

## Mqo, a Tricarboxylic Acid Cycle Enzyme, Is Required for Virulence of *Pseudomonas syringae* pv. tomato Strain DC3000 on *Arabidopsis thaliana*<sup>∇†</sup>

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**Plant pathogenic bacteria, such as *Pseudomonas syringae* pv. tomato strain DC3000, the causative agent of tomato bacterial speck disease, grow to high levels in the apoplastic space between plant cells. Colonization of plant tissue requires expression of virulence factors that modify the apoplast to make it more suitable for pathogen growth or facilitate adaptation of the bacteria to the apoplastic environment. To identify new virulence factors involved in these processes, DC3000 Tn5 transposon insertion mutants with reduced virulence on *Arabidopsis thaliana* were identified. In one of these mutants, the Tn5 insertion disrupted the malate:quinone oxidoreductase gene (*mgo*), which encodes an enzyme of the tricarboxylic acid cycle. *mgo* mutants do not grow to wild-type levels in plant tissue at early time points during infection. Further, plants infected with *mgo* mutants develop significantly reduced disease symptoms, even when the growth of the *mgo* mutant reaches wild-type levels at late stages of infection. Mutants lacking *mgo* function grow more slowly in culture than wild-type bacteria when dicarboxylates are the only available carbon source. To explore whether dicarboxylates are important for growth of DC3000 in the apoplast, we disrupted the *dctA1* dicarboxylate transporter gene. DC3000 mutants lacking *dctA1* do not grow to wild-type levels in planta, indicating that transport and utilization of dicarboxylates are important for virulence of DC3000. Thus, *mgo* may be required by DC3000 to meet nutritional requirements in the apoplast and may provide insight into the mechanisms underlying the important, but poorly understood process of adaptation to the host environment.**

One important aspect of interactions between plant pathogens and their hosts is the ability of the pathogen to obtain nutrients within the plant tissue. Nutrient acquisition is essential for growth within the host, since both cell division and DNA replication can be influenced by nutrient availability. Bacterial plant pathogens differ in the strategies they use to get necessary nutrients during infection. Some pathogens, such as *Agrobacterium tumefaciens*, elicit production of specific carbon and nitrogen sources by the plant (1). Other pathogens may rely on metabolites that are readily available in the plant apoplast or may stimulate the release of water or nutrients from surrounding plant cells (27).

Little is known about how pathogenic *Pseudomonas syringae* strains acquire nutrients when growing in their hosts. *P. syringae* strains are gram-negative gammaproteobacteria, which as a group cause disease on many agriculturally important plants. For example, *P. syringae* pv. tomato strain DC3000 causes disease on tomato, *A. thaliana*, and several agriculturally important *Brassicaceae*, such as turnip, mustard, collard, and cauliflower (8, 51). Initially, DC3000 colonizes plant surfaces and then enters the plant tissue through natural openings (such as

stomata) or wounds (34, 38). DC3000 then establishes itself in the plant apoplast, the intercellular space between plant cells (38). Once in the apoplast of susceptible hosts, DC3000 multiplies to high levels, and the infected plants develop disease symptoms, including chlorosis (yellowing) of the leaf tissue and necrotic spots or patches called lesions (38, 49). Pseudomonads, such as *P. aeruginosa* and *P. fluorescens*, preferentially utilize tricarboxylic acid (TCA) cycle intermediates (20, 29, 33, 44), and DC3000 utilizes these carbon sources in culture (19). Some studies to investigate nutrient acquisition of DC3000 have been carried out (3, 7, 40); however, it is not clear what carbon sources DC3000 utilizes when growing in plant tissue.

Several virulence factors are necessary for DC3000 to enter, grow inside the plant, and cause disease. Like many other bacterial pathogens, DC3000 uses a type III secretion system (TTSS) (15), which is encoded by the *hrp/hrc* genes, to inject effector proteins into plant cells (16, 27). Many of these effectors suppress host defenses, and it is likely that some may be involved in modulating the apoplastic environment or nutrient acquisition (16). DC3000 also produces the phytotoxin coronatine, which promotes entry of the bacteria into the plant apoplast by stimulating the opening of stomata (34) and is required for bacterial growth in the apoplast by suppressing salicylic acid (SA)-dependent host defenses (4, 45). Coronatine also promotes disease symptom development via an SA-independent mechanism (4). While much emphasis has been placed on exploring how type III-secreted effectors and coronatine promote DC3000 virulence, other factors are also likely to be important during pathogenesis.

