by *Pythium ultimum* on cucum-

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ber and other crops (27,47). *E. cloacae* colonizes the spermosphere and rhizosphere of several plant species, including cucumber (37,62). We report here the isolation of *E. cloacae* C10, a mutant of strain 501R3 that contains a single mini-Tn5 Km insertion within *degS*. The major objective of this study was to determine the role of *degS* in seed and root environments occupied by *E. cloacae* during colonization and biological control of damping-off.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. A description of strains and plasmids used in this study is given in Table 1. *E. cloacae* 501R3 is a prototrophic, spontaneous rifampicin (Rif)-resistant mutant of *E. cloacae* EcCT501 (47,68) while *E. cloacae* C10 is a prototrophic, mini-Tn5 Km mutant of 501R3. *E. cloacae* C10 was constructed and isolated as described previously (64). All strains were grown in Luria-Bertani (LB) broth or agar (45), nutrient broth or agar, or M56 basal salts broth or agar (12) plus a reduced-carbon source to stationary phase at 250 rpm and 35°C unless indicated otherwise. The growth medium was supplemented with ampicillin (Ap, 50 µg/ml), kanamycin (Kan, 75 µg/ml), Rif (100 µg/ml), tetracycline (Tc, 12.5 µg/ml), or chloramphenicol (Cam, 25 µg/ml) where appropriate (Table 1).

Molecular techniques. Genomic and plasmid DNA isolation, restriction enzyme digestion, ligation, electrophoresis, hybridization, transformation, and electroporation procedures were performed as previously described (72).

Plasmid pC10-1 was constructed by digesting *E. cloacae* C10 genomic DNA with *SalI* followed by ligation of the resulting 7-kb *SalI* fragment to *XhoI*-digested pGEM-7Zf(+). Plasmid pBCC10-1 was constructed by digesting C10 genomic DNA with *BglIII* and *ClaI* followed by ligation of the resulting 3.5-kb *BglIII-ClaI* fragment to *BglIII-ClaI*-digested pGEM-7Zf(+). Plasmids pC10-1 and pBCC10-1 were isolated after electroporation of the respective ligation mixtures into *Escherichia coli* DH5α and selection on LB agar containing Ap and Kan (Fig. 1). Primers designed to the right (MT5R2096: 5'-GGG CCT TGA TGT TAC CGA GAG C-3') and the left (MT5L294: 5'-TAA GCG TGC ATA ATA AGC CCT ACA-3') ends of mini-Tn5 Km were used to sequence outward from mini-Tn5 Km in pBCC10-1 and pC10-1. Wild-type *degS* and all transcriptional control elements were polymerase chain reaction (PCR) amplified (GeneAmp PCR System 9700 thermocycler; Applied Biosystems, Carlsbad, CA) from strain 501R3 using primers degSUP (5'-CGG CGC TAC AGG GGG CTA CG-3') and degSDOWN (5'-TTT GAA CAA CAC GCG ATG GAA GG-3') and *Pfu* polymerase. Primer degSUP is located

at residues -343 to -324 relative to the translation start site and degSDOWN is located at residues -139 to -117 relative to the translation termination site. The resulting 1.55-kb PCR product was cloned into the *SmaI* site of pGEM-7Zf(+) generating pGRDS1.5. The complete nucleotide sequence of pGRDS1.5 was determined on both strands for confirmation. Plasmid pKRDS1.5 was constructed by ligating the 1.55-kb *XbaI-HindIII* fragment containing *degS* from pGRDS1.5 into pRK415. This placed *degS* in the opposite transcriptional orientation relative to the *lac* promoter of pRK415. Plasmid pBRDS1.5 was constructed by ligating the 1.55-kb *BamHI-HindIII* fragment from pKRDS1.5 into *BamHI-HindIII* digested pBeloBac11, positioning *degS* in the opposite transcriptional orientation relative to the *lac* promoter of pBeloBac11. Plasmid pGEDS1.2 was constructed by digesting pLC259 (1) with *XbaI* and *HindIII* followed by ligation of the resulting 1.2-kb *XbaI-HindIII* fragment containing *degS* from *E. coli* to *XbaI-HindIII*-digested pGEM-7Zf(+). To construct pBEDS1.2, the 1.2-kb *SphI-HindIII* fragment from pGEDS1.2 was ligated to *SphI-HindIII*-digested pBeloBac11, positioning *E. coli degS* in the opposite transcriptional orientation relative to the *lac* promoter of pBeloBac11.

DNA sequencing was carried out by PCR-mediated Taq Dye Deoxy terminator cycle sequencing. Sequences were analyzed using the DNA analysis programs of DNASTar (DNASTar, Inc., Madison, WI). BLAST searches of databases were conducted with translated sequences by using the BLASTP program (5) (National Center for Biotechnology Information; <http://blast.ncbi.nlm.nih.gov/Blast>).

Nucleotide sequence accession number. The GenBank/EMBL/DDBJ accession number for the complete sequence of *degS* and adjoining region is AY192579.

