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Loss of glutamate dehydrogenase in *Ralstonia solanacearum* alters dehydrogenase activity, extracellular polysaccharide production and bacterial virulence

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ABSTRACT

Metabolism in *Ralstonia solanacearum*, which causes lethal wilt on Solanaceous plants, is poorly understood. In this study, we selected a Tn5-inserted mutant of *R. solanacearum* SL341 showing various phenotypic changes and altered virulence. When the *gdhA* gene encoding NAD(P)$^+$-dependent glutamate dehydrogenase was disrupted, the *gdhA* mutant of SL341 (SL341P2) was defective in red colony development on tetrazolium chloride-amended medium and showed less extracellular polysaccharide (EPS) production. The growth rate of the *gdhA* mutant on rich medium did not differ from that of the wild-type strain; however, its growth on minimal medium with glutamate as the sole carbon source was completely inhibited. SL341P2 was also defective in the oxidation of several carbon sources compared to the wild type. All the observed defects of SL341P2 *gdhA* mutant were fully or partially restored by providing the *gdhA* gene *in trans*. The *gdhA* mutant showed reduced virulence after soil-soaking inoculation of tomato plants, both on susceptible tomato cultivar Moneymaker and on the well-known bacterial-wilt-resistant cultivar Hawaii 7996. The delayed disease development by the *gdhA* mutant was due to slower multiplication of the mutant bacteria than wild type in tomato plants. Taken together, these results indicate that GdhA is required for diverse metabolic functions in *R. solanacearum*, including normal production of the virulence factor EPS, as well as normal bacterial growth *in planta* and full virulence on tomato plants.

Introduction

Ralstonia solanacearum is a formidable plant pathogen that causes lethal wilt on a broad range of crops, including more than 200 plant species from 50 botanical families with a global distribution. The important host crops of *R. solanacearum* include tomato, potato, peppers, tobacco, eggplant and banana [17]. The diversity of *R. solanacearum* strains has been described using various methods [18], and the pathogen has now been classified into four phylotypes based on geographic origin and molecular typing [9]. The nature of the disease symptoms and the wide host range of *R. solanacearum* strains have been investigated with the aim of increasing our understanding of bacterial virulence towards host plants. Genomes of several strains of *R. solanacearum* have been analyzed [29,32], and its virulence mechanisms have been investigated extensively [16]. For example, extracellular polysaccharide (EPS) and cell-wall degrading enzymes [8,31] are known to be major virulence factors.

When this pathogen infects plant roots, the bacteria move toward the plant xylem. While bacteria multiply in intercellular spaces and xylem which are nutrient poor environments [19], *R. solanacearum* adapts to the apoplastic environment to overcome the unfavorable conditions [6,11,12]. However, the overall process for *R. solanacearum* adaptation in the apoplastic environment remains unclear [14]. The metabolic capacity of *R. solanacearum* as a member of β-Proteobacteria should be investigated to understand both bacterial virulence and adaptation to the host environment.

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Previous studies showed that tryptophan [5] and methionine [28] are essential amino acids required for full virulence in *R. solanacearum*. A variety of organic substrates can be used by *R. solanacearum* [15]; however, the associated metabolic pathways have not been characterized thoroughly.

Here, we characterize a mutant of *R. solanacearum* carrying a transposon insertion in the *gdhA* gene. *gdhA* encodes glutamate dehydrogenase (GDH), which is found in all domains of life and catalyzes the reversible reaction between 2-oxoglutarate and glutamate [34]. In *R. solanacearum* GMI1000, L-Glutamate can be produced through two separate pathways, which involve the action of two enzymes. In addition to GDH, one pathway (GOGAT pathway) involves the GltB/D enzyme, which combines 2-oxoglutarate and L-glutamine to generate L-glutamate (Fig. 1). Another pathway associated with glutamate metabolism include the GlsA enzyme, which catalyzes the reversible reaction between glutamate and glutamine. Glutamine synthetase (GS pathway, encoded by *glnA1/A2*) catalyzes L-glutamate to form L-glutamine, which is the only pathway for glutamine biosynthesis. The presence of glutamate is a prerequisite for both GlsA and GS. The *gdhB* gene, a paralogue of *gdhA*, has not been identified in the GMI1000 genome; thus, *gdhA* is the only dehydrogenase gene that catalyzes the reversible oxidative deamination of 2-oxoglutarate to glutamate.

Based on our initial observation of the reduced EPS production of a *gdhA* mutant of *R. solanacearum*, we explored whether GDH activity and primary metabolism of glutamate are associated with bacterial virulence and metabolic adaption of *R. solanacearum* in host plant environments. Overall, our results suggest that *R. solanacearum* utilizes multiple nitrogen sources from the plant apoplast to support its full virulence.