To identify additional factors involved in pathogenesis, we

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TABLE 1. Bacterial strains and vectors used in this study

Strain or plasmid	Characteristics <sup>a</sup>	Source or reference
<b>Strains</b>		
<i>P. syringae</i>		
DC3000	Derivative of NCPPB1106; Rif <sup>r</sup>	8
AK4C9	<i>mgo</i> ::Tn5 <i>uidA</i> ; Rif <sup>r</sup> Km <sup>r</sup>	This study
<i>mgo</i> ::KO	<i>mgo</i> gene replaced with Sm/Spec <sup>r</sup> gene; Rif <sup>r</sup> Spec <sup>r</sup>	This study
DC3000 pMQO	DC3000 with pMQO clone; Rif <sup>r</sup> Tet <sup>r</sup>	This study
DC3000 pME6031	DC3000 with pME6031; Rif <sup>r</sup> Tet <sup>r</sup>	This study
<i>mgo</i> ::KO pMQO	<i>mgo</i> ::KO with pMQO clone; Rif <sup>r</sup> Spec <sup>r</sup> Tet <sup>r</sup>	This study
<i>mgo</i> ::KO pME6031	<i>mgo</i> ::KO with pME6031; Rif <sup>r</sup> Spec <sup>r</sup> Tet <sup>r</sup>	This study
<i>dctA1</i> ::KO	<i>dctA1</i> gene replaced with Sm/Spec <sup>r</sup> gene; Rif <sup>r</sup> Spec <sup>r</sup>	This study
<i>dctA2</i> ::pJP5603	<i>dctA2</i> ::disrupted with pJP5603; Rif <sup>r</sup> , Km <sup>r</sup>	This study
<i>dctA1</i> ::KO <i>dctA2</i> ::pJP5603	<i>dctA1</i> ::KO; <i>dctA2</i> ::pJP5603; Rif <sup>r</sup> Spec <sup>r</sup> Km <sup>r</sup>	This study
<i>E. coli</i>		
DH5α <i>λpir</i>	<i>recA</i> , <i>lacZ</i> ΔM15, <i>λpir</i>	35
MM294A (pRK600)	Triparental mating helper strain; Cm <sup>r</sup>	12
<b>Plasmids</b>		
pHP45Ω	Source of Ω cassette; Sm/Spec <sup>r</sup> gene	37a
pRK415	Broad-host-range vector; Tet <sup>r</sup>	21a
pJP5603	Suicide vector; Km <sup>r</sup>	36a
<i>pmgo</i> -pRK415	Construct used to make <i>mgo</i> ::KO; Tet <sup>r</sup>	This study
<i>pmgo</i> ::Spec-pRK415	Construct used to make <i>mgo</i> ::KO; Spec <sup>r</sup> Tet <sup>r</sup>	This study
<i>pmgo</i> ::KO	Construct used to make <i>mgo</i> ::KO strain; Spec <sup>r</sup> Km <sup>r</sup>	This study
<i>pdctA1</i> up-pJP5603	Construct used to make <i>dctA1</i> ::KO plasmid; Tet <sup>r</sup>	This study
<i>pdctA1</i> down-TOPO	Construct used to make <i>dctA1</i> ::KO plasmid; Km <sup>r</sup>	This study
<i>pdctA1</i> ::KO	Construct used to make <i>dctA1</i> ::KO strain; Spec <sup>r</sup> Tet <sup>r</sup>	This study
<i>pdctA2</i> ::pJP5603	Construct used to make <i>dctA2</i> ::pJP5603 strain; Km <sup>r</sup>	This study
pCR-Blunt II TOPO	Cloning vector; Km <sup>r</sup>	Invitrogen
pME6031	Replicating vector, stable in <i>P. syringae</i> ; Tet <sup>r</sup>	18
pMQO	Wild-type <i>mgo</i> gene in pME6031; Tet <sup>r</sup>	This study
pDctA1	Wild-type <i>dctA1</i> gene in pME6031; Tet <sup>r</sup>	This study