In vitro growth assays. To determine relative growth of *Enterobacter cloacae* strains at 35 and 43°C, strains were grown overnight in LB broth, harvested by centrifugation, washed in sterile distilled water (SDW), and resuspended to an optical density at 540 nm (OD₅₄₀) of 1.00 in SDW (Spectronic 20 D+ spectrophotometer; Spectronic Instruments, Inc., Rochester, NY), and 200 µl of this suspension used to inoculate 5 ml of LB broth. Cultures were incubated concurrently at 35 and 43°C and growth was monitored spectrophotometrically at OD₅₄₀. Experiments were performed at least twice with three replicate tubes per strain per growth temperature. Relative growth by *E. cloacae* strains on complete synthetic cucumber root exudate (SRE) (36) or individual reduced-carbon components of the SRE medium in M56 basal salts was determined spectrophotometrically. For this, strains were grown overnight in the same medium to be tested, washed and resuspended in M56 basal salts without a reduced-carbon source, and 200 µl of suspension was added to 5 ml of this

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics ^z	Reference or source
Strains		
<i>Enterobacter cloacae</i> 501R3	Spontaneous Rif ^r mutant of <i>E. cloacae</i> EcCT501	68
<i>E. cloacae</i> C10	Mini-Tn5 Km mutant of 501R3 <i>degS</i> ::mini-Tn5 Km; Rif ^r , Kan ^r	This study
<i>Escherichia coli</i> DH5α	(Φ80 _{dlacZAM15}) Δ (<i>lacZYA-argF</i>) U169 <i>glnV44 deoR gyrA96 recA1 relA91 endA1 thi-1 hsdR17</i>	72
Plasmids		
pRK415	Tc ^r ; cloning vector	32
pGEM-7Zf(+)	Ap ^r ; cloning vector	Promega Corp.
pBeloBac11	Cam ^r ; cloning vector	New England Biolabs
pLC259	Cam ^r ; <i>E. coli degS</i> and <i>degS</i> promoter in pSU21	1
pC10-1	Ap ^r ; Kan ^r ; 7-kb <i>SalI</i> fragment from C10 containing <i>degS</i> ::mini-Tn5 Km <i>mdh</i> in pGem-7Zf(+)	This study
pBCC10-1	Ap ^r , Kan ^r ; 3.5-kb <i>BglIII-ClaI</i> fragment from C10 containing <i>degQ degS</i> ::mini-Tn5 Km in pGem-7Zf(+)	This study
pGEDS1.2	Ap ^r ; 1.2-kb <i>XbaI-HindIII</i> fragment from pLC259 in pGem-7Zf(+) containing <i>E. coli degS</i>	This study
pBEDS1.2	Cam ^r ; <i>E. coli degS</i> from pSU21 in pBeloBac11	This study
pGRDS1.5	Ap ^r ; 1.55-kb wild-type <i>degS</i> PCR product from 501R3 in pGem-7Zf(+)	This study
pKRDS1.5	Tc ^r ; 1.55-kb <i>XbaI-HindIII</i> fragment from pGRDS1.5 containing wild-type <i>degS</i> in pRK415	This study
pBRDS1.5	Cam ^r ; 1.55-kb <i>BamHI-HindIII</i> fragment from pKRDS1.5 containing wild-type <i>degS</i> in pBeloBac11	This study

^z Ap^r, Cam^r, Kan^r, Rif^r, and Tc^r = ampicillin, chloramphenicol, kanamycin, rifampicin, and tetracycline resistant, respectively.

medium. Compounds tested individually as substrate are listed in Table 2. Growth by strain 501R3 was considered greater than that of C10 if the OD₅₄₀ was more than one standard deviation greater at two or more time points. All experiments were analyzed independently.

Seedling colonization assays. *E. cloacae* strains were grown, washed, and resuspended in SDW, and 40- μ l suspensions of bacteria containing $\approx 10^4$ CFU in SDW were applied to single cucumber (*Cucumis sativum* cv. Marketmore 76), pea (*Pisum sativum* cv. Sugar snap), or sunflower (*Helianthus giganteus*) seed as aqueous suspensions as previously described (68). SDW (40 μ l) without bacteria was applied to the controls. Natural Hatboro loamy sand soil (59.4% sand, 28.8% silt, 11.8% clay, and 3.04% organic matter, pH 4.5) + 1.0 ml of SDW was placed in 14-ml snap-cap tubes 24 h prior to sowing seed. Seed were placed on the soil surface, spotted with the bacterial suspension or SDW, and then pushed into the soil so that the seed were covered with ≈ 1 cm of soil. Caps were placed loosely on the tubes to allow gas exchange and the tubes were incubated at 22°C. CFU were determined at various times by adding SDW, sonicating for 5 min (Model 810; Branson Ultrasonics Corp., Danbury, CT), and spiral plating the entire contents of the tubes onto LB agar containing the appropriate antibiotics. Experiments were performed twice with six replicates per treatment. Mean log₁₀ CFU per seedling for each seed type for each strain was determined, analysis of variance (ANOVA) carried out, and differences among means estimated using the Student's *t* test in SAS (SAS Institute, Cary, NC). Repeated experiments were not combined prior to analysis (*n* = 6). There was a significant experiment effect for experiments conducted with cucumber (*P* = 0.003) and pea (*P* < 0.0001) seed but not with sunflower seed (*P* = 0.46).

Root colonization assays. *E. cloacae* strains were grown for 48 h in LB broth plus appropriate antibiotics, washed in SDW, and diluted in SDW prior to application to cucumber seed in a gelatin formulation (63). Cucumber seed treated with 10⁷ to 10⁸ CFU/seed of *E. cloacae* strains in the gelatin formulation, or SDW only in the gelatin formulation, were sown in the natural Hatboro loamy sand soil or in nonsterile soilless mix (Redi-Earth Peat-Lite Mix; Scott's Horticultural Products, Marysville, OH) in 6.5-cm-diameter by 25-cm-deep pots (one seed per pot), and incubated in a growth chamber at 22°C with a 12-h photoperiod. At sampling time, the entire root system and tightly adhering planting medium were placed in SDW and sonicated, and CFU per root system was determined as described (60). Mean log₁₀ CFU per root system was determined for each treatment and ANOVA carried out. For experiments where colonization between the two strains 501R3 and C10 was compared in natural soil, differences among means were estimated using the Student's *t* test. The experiment was performed twice with six replicates per treatment and results combined prior to analysis (*n* = 2). There was no significant experiment effect (*P* = 0.44). For experiments where colonization by seven *E. cloacae* strains was compared, differences among means were estimated using a protected least significant difference (LSD) test in SAS. Experiments were performed twice with six replicates per treatment. Repeated experiments were not combined prior to analysis (*n* = 6) because there was a significant experiment effect (*P* < 0.0001).