Materials and methods

Bacterial strains and culture conditions

Bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were routinely grown at 37 °C with shaking at 200 rpm in Luria–Bertani (LB) broth containing appropriate antibiotics. The following antibiotic concentrations were used for *E. coli* strains: tetracycline, 15 μg ml^{-1}; kanamycin, 50 μg ml^{-1}; and ampicillin, 100 μg ml^{-1}. *R. solanacearum* strain SL341, a strain of race 1 (phylotype I) which is closely related to GMI1000 [20], and its mutant strains, were routinely grown at 28 °C on the following media, including CPG broth (0.1% casamino acid, 1% peptone and 0.5% glucose), TTC medium (CPG broth supplemented with 0.005% 2,3,5-triphenyltetrazolium chloride (TTC) and 1.5% agar), and MG medium (1% mannitol, 0.2% L-glutamic acid, 0.05% KH_2PO_4, 0.02% NaCl, 0.02% $MgSO_4$ and 1.5% agar, adjusted to pH 7.0). The following antibiotic concentrations were used in CPG and TTC media: tetracycline, 20 μg ml^{-1}; kanamycin, 50 μg ml^{-1}. The minimal medium M9 was used to investigate the growth of *R. solanacearum* strains, and YDC medium (1% yeast extract, 2% dextrose, 2% $CaCO_3$ and 1.5% agar) was used for triparental mating between *R. solanacearum* and *E. coli* strains.

Recombinant DNA techniques and transposon mutagenesis

Plasmid preparation, restriction endonuclease digestion, DNA ligation, agarose gel electrophoresis and other standard recombinant DNA technology were performed as described previously [33]. Total DNA of *R. solanacearum* wild-type strain SL341 and its mutants were prepared using a standard method with a bacterial genomic DNA purification kit (Elpis-Biotech, Daejeon, Korea).

Transposon mutagenesis of *R. solanacearum* SL341 was conducted to generate random Tn5 insertion mutants using the EZ-Tn5™<KAN-2>Tnp Transposome™ kit (Epicentre, USA), as per the manufacturer's instructions. The transposon-inserted mutants were selected on CPG agar supplemented with kanamycin, and all of the mutants were stored in 40% glycerol at −80 °C until phenotype screening.

Southern hybridization

To verify the single insertion of the transposon in selected mutants, Southern blot analysis of digested genomic DNA from *R. solanacearum* strains was performed using nylon membranes. The *gdhA* gene amplified from SL341 genomic DNA using Tn90-F1 (5′-CCAAAGGAAGAGGAAGACAG-3′) and Tn90-R1 (5′-CCGATCAGGGGTACAGACCG-3′) as primers and a kanamycin resistance cassette from Tn5 were used as probes. Probes were labeled with a digoxigenin-dUTP DNA-labeling Kit (Roche, Mannhein, Germany) and were detected with a chemiluminescent substrate, disodium 3-(4-methoxyspiro-{1,2-dioxetane-3,2′-(5′-chloro)tricycle[3.3.1.1^{3,7}]decan}-4-yl)phenyl phosphate (Roche), as per the manufacturer's instructions. Hybridization of the membrane with the probe was performed under high-stringency conditions at 68 °C.

Identification of Tn insertion sites and complementation

To identify the Tn5 insertion sites of selected mutants, extracted genomic DNA was directly used for DNA sequencing using transposon primers KAN-2 FP-1 (5′-ACCTACAACAAAGCTCTCATCAACC-3′) and KAN-2 RP-1 (5′-GCAATGTAACATCAGAGATTTTGAG-3′). In

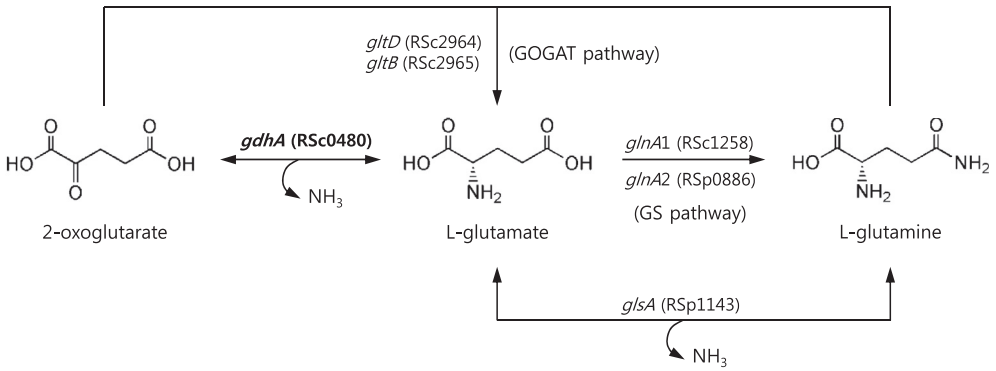

Fig. 1. The ammonium assimilatory pathways in *R. solanacearum*. *gdhA*, NAD(P)+-dependent glutamate dehydrogenase; *gltBD*, glutamate synthase protein (GOGAT); *glsA*, glutaminase; *glnA*, glutamine synthetase protein (GS).