<sup>a</sup> Cm<sup>r</sup>, chloramphenicol resistance; Tc<sup>r</sup>, tetracycline resistance; Rif<sup>r</sup>, rifampin resistance; Spec<sup>r</sup>, spectinomycin resistance; Km<sup>r</sup>, kanamycin resistance.

undertook a genetic screen to identify novel virulence factors (5, 24). DC3000 mutants with reduced virulence were identified by assaying for their ability to elicit disease symptoms on *A. thaliana* and tomato plants (24, 37). One of these mutants, AK4C9, had reduced virulence on both hosts. The gene disrupted in this mutant is the malate:quinone oxidoreductase gene (*mgo*), which encodes an enzyme of the TCA cycle. *mgo* mutants grow more slowly than wild-type DC3000 in planta and in culture when dicarboxylates are the only carbon source, suggesting that dicarboxylates are important for the growth of DC3000 in the apoplast. In the present study, we explore the role of Mqo and a dicarboxylate transporter, DctA1, in DC3000 pathogenesis.

#### MATERIALS AND METHODS

**Plant growth and infections.** *A. thaliana* ecotype Columbia plants were grown from seed in a growth chamber with 8 h of light at 22°C and 75% relative humidity. For dip-inoculation infections, 3.5-4-week-old plants were dipped in a solution of bacteria with an optical density at 600 nm (OD<sub>600</sub>) between 0.25 and 0.4, containing 10 mM MgCl<sub>2</sub> and 0.02% Silwet (26). The plants were covered with a clear plastic dome for 24 h after the dip treatment. For day 0, the leaves were removed 2 h after inoculation. Leaves collected on day 0 were surfaced sterilized with a solution of 15% hydrogen peroxide for 5 min and then washed three times in sterile distilled water, prior to manual grinding of tissue in 10 mM MgCl<sub>2</sub>. For *dctA1*::KO infections, surface sterilization was also included on day 1 where indicated. A similar protocol was used to dip inoculate tomato plants.

For *mgo*::KO infections on *sid2* mutant plants, *sid2-2* (50) plants were used. Hypersensitive response (HR) assays were performed as described previously (49), where a range of bacterial concentrations (5 × 10<sup>6</sup>, 1 × 10<sup>7</sup>, 5 × 10<sup>7</sup>, and 1 × 10<sup>8</sup> CFU/ml) resuspended in 10 mM MgCl<sub>2</sub> were used to syringe infiltrate tobacco leaves (*Nicotiana tabacum* cv. Xanthi N/N). Leaves were scored for

macroscopic tissue collapse, a finding indicative of an HR, at 18 h after inoculation.

**Bacterial strains and vectors.** The bacterial strains and plasmids used in the present study are shown in Table 1. *P. syringae* strains were routinely grown at 28 to 30°C in King's broth (KB) medium (23) unless otherwise described. *Escherichia coli* strains were grown on Luria-Bertani (LB) medium (42) at 37°C. Antibiotics were used at the following concentrations (in μg/ml) for *P. syringae* strains: rifampin, 100; kanamycin, 25; spectinomycin, 100; and tetracycline, 16. Antibiotics were used at the following concentrations for *E. coli* strains: kanamycin, 25 μg/ml; spectinomycin, 80 μg/ml; chloramphenicol, 20 μg/ml; and tetracycline, 10 μg/ml. Plasmids were transferred from *E. coli* DH5α (containing *λpir*) (35) to *P. syringae* strains by triparental mating (11) with the helper strain MM294A(pRK600) (12).