Disease control assays. *E. cloacae* strains were grown overnight in LB broth containing the appropriate antibiotics, washed with SDW, resuspended in SDW, and applied to cucumber seed in the gelatin formulation. Populations of *E. cloacae* strains in seed treatments were adjusted by diluting the original suspensions with

Fig. 1. A, Physical maps of the portion of the *Enterobacter cloacae* C10 genome containing mini-Tn5 Km and of plasmids pBCC10-1 and pC10-1. The location and direction of transcription of *degS*, *degQ*, and *mdh* are indicated by arrows. The box indicates the approximate site of the mini-Tn5 Km insertion. Kan^r, location of the gene encoding kanamycin resistance; B, *Bam*HI; Bg, *Bgl*II; C, *Clat*; H, *Hind*III; S, *Sal*I; and *lacp*, *lac* promoter. The location of *degS* polymerase chain reaction primers are indicated by arrows (1 = *degS*UP, 2 = *degS*DOWN). Features are approximately to scale. **B,** Predicted amino acid sequence for *E. cloacae* 501R3 *degS*. The transmembrane domain is represented by large, bold italicized letters while the PDZ domain is represented by large, bold letters.

SDW prior to addition to the gelatin. Seed coated with gelatin and SDW but no bacteria were used as controls. *Pythium ultimum* isolate Puzc (63) was grown on corn meal agar at 25°C for 3 days, flooded with soil extract (6), and incorporated into soilless mix. Soilless mix, soilless mix amended with sporangia of *P. ultimum* (24 sporangia/ml) or with SDW, treated cucumber seed, and a second layer of soilless mix amended with sporangia of *P. ultimum* or SDW were added as sequential layers to small cups as described (60,66). Eight replicate cups were sown with five seeds each and incubated in a growth chamber at 22°C for 14 days with a 12-h photoperiod. Mean plant stand per cup was determined visually, ANOVA carried out, and differences among means estimated using an LSD test (SAS). Due to growth chamber space constraints, experiments were performed where biological control by strains 501R3 and C10 was compared and where biological control by strains 501R3(pBeloBAC11), C10(pBeloBAC11), 501R3(pBRDS1.5), C10(pBRDS1.5), and C10(pBEDS1.2) was compared. Experiments were performed twice with each set of strains. Experiments with strains 501R3 and C10 were analyzed

independently ($n = 8$) because there was a significant experiment effect ($P = 0.009$). Experiments comparing plasmid containing strains were combined prior to analysis ($n = 2$) because there was no significant experiment effect ($P = 0.13$).

Environmental stress assays. Acid resistance of *E. cloacae* strains 501R3 and C10 was determined as described (13). Strains were grown overnight in LB broth plus 100 mM MES, pH 5.5; diluted 1:1000 in EG broth, pH 2.5 (78) or in LB broth plus 100 mM MES, pH 7.0; and incubated at 35°C prior to dilution plating at various times onto LB agar containing appropriate antibiotics to determine CFU per milliliter. Experiments were performed three times with three replicates per treatment and combined prior to determination of mean CFU per milliliter $\pm$ standard deviation ($n = 3$).

Sensitivity of *E. cloacae* strains 501R3 and C10 to certain plant-derived phenolic compounds was determined with 1.0, 2.0, 5.0, 10.0, or 20.0 mM concentrations of acetosyringone, acetovanillone, and 4-hydroxyacetophenone (Sigma-Aldrich, St. Louis). M56 basal salts broth (5 ml) plus 0.5% glucose plus an

TABLE 2. Growth and transport of reduced carbon compounds detected in cucumber root exudate by *Enterobacter cloacae* strains C10 and 501R3

Compound ^v	Amount (μ g per plant) ^w	Relative growth ^x	<i>Escherichia coli</i> transport gene ^y	Location of gene product in <i>E. coli</i> ^y
Carbohydrate				
Arabinose	1.0	501R3 > C10	<i>araF</i> <i>araG</i> <i>araH</i> <i>araE</i>	Periplasm Cytoplasm Inner membrane Inner membrane
Fructose	0.5	501R3 = C10	<i>fruB</i> <i>fruK</i> <i>fruA</i>	Inner membrane Inner membrane Inner membrane
Galactose	2.0	501R3 > C10	<i>mglB</i> <i>mglA</i> <i>mglC</i>	Periplasm Cytoplasm Inner membrane
Glucose	5.2	501R3 = C10	<i>ptsG</i>	Inner membrane
Maltose	0.4	501R3 = C10	<i>lamB</i>	Outer membrane
Mannitol	0.7	501R3 = C10	<i>mtlA</i> <i>mtlD</i>	Inner membrane Cytoplasm
Mannose	1.1	501R3 = C10	<i>manX</i> <i>manY</i> <i>manZ</i>	Inner membrane Inner membrane Inner membrane
Rhamnose	2.4	501R3 = C10	<i>rhaT</i>	Inner membrane
Sucrose	11.8	501R3 = C10	ND	ND
Trehalose	0.8	501R3 = C10	ND	ND
Xylose	1.3	50R3 > C10	<i>xylF</i> <i>xylG</i> <i>xylH</i>	Periplasm Cytoplasm Inner membrane
Organic acid				
Citrate	12.7	501R3 = C10	<i>fecB</i> <i>fecC</i> <i>fecD</i> <i>fecE</i>	Periplasm Inner membrane Inner membrane Cytoplasm
Malate	4.4	50R3 = C10	<i>dctA</i>	Inner membrane
Succinate	83.4	501R3 $\geq$ C10 ^z	<i>dctA</i>	Inner membrane
Amino acid				
Glutamine	13.0	501R3 = C10	<i>glnH</i> <i>glnP</i> <i>glnQ</i>	Periplasm Inner membrane Cytoplasm
Proline	188.1	501R3 > C10	<i>proV</i> <i>proW</i> <i>proX</i>	Cytoplasm Inner membrane Periplasm
Serine	40.9	501R3 > C10	<i>sdaC</i> <i>sstT</i> <i>tdcC</i>	Inner membrane Inner membrane Inner membrane

^v The following compounds were in the synthetic cucumber root exudate but not tested individually: cellibiose, gentiobiose, isomaltose, lactose, and isocitrate. The following compounds in synthetic cucumber root exudate were not tested because they did not support growth by 501R3: α -ketoglutarate, arginine, γ -aminobutyrate, glycine, histidine, isoleucine, leucine, lysine, methionine, ornithine, phenylalanine, threonine, tryptophane, and tyrosine. Aspartate was not tested because it appeared that *degS* suppressor mutations arose due to very slow growth on this compound.

^w Data are from Liu et al. (36). All amino acids reported were the L-stereoisomer.

^x Growth was tested on M56 salts plus 0.1 and 0.05% carbohydrate or organic acid or 0.5% amino acid. Growth by 501R3 was considered greater than that by C10 if the optical density at 540 nm was more than one standard deviation greater at two or more time points.

^y Information regarding the transport gene or the cellular location of gene product was from the Ecocyc website for *E. coli* K12 substr. MG1655. ND = not determined.

^z *Enterobacter cloacae* 501R3 had faster growth than strain C10 in four experiments and similar growth in four experiments. ND = not determined.

individual phenolic compound was inoculated with 50 μ l of a mid-log phase culture of 501R3 or C10 grown in M56 basal salts plus 0.5% glucose and rated for optical density after incubation overnight at 35°C with shaking. Experiments were performed twice with two replicates per treatment and analyzed independently. Controls included 100 μ l of dimethyl sulfoxide (DMSO) without plant phenolic compound. These phenolic compounds were dissolved in DMSO and stored at -20°C as 0.1 and 1.0 M stock solutions prior to use.

For oxidative stress experiments, sensitivity to hydrogen peroxide (H_2O_2), was determined essentially as described (42,73). Filter disks (10 mm in diameter, Whatman no. 1) were wetted with 20 μ l of H_2O_2 (1.5, 3.0, 6.0, or 12.0%; vol/vol), or SDW, and placed on soft agar (4 ml of nutrient broth plus 0.8% agar containing 100 μ l of a mid-log phase culture of either C10 or 501R3 grown in LB broth) overlaying a nutrient agar plate. Each plate contained four disks (one control plus three concentrations of H_2O_2). Zones of inhibition were measured after overnight incubation at 35°C and mean zone of inhibition $\pm$ standard deviation determined. The experiment was performed twice with four replicates per treatment. Experiments were analyzed independently ($n = 4$).

The desiccation survival assay was a modification of the procedure of Ophir and Gutnick (48). Strains 501R3 and C10 were grown overnight in LB broth containing appropriate antibiotics, washed with sterile 0.85% saline (wt/vol), resuspended in sterile 0.85% saline, and the OD₅₄₀ adjusted to 0.7. Cell suspensions (100 μ l) were spotted onto 13-mm-diameter Whatman no. 1 filter disks and the disks placed in empty petri dishes in a desiccation chamber and incubated at 22°C. Desiccation chambers were maintained at 20 to 25% relative humidity. Controls were placed in a desiccation chamber with wet paper towels and no desiccant. Mean CFU per filter disk $\pm$ standard deviation was determined at various times after dilution plating onto LB agar containing appropriate antibiotics. The experiment was performed twice with four replicates per treatment and results were combined prior to analysis ($n = 2$).

Osmosensitivity with strains 501R3 and C10 was determined on nutrient agar or on nutrient agar supplemented with 1.0, 2.0, or 3.0 M sorbitol; 1.0, 2.0, or 3.0 M glucose; 0.5, 1.0, or 1.5 M NaCl; or 0.5, 1.0 or 1.5 M KCl as described (2). Plates were incubated at 35°C and examined for growth after 72 h. The experiment was performed twice, with two replicate plates per treatment. Experiments were analyzed independently.

RESULTS

***E. cloacae* C10 contains a transposon insertion in *degS*.** *E. cloacae* strain C10 was selected for further study after a preliminary experiment indicated that it was reduced in colonization of cucumber seedlings. Southern hybridization analysis demonstrated the presence of a single mini-Tn5 Km insertion in the genome of strain C10 (data not shown). Subsequent subcloning of the C10 genome containing portions of mini-Tn5 Km yielded plasmids pBCC10-1 and pC10-1 (Fig. 1A; Table 1). Sequencing outward from mini-Tn5 Km in both plasmids using primers specific for the ends of mini-Tn5 Km provided sequence of the entire open reading frame (ORF) affected by the transposon insertion. Subsequent sequence analysis indicated that the transposon was inserted in a region of the C10 genome with a high degree of sequence similarity to *degS* (*hhoB*) from *Escherichia coli* K12. The complete sequence of *degS* contained an ORF of 1,065 nucleotides, which is sufficient to encode a polypeptide of 355 amino acids. Comparison of this *Enterobacter cloacae* sequence with the *degS* sequence from *Escherichia coli* K12 indicated 69.9% DNA sequence identity and 88.7% amino acid sequence identity. DegS is an inner-membrane bound, serine protease in *E. coli* (4,79). As with *degS* in *E. coli*, analysis of the

predicted amino acid sequence revealed the presence of conserved His96, Asp126, and Ser201 residues, composing a putative catalytic triad characteristic of serine proteases (50,79). Similar to *degS* in *E. coli*, the predicted amino acid sequence contained a PDZ domain and a transmembrane domain (Fig. 1B) (79).