**DNA manipulation and sequencing.** Plasmid DNA was isolated by using a Promega Wizard miniprep kit, and bacterial genomic DNA was isolated by using the genomic DNA purification kit (Promega, Madison, WI). Sequencing reactions were performed by using ABI BigDye v3.1, with 4 μl of BigDye mix used in a 12-μl volume reaction (Advanced Biosystems, Inc., Foster City, CA). A total of 150 to 300 ng of template was used for each sequencing reaction. Automated sequencing was performed on an ABI sequencer at the Washington University Biology Department sequencing facility (Washington University, St. Louis, MO). For semiquantitative PCR assays of gene expression, DC3000 RNA was isolated from cultures grown in KB or minimal medium by using the Qiagen RNeasy minikit (Qiagen, Hilden, Germany). cDNA was synthesized by using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA).

To identify the location of the Tn5 insertion in the AK4C9 mutant, the genomic fragment containing the Tn5 was identified by digesting total AK4C9 genomic DNA with PstI, followed by ligation of these fragments into the cloning vector pBluescript KS(+) cut with PstI. The resulting plasmids were introduced into *E. coli*, and the transformants were selected for kanamycin resistance (Kan<sup>r</sup>) provided by the *aph* gene on the Tn5 (24). An ~4-kb genomic DNA fragment was identified that contained the Tn5 transposon and was sequenced to identify the location of the Tn5 transposon.

To make the *mgo*::KO strain, 2 kb of genomic sequence flanking each side of

the *mgo* coding sequence was amplified with the following primers (restriction enzyme sites are underlined): *mgo3EcoRI*, 5'-ATA GGA ATT CGG CGA TAC CAA ACG CTA CGG-3'; *mgo3KpnI*, 5'-TCA GGA TCG TGG TAC CGG TCT TCG CGC AGT TCA TCC-3'; *mgo5KpnI*, 5'-CGC GAA GAC CGG TAC CAC GAT CCT GAG GCT GGA TGC-3'; and *mgo5XbaI*, 5'-TAT GTC TAG AGG AGT TGA TCG ACG GCT TGG-3'. The resulting PCR products (which contained 26 nucleotides of overlapping sequence allowing them to anneal to each other) were mixed together and amplified 10 times, using the *mgo3EcoRI* and *mgo5XbaI* primers, to produce a 4-kb fragment containing both flanking regions without the *mgo* coding sequence in between. The PCR product was digested with *EcoRI* and *XbaI* and ligated into the pRK415 vector, also digested with *EcoRI* and *XbaI*, to generate *pmgo*-pRK415 (Table 1). The spectinomycin resistance (*Spec<sup>r</sup>*) cassette from the omega fragment was amplified from pHP45Ω with the primers *Spec1f* (5'-GTC CGG TAC CCC AAG CTC TCG GGT AAC ATC-3') and *Spec1r* (5'-GTC CGG TAC CTT CGC CAA CTA TTG CGA TAA C-3') containing the *KpnI* recognition site (underlined) on the 5' end of each primer and inserted in between the *mgo* flanking DNA sequences to generate *pmgo::Spec*-pRK415 (Table 1). The fragment containing the *mgo* flanking sequence and *Spec<sup>r</sup>* gene was removed from pRK415 by digestion with *EcoRI* and *XbaI* and inserted into the suicide vector pJP5603 cut with *EcoRI* and *XbaI*, to generate *pmgo::KO* (Table 1). This plasmid was introduced into DC3000 by triparental mating using the helper strain MM294A (Table 1). DC3000 transconjugates were screened for *Spec<sup>r</sup>* and kanamycin sensitive (*Kan<sup>s</sup>*), and gene-specific replacement of the *mgo* gene with the *Spec<sup>r</sup>* gene was verified by PCR.

To construct the *mgo* complementing clone, pMQO, the following primers were used to amplify the *mgo* coding sequence and promoter from genomic DNA: *Mqosmallreverse*, 5'-ATA CCT CGA GTC ATC GCT ATA ATC TTC CCC CGA TT-3' (*XhoI* site underlined); and *mgo*+*promf1*, 5'-ATA TGA ATT CAG CTC TGA CGG CAG CAA CCA AAA AG-3' (*EcoRI* site underlined). The resulting 2.3-kb PCR product was digested with *EcoRI* and *XhoI* and cloned into the shuttle vector pME6031 (18) cut with the same enzymes. The clone was sequenced and found to have two nucleotide changes, one in the coding sequence but not predicted to change the amino acid sequence, and one T-to-C change 138 bp upstream of the *mgo* start codon. Because these changes would most likely not affect Mgo function, this clone was used in complementation tests and was demonstrated to confer wild-type Mgo activity (see Results).

The *dctA1::KO* strain was made using a strategy similar to that used to generate the *mgo::KO* mutant. A 1-kb region directly upstream of the *dctA1* gene and a 1-kb region directly downstream of *dctA1* were cloned into the pJP5603 suicide vector. These regions were amplified by using the following primers: *dctA1upF*, 5'-TCA TAA AGC TTG CGA CAG CTT GAG TGA AGA GCA T-3'; *dctA1upR*, 5'-ATT GGA TCC GTC GTC GGC ATC TTC ACC TGA T-3' (with the *HindIII* and *BamHI* restriction sites, respectively, underlined); *dctA1downF*, 5'-TAT GGA TCC TCT GAT AGC TTC AGG CCA TGA AAA A-3'; and *dctA1downR*, 5'-TAA GAA TTC GTA CTC CAC ACC CAG CTC TTT CAG A-3' (with the *BamHI* and *EcoRI* restriction sites, respectively, underlined). The *Spec<sup>r</sup>* cassette from the omega fragment was cloned in between these two fragments. The resulting plasmid, *pdctA1::KO*, was introduced into DC3000 by triparental mating as described above. DC3000 transconjugates were screened for *Spec<sup>r</sup>* and *Kan<sup>s</sup>*, and gene-specific replacement of the *dctA1* gene with the *Spec<sup>r</sup>* gene was verified by PCR.

The *dctA1* complementing clone (pDctA1, Table 1) was constructed similar to the way the *mgo* complementing clone was made. The following primers were used to amplify the *dctA1* coding sequence and promoter from genomic DNA: *dctA1F*, 5'-GAA AGC TAT GGC GCA CAA CTG AC-3'; and *dctA1R*, 5'-CTG ATG TTC AGC GGC GAG AC-3'. The resulting 1.9-kb PCR product was cloned into the pCR-Blunt II TOPO vector and then subcloned into the shuttle vector pME6031 by using *HindIII* and *XhoI* restriction enzymes (18). The clone was sequenced and found to have no nucleotide changes.

To generate *pdctA2::pJP5603*, a 667-bp fragment internal to the *dctA2* coding sequence was amplified with the primers *DctA2KOF* (5'-AAA ACT TCC AGG TCC CGC TGG TAC G-3') and *DctA2KOR* (5'-CGA GTG ACA GCA ACG TAC CAA TTC C-3') and cloned into the pCR-Blunt II TOPO vector. The PCR product was digested out of the TOPO vector with *BamHI* and *XbaI* and ligated into pJP5603 cut with the same enzymes. The resulting plasmid, *pdctA2::pJP5603*, was introduced into DC3000 by triparental mating using the helper strain MM294A (Table 1) to make strain *dctA2::pJP5603*. DC3000 transconjugants were screened for *Kan<sup>s</sup>*, and insertional disruption of the *dctA2* gene with pJP5603 was verified by PCR. To make the *dctA1::KO* *dctA2::pJP5603* double mutant strain, *pdctA2::pJP5603* was introduced into *dctA1::KO* by triparental mating.