Analysis of the DNA sequence upstream of *degS* indicated the presence of an ORF with a high degree of similarity to *degQ* (*hhoA*) from *E. coli* (Fig. 1A), encoding another putative serine protease (79). An ORF with the opposite orientation that had a high degree of sequence similarity to *mdh* from *E. coli* K12 was detected downstream of the *degS* gene. The *degQ*, *degS*, and *mdh* genes have identical organization and relative direction of transcription in *E. coli* K12 and *Enterobacter cloacae* 501R3 (9,79). Sequence and biochemical analyses indicated that the transposon insertion in *degS* did not impact expression of *degQ* and *mdh*. (i) The *degQ* ORF is upstream and physically separated from the *degS* ORF (Fig. 1A). (ii) C10 grows on acetate as a sole carbon source, suggesting that expression of *mdh* is unaffected. In *Escherichia coli*, *mdh* mutants cannot grow on acetate as a sole carbon source (16).

Wild-type *degS* from *E. coli* K12 or *Enterobacter cloacae* 501R3 genetically restored the slow growth on LB broth phenotype expressed by strain C10 that is characteristic of *degS* mutants. As with *Escherichia coli degS* mutants (56), growth by strain C10 was significantly slower than that of strain 501R3 at 43°C but similar to that of 501R3 at 35°C in LB broth (Fig. 2A).

Fig. 2. Relative growth by *Enterobacter cloacae* strains on Luria-Bertani broth at 35 and 43°C. **A and B.** Growth curves were from a single experiment but presented separately to improve clarity. Data points are the mean of three replicates with error bars representing one standard deviation from the mean. The experiment was conducted three times; results shown are from a representative experiment.

Growth by strain C10 was partially restored at 43°C when *degS* from *Enterobacter cloacae* or *Escherichia coli* was introduced into C10 on plasmids pBRDS1.5 and pBEDS1.2, respectively (Table 1; Fig. 2B). Strains C10(pBeloBAC11), 501R3(pBeloBAC11), C10(pBRDS1.5), and C10(pBEDS1.2) were all essentially similar in growth at 35°C.

***degS* is important for seed and root colonization.** *Enterobacter cloacae* C10 was significantly reduced in short-term colonization of cucumber, pea, and sunflower seedlings relative to strain 501R3 in natural soil (Table 3). Populations of both strains 501R3 and C10 increased on cucumber seed over the 70-h experiment. However, populations of strain 501R3 increased 562-fold whereas populations of strain C10 increased only 40-fold. Populations of C10 were significantly lower than those of 501R3 at 18, 45, and 70 h on cucumber. Populations of C10 increased 8,913-fold on pea (significantly lower than 501R3 at 70 h) and 19-fold on sunflower seed (significantly lower than 501R3 at 18 and 70 h) whereas strain 501R3 increased 39,810-fold on pea and 77-fold on sunflower seed. Populations of strain C10 were significantly lower than those of strain 501R3 at one or more time points in repeated experiments with each seed type. Rif-resistant bacteria were not detected in no-bacteria control treatments in any experiment.

TABLE 3. Growth of *Enterobacter cloacae* strains 501R3 and C10 on various seed types in natural soil^y

Seed, time (h)	Log ₁₀ CFU per seedling		<i>P</i> > <i>F</i> ^z
	501R3	C10	
Cucumber			
0	3.68	3.27	0.08
18	5.15	4.64	0.03*
45	5.34	4.77	0.01*
70	6.43	4.87	0.0001*
Pea			
0	3.58	3.31	0.24
18	7.16	6.87	0.21
45	7.29	6.93	0.12
70	8.18	7.26	0.0001*
Sunflower			
0	4.89	4.94	0.85
18	5.81	5.18	0.02*
45	ND	ND	ND
70	6.78	6.23	0.03*

^y Populations were determined by spiral plating at the indicated time points. Values are the mean of six replicate samples from a single experiment. ND = not determined.

^z *P* > *F* log₁₀ CFU per seedling in this row; * = a significant difference (*P* ≤ 0.05) in population between strains at this time point on this seed-type. An independent analysis was conducted for each seed type.

TABLE 4. Colonization of cucumber root system by strains of *Enterobacter cloacae* in soilless mix^z

Strain	Log ₁₀ CFU/seed	Log ₁₀ CFU/root system	
		Day 19	Day 47
501R3	8.16 ± 0.23	6.50 AB	6.04 C
C10	8.30 ± 0.09	6.28 BC	5.60 D
501R3(pBeloBAC11)	8.01 ± 0.33	6.35 ABC	6.71 A
501R3(pBRDS1.5)	7.89 ± 0.19	6.41 ABC	5.58 D
C10(pBeloBAC11)	7.82 ± 0.42	5.53 D	4.33 E
C10(pBRDS1.5)	8.27 ± 0.34	6.18 BC	5.59 D
C10(pBEDS1.2)	8.32 ± 0.23	6.76 A	6.74 A

^z Plants were grown in deep pots filled with soilless mix. Log₁₀ CFU per seed or per root system was determined by spiral plating onto selective media for each strain. Values are the mean of six replicates and expressed as log₁₀ CFU per seed or log₁₀ CFU per root system. Means were separated by standard deviation or least significant difference where values followed by the same letter were not significantly different (*P* ≤ 0.05). Comparisons of means were made within and between columns. Least significant difference for this experiment was 0.423.

Strain C10 was substantially impaired in long-term colonization of cucumber roots. Populations of strain C10 in natural soil were similar to those of strain 501R3 at 21 days but 149-fold lower at 43 days in the cucumber rhizosphere when similar populations of strains 501R3 and C10 (7.19 ± 0.27 log₁₀ CFU/seed versus 7.05 ± 0.38 log₁₀ CFU/seed, respectively) were applied in seed treatments. Populations of strain 501R3 and C10 were 5.62 log₁₀ CFU/root system and 4.99 log₁₀ CFU/root system (*P* = 0.08) at 21 days, and 5.37 log₁₀ CFU/root system and 3.19 log₁₀ CFU/root system (*P* < 0.0001) at 43 days, respectively. Results were from two experimental runs combined prior to analysis.

Strain C10 was also impaired in long-term colonization of cucumber roots in soilless mix (Table 4). However, differences in populations between strains C10 and 501R3 were not as dramatic as those in natural soil. Populations of strain C10 were significantly different but only 2.75-fold lower than those of 501R3 at 47 days in soilless mix. Differences between these strains were magnified when they each contained the empty cloning vector. Strain C10(pBeloBAC11) was 7-fold lower and 240-fold lower in population than 501R3(pBeloBAC11) at 19 days and 47 days, respectively. Wild-type *degS* from *E. cloacae* on pBRDS1.5 or from *Escherichia coli* on pBEDS1.2 partially restored colonization by C10. Populations of C10(pBRDS1.5) and of C10(pBEDS1.2) were significantly greater than those of C10 containing the empty cloning vector pBeloBAC11 at 19 and 47 days (Table 4). Similar observations were obtained from a second experiment conducted in soilless mix. Rif-resistant bacteria were not detected in control treatments containing seed treated with gelatin but no bacteria in any root colonization experiment.

degS plays a role in disease control. *Enterobacter cloacae* C10 was slightly reduced in control of damping-off of cucumber in experiments conducted in soilless mix. In two experiments, only the wild-type strain 501R3 provided significant (*P* ≤ 0.05) control of damping-off relative to the no-pathogen treatment at all levels of pathogen inoculum where there was significant disease pressure. Treatments containing strain C10 were similar (*P* > 0.05) to those containing the pathogen control at at least one level of pathogen inoculum in both experiments. C10 containing wild-type *degS* from *E. cloacae* or *Escherichia coli* was improved,

TABLE 5. Suppression of damping-off of cucumber caused by *Pythium ultimum* with *Enterobacter cloacae* 501R3 and C10 containing various plasmids^w

Treatment ^x	Log ₁₀ CFU/seed ^y	Mean plant stand per pot ^z	
		0	24
High population			
Gelatin only	BDT	4.81 A	0.56 EF
501R3(pBeloBAC11)	8.89 ± 0.11	4.81 A	2.38 BC
C10(pBeloBAC11)	8.62 ± 0.23	4.75 A	0.31 F
501R3(pBRDS1.5)	9.04 ± 0.09	4.94 A	2.06 C
C10(pBRDS1.5)	9.08 ± 0.09	4.81 A	3.44 B
C10(pBEDS1.2)	8.97 ± 0.09	4.56 A	2.06 C
Low population			
501R3(pBeloBAC11)	6.94 ± 0.01	ND	1.75 CD
C10(pBeloBAC11)	6.23 ± 0.09	ND	0.75 DEF
501R3(pBRDS1.5)	6.74 ± 0.22	ND	1.63 CDE
C10(pBRDS1.5)	7.13 ± 0.10	ND	2.06 C
C10(pBEDS1.2)	7.04 ± 0.18	ND	1.38 CDEF

^w Plasmid pBeloBAC11 is the empty cloning vector, pBRDS1.5 contains wild-type *E. cloacae* 501R3 *degS* in pBeloBAC11, and pBEDS1.2 contains wild-type *Escherichia coli* *degS* in pBeloBAC11.

^x BDT = rifampicin-resistant bacteria were below the detectable threshold.

^y Treatments contained high or low populations of *Enterobacter cloacae* strains.

^z Values are the mean of eight replicate pots, each containing five seeds, and expressed as mean plant stand per pot in an experiment conducted in soilless mix. Means were separated by least significant difference, with numbers followed by the same letter not significantly different (*P* ≤ 0.05). Least significant difference for this experiment was 1.11. Results are from two experiments combined prior to analysis. Quantity of sporangia of *P. ultimum* per cubic centimeter of soilless mix inoculum. ND = not determined.

providing disease control with plant stands statistically greater than the gelatin-only control in the presence of *P. ultimum* when applied as a seed treatment at the high population density (Table 5). Disease control by C10 containing the empty cloning vector pBeloBAC11 was similar to the gelatin-only control in the presence of *P. ultimum* when applied to cucumber seed at lower and high population densities. 501R3 containing pBeloBAC11 provided significant disease control when applied as a seed treatment at both population densities.

degS is important for growth on SRE. Growth by strain C10, containing a mutation in *degS*, was significantly slower than that of the wild-type strain 501R3 on SRE at 35°C (Fig. 3). SRE was constructed based on the composition of reduced-carbon compounds detected in cucumber root exudate (36). Growth by strain C10 was essentially restored when wild-type *degS* from *Enterobacter cloacae* 501R3 or *Escherichia coli* was introduced into C10 on plasmids pBRDS1.5 or pBEDS1.2, respectively. Growth by C10 containing the empty cloning vector pBeloBAC11 was barely detectable whereas growth by 501R3(pBeloBAC11) was the same as that of 501R3 (Fig. 3). Of the 17 individual reduced-carbon components tested in the SRE medium, 5 clearly supported only diminished growth by strain C10 (Table 2). Growth on these compounds was increased in all cases when *degS* on pBRDS1.5 or pBEDS1.2 was introduced into C10 (data not shown). Wild-type growth by strain C10 on certain of these compounds indicates that the mutation in *degS* has a specific impact on the cell and does not result in a generalized growth deficiency.