**Bioinformatics, nucleotide sequences, and accession numbers.** BLASTP searches were performed with the National Center for Biotechnology Information (NCBI) servers (version 2.2.18) to search nonredundant databases and DC3000 specific sequences (2). The NCBI BLAST 2 sequences program (version 2.2.18) was used for BLASTP comparisons of amino acid sequences. DC3000 sequence information was obtained from *Pseudomonas*-Plant Interaction (PPI; <http://pseudomonas-syringae.org> [31]) and The J. Craig Venter Institute comprehensive microbial resource (<http://cmr.jcvi.org/cgi-bin/CMR/CmrHomePage.cgi>). The accession numbers for the following proteins were as follows: *Pseudomonas syringae* pv. tomato strain DC3000 Mgo, AAO54665; *P. aeruginosa* PAO1 MgoA, AAG06840; *P. aeruginosa* PAO1 MgoB, AAG08028; *P. citronellolis* Mgo, AAW88350; *Sinorhizobium meliloti* Mdh, AAG41996; *P. syringae* pv. tomato strain DC3000, ME, AAO57390; *E. coli* strain K-12 substrain MG1655 ME, NP\_415996; *S. meliloti* DctA, P20672; *P. syringae* pv. tomato strain DC3000 DctA1, AAO55202; and *P. syringae* pv. tomato strain DC3000 DctA2, AAO57520.

**Growth of DC3000 in minimal medium cultures.** To assess the ability of the *mgo::KO* mutant to grow on different carbon sources, mutant and wild-type DC3000 strains were grown first in KB medium with rifampin in overnight cultures. Cells were precipitated from 3 ml of each overnight culture by centrifugation and washed once with 10 mM MgCl<sub>2</sub>, and then approximately 8 × 10<sup>7</sup> bacteria were resuspended in 5 ml of minimal medium with an appropriate carbon source. For each time point, 100 μl of each culture was removed and added to a microtiter well. The OD<sub>600</sub> was measured by using a SPECTRAMax Plus spectrophotometer (Molecular Devices, Union City, CA).

HS minimal medium was prepared as described previously (43), with appropriate carbon sources added in place of sucrose. D,L-Malate (Sigma); citric acid, trisodium, and dihydrate (Amresco, Solon, OH); succinic acid (Sigma); D-sucrose (Fisher Scientific, Pittsburgh, PA); and sodium acetate (Fisher Scientific, Pittsburgh, PA) were added to the prepared Hoitink-Sinden (HS) medium to a final concentration of 10 mM, and the pH was adjusted to 6.5. The medium was then filter sterilized and used for growth assays.

## RESULTS

**DC3000 Mgo is required for wild-type growth and disease symptom production in *A. thaliana*.** To identify novel virulence factors that promote DC3000 pathogenesis, a Tn5 transposon mutagenesis screen was undertaken (5, 24). One of the DC3000 mutants identified, AK4C9, had reduced virulence on *A. thaliana* plants. Growth of AK4C9 in planta was reduced compared to the wild type (Fig. 1a). Plants inoculated with AK4C9 developed significantly fewer disease lesions and very little chlorosis compared to plants infected with wild-type DC3000 (data not shown). AK4C9 also had reduced virulence on tomato plants (data not shown). The AK4C9 mutant elicited a normal HR on tobacco, indicating that type III secretion is not impaired in this mutant (data not shown).

The Tn5 transposon insertion in AK4C9 was mapped to a region of the DC3000 genome ~200 bp upstream of the PSPTO\_1136 coding region, which is predicted to encode malate:quinone oxidoreductase (Mgo; Fig. 2). Mgo is an enzyme that can function as part of the TCA cycle, converting malate to oxaloacetate. DC3000 Mgo exhibits 77% amino acid identity and 86% similarity to *P. aeruginosa* MgoB. A presumptive FAD binding site and a βαβ fold ADP-binding motif, found in other Mgos (see Fig. S1 in the supplemental material) (25, 36), are present in the amino-terminal region of the DC3000 Mgo protein. Another enzyme capable of catalyzing this reaction is malate dehydrogenase (Mdh). Bacterial species differ on whether they have both a Mgo and Mdh, or just one of the two enzymes. For instance, *E. coli* has both an Mdh and an Mgo enzyme (46), while *P. aeruginosa* has two Mgos, MgoA, and MgoB, but no Mdh (13, 25). *P. syringae* pv. *syringae* B728a and *P. syringae* pv. *phaseolicola* 1448A also appear to have two Mgos, in contrast to the single Mgo in DC3000.