degS is not important for tolerance to acidic conditions, plant-derived phenolic compounds, oxidative stress caused by H₂O₂, dessication, or high osmoticum. *degS* was not required for acid resistance by *Enterobacter cloacae*. When strains C10 and 501R3 at 7.98 ± 0.14 and 8.27 ± 0.08 log₁₀ CFU/ml, respectively, were applied to EG medium (pH 2.5), populations of these strains were reduced to 3.31 ± 0.90 and 4.22 ± 0.63 log₁₀ CFU/ml, respectively, after 15 min. Populations of both strains were below detectable limits (2.00 log₁₀ CFU/ml) at 30, 45, and 60 min.

degS was not required for general resistance to phenolic compounds or H₂O₂ by *E. cloacae*. In two experiments, *E. cloacae* 501R3 and C10 grew in LB broth containing 1.0, 2.0, 5.0, 10.0, and 20.0 mM acetosyringone. Both strains also grew in LB broth containing 1.0, 2.0, 5.0, or 10.0 mM acetovanillone whereas both failed to grow in the presence of 20.0 mM acetovanillone. Strain C10 was slightly more sensitive to 4-hydroxyacetophenone than strain 501R3. Strain C10 did not grow in the presence of

10.0 mM 4-hydroxyacetophenone whereas strain 501R3 did. Both strains failed to grow in LB broth containing 20.0 mM 4-hydroxyacetophenone. Both strains also grew in LB broth containing 1.0, 2.0, and 5.0 mM 4-hydroxyacetophenone. Sensitivity of both strains was similar for the reactive oxygen species H₂O₂. Zones of inhibition on nutrient agar plates with filter paper saturated with 1.5, 3.0, 6.0, and 12.0% H₂O₂ for 501R3 and C10 were 5.0 ± 0.8 and 7.0 ± 0.5 , 8.5 ± 0.5 and 8.5 ± 0.9 , 9.8 ± 0.8 and 8.8 ± 0.4 , and 10.3 ± 0.5 and 9.3 ± 0.8 mm, respectively, in the first experiment. In the second experiment zones of inhibition for 501R3 and C10 in the presence of 1.5, 3, and 6% H₂O₂ were 3.9 ± 0.2 and 4.3 ± 0.3 , 5.6 ± 0.2 and 5.4 ± 0.2 , and 7.0 ± 0.4 and 7.0 ± 0.44 mm, respectively.

degS was not required for dessication survival of *E. cloacae*. Survival of both strains 501R3 and C10 on filter paper incubated at 20 to 25% relative humidity was similar over a 120-h experiment (Table 6). Both strains had significant but similar declines in population relative to the nondessicated control. *degS* was also not required for osmotolerance by *E. cloacae*. Growth by strains 501R3 and C10 were similar on nutrient agar amended with 1.0, 2.0, and 3.0 M sorbitol; 1.0, 2.0, and 3.0 M glucose; 0.5, 1.0, and 1.5 M NaCl; and 0.5, 1.0, and 1.5 M KCl in two experiments.

DISCUSSION

DegS played an important role for *E. cloacae* while in the spermosphere and rhizosphere during colonization and during control of *P. ultimum* damping-off of cucumber. The DegS protein is of pivotal importance in the RpoE-mediated extracytoplasmic stress response pathway in enteric bacteria, functioning as a sensor of stress in the cell envelope (75,80). Plant-associated bacteria, such as *E. cloacae*, are subjected to stress in the spermosphere and rhizosphere resulting from reactive oxygen species, acid conditions, nonavailability of water, and changes in osmolarity (73,77). These stress factors interact with the cell envelope because it contains the outermost structures of the bacterium and is in direct contact with the environment. It appears that DegS does not function in sensing stress in the cell envelope caused by these extracytoplasmic factors while *E. cloacae* is in the spermosphere and rhizosphere. Strain C10, containing a mutation in *degS*, was not increased in sensitivity to acidic conditions, plant-derived phenolic compounds, oxidative stress caused by H₂O₂, dessication, or high osmoticum relative to the wild-type strain 501R3. It is possible that the Cpx or Bae two-component stress response mechanisms or another sensor for the RpoE-mediated stress response detect stress caused by these stress factors (55,68, 70,71). Rather, our data suggest that DegS played an important role during colonization and biocontrol of damping-off disease by sensing stress on *E. cloacae* resulting from the complex nutritional environments in the spermosphere and rhizosphere. Strain C10 was decreased in growth on SRE medium, a complex nutritional environment comprising all 37 reduced-carbon compounds detected in cucumber root exudate (36).

Fig. 3. In vitro growth of *Enterobacter cloacae* strains on 0.1% synthetic cucumber root exudate medium at 250 rpm and 35°C. Data points are the mean of three replicates with error bars representing one standard deviation from the mean. The experiment was conducted twice; results shown are from an individual experiment and representative of both experiments.

TABLE 6. Dessication survival of *Enterobacter cloacae* strains^z

Time (h)	Log ₁₀ CFU filter disk ⁻¹			
	Desiccated		Nondessicated	
	501R3	C10	501R3	C10
0	8.30 ± 0.03	8.42 ± 0.05	8.30 ± 0.03	8.42 ± 0.05
24	4.92 ± 0.44	4.73 ± 0.75	8.38 ± 0.16	8.34 ± 0.17
48	4.63 ± 0.21	4.42 ± 0.12	8.37 ± 0.06	8.35 ± 0.23
120	3.27 ± 0.95	2.97 ± 0.83	8.32 ± 0.16	8.06 ± 0.54

^z *E. cloacae* strains were incubated on sterile filter paper in a desiccation chamber at 20 to 25% relative humidity (desiccated) or in a desiccation chamber with moist paper towels (nondessicated). Populations were determined by dilution plating. Results are from two experiments combined prior to determination of mean log₁₀ CFU ± standard deviation.

It is interesting to hypothesize that the complex nutritional environment associated with the SRE medium caused extracytoplasmic stress in the cell envelope by forcing adaptation of the cell, through the expression of a number of nutrient uptake systems, to utilize the array of available reduced-carbon compounds. This expression of these nutrient uptake systems with protein components functioning in the periplasm and outer membrane resulted in increased protein traffic in the periplasm and, ultimately, extracytoplasmic stress. It has been shown that the adaptation of *Salmonella enterica* serovar Typhimurium to different nutritional environments where transport of the new nutrient involves periplasmic protein or outer membrane protein components resulted in increased RpoE expression (34). Also, artificial or inappropriate overproduction of certain outer-membrane proteins upregulated RpoE activity in *Escherichia coli* (1,80). The data presented here are generally consistent with this hypothesis. (i) With the exception of succinate, all of the compounds that supported slower growth by strain C10 are transported into the cell in *E. coli* K12 or *Enterobacter* sp. 638 by transport mechanisms that contain a periplasmic protein component (Table 2) (J. Lydon, D. K. Lakshman, and D. P. Roberts, *unpublished*). Relative growth rate results with succinate were inconsistent. In addition, there are hypothetical genes encoding putative extracytoplasmic receptor proteins for C₄ dicarboxylic compounds that may play a role in succinate uptake (29,34,53). (ii) All sugars taken into the cell via the sugar phosphotransferase system supported wild-type growth by C10. The sugar phosphotransferase system is composed solely of inner membrane and cytoplasmic protein components (20).

Not all reduced-carbon compounds taken into *E. coli* K12 by transport mechanisms involving periplasmic or outer-membrane protein components supported slower growth by strain C10. Citrate, maltose, and glutamine all supported wild-type growth rates by strain C10 (Table 2). It is possible that periplasmic proteins functioning in uptake of these compounds, if present in our strains of *Enterobacter cloacae*, lack the COOH amino acid sequences responsible for binding the DegS PDZ domain (80). In the case of citrate, FecB, the periplasmic component of the citrate transport system, was not detected in *Enterobacter* sp. 638 and may not be present in *E. cloacae* 501R3 (J. Lydon, D. K. Lakshman, and D. P. Roberts, *unpublished*).

The emerging picture is that the spermosphere and rhizosphere nutritional environments are highly competitive and biochemically complex, with nutrient availability being spatially and temporally heterogeneous on micro- and macroscales (7,11,28,31,35). Our working model for colonization by biocontrol bacteria is that, for them to be competitive and become spatially distributed throughout the rhizosphere, they must be capable of utilizing a large number of biochemically diverse reduced-carbon compounds for growth and also be capable of adapting to the nutritional profiles found in localized microenvironments so that they can not only utilize the reduced-carbon compounds that are available but also selectively use the reduced-carbon compounds that provide them the greatest competitive advantage over the indigenous microflora. Certain reduced-carbon compounds in the complex mixture of compounds released from seed and roots support faster growth rates, higher populations, and higher levels of metabolic activity by biocontrol bacteria when in association with subterranean plant parts (59,61,62). For example, hexose sugars had the greatest stimulatory activity on the enteric bacterium *E. cloacae* (59,61).

Potential with this model, studies have demonstrated the consistent of biocontrol bacteria, such as *E. cloacae* and certain pseudomonads, to grow on a large number of the vast array of reduced-carbon compounds detected in seed and root exudate (39,64,68). Work with isogenic mutants of *E. cloacae* and other bacterial biocontrol agents has demonstrated that diverse classes of reduced-carbon compounds in exudate are used, and important,

for growth in the spermosphere and rhizosphere (36,39,59,61,62,67). Additionally, evidence suggests that biocontrol pseudomonads adapt gene expression to nutritional conditions while in soil and in the rhizosphere (44,54,74), although the mechanisms by which these bacteria adapt have not been fully described.

Mechanisms have been elucidated that detail how bacteria adapt physiologically to utilize preferred reduced-carbon sources in vitro (46). For example, the sugar phosphotransferase system in enteric bacteria relies on cAMP and the catabolite-activating protein (CAP) for expression of pathways involved in utilization of nonpreferred reduced-carbon sources when the preferred, phosphotransferase sugars are available in insufficient quantities (20,52). There is evidence that this cAMP-CAP mechanism plays a role in colonization of plant rhizosphere by *E. cloacae* (65). However, mechanisms such as the sugar phosphotransferase system have been studied primarily in relatively simplistic media in batch culture. Additional mechanisms may be required for adaptation by bacteria to highly complex nutritional substrates such as those found in the spermosphere and rhizosphere. Work presented here demonstrating the involvement of the stress-response protein DegS in growth on complex media and during colonization of the nutritionally complex spermosphere and rhizosphere environments suggests that this is the case. It is interesting to speculate that DegS and the RpoE regulon function intimately in conjunction with mechanisms such as the sugar phosphotransferase system to allow adaptation of bacteria to growth on highly complex nutritional substrates.

We demonstrate here, for the first time, a role for *degS* in enabling plant-associated bacteria to cope with the complex nutritional environment found in plant spermosphere and rhizosphere. By extension, data reported here also suggest a role for the RpoE-mediated stress response in coping with this nutritional environment. DegS is not known to initiate stress response mechanisms in enteric bacteria other than the RpoE-mediated stress response. Although we have not yet demonstrated the direct interaction of DegS with the *rpoE* regulon, PCR and sequencing of PCR products indicate the presence of the *rpoE* regulon in *E. cloacae* 501R3 (H. Kong, J. Lydon, D. K. Lakshman, and D. P. Roberts, *unpublished*). Prior studies have investigated roles for *rpoE* (*algU* and *algT*) in plant associations by bacteria (33,43,73). However, these studies reported roles for *rpoE* in adaptation of pseudomonads to osmotic, heat, and oxidative stresses and in adaptation to dry soil conditions but not in adaptation to complex nutritional environments (33,73).

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