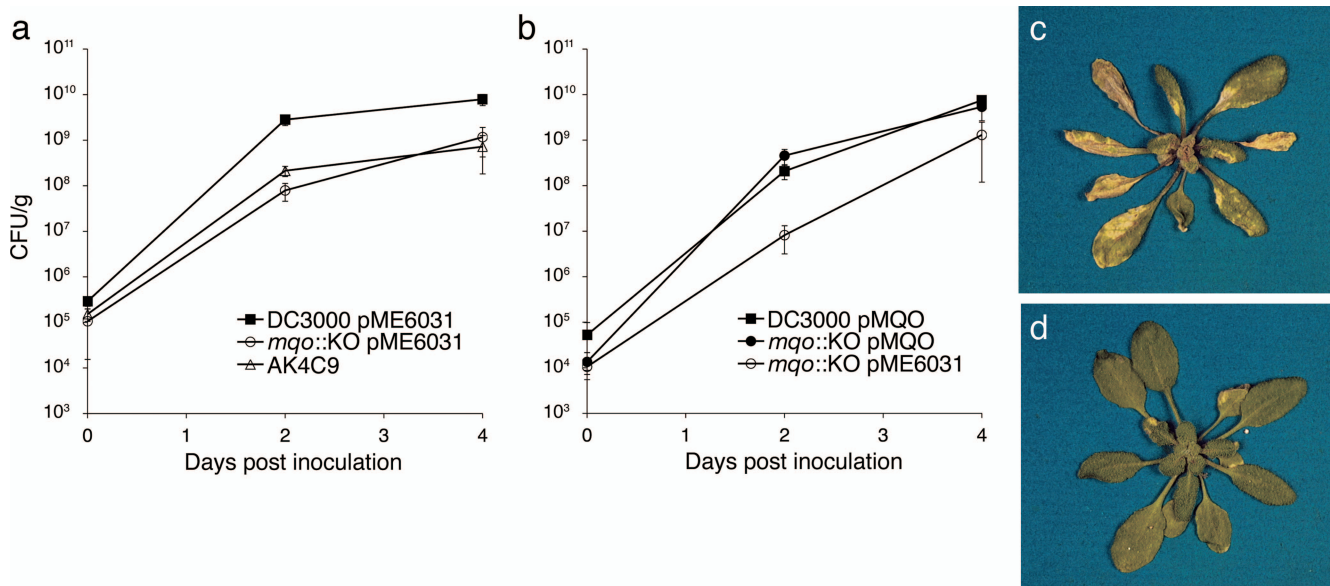


FIG. 1. In planta growth and disease symptom phenotypes of DC3000 *mgo* mutants. *mgo* is required for normal growth and disease symptom production by *P. syringae* strain DC3000 on *A. thaliana*. (a) Growth of wild-type, *mgo*::KO, and AK4C9 bacteria (in CFU/gram of leaf tissue) on *A. thaliana* plants. DC3000 and *mgo*::KO strains contained the empty vector pME6031. Four samples were used for each time point. (b) Growth of wild-type and the *mgo*::KO mutant carrying the pMQO complementing clone, and growth of *mgo*::KO with the empty vector control, pME6031. Three samples were used for each time point. (c and d) Disease symptoms of a plant dip inoculated with wild-type bacteria (c) and a plant infected with the *mgo*::KO mutant (d) at 5 days after inoculation. Note that in panel a the *mgo*::KO and AK4C9 strains did not reach wild-type levels of growth on day 4, but in panel b the *mgo*::KO strain approached wild-type levels of growth on day 4. Error bars represent the standard error of the mean. Similar results were obtained for at least three additional independent experiments.

Bioinformatic searches failed to identify a *mdh* gene in DC3000, suggesting that the single Mqo in DC3000 may be the primary enzyme responsible for conversion of malate to oxaloacetate.

To confirm that the Tn5 transposon insertion in the presumptive *mgo* promoter region was responsible for the reduced virulence phenotype of the AK4C9 mutant, we created

a gene replacement knockout strain (*mgo*::KO) of DC3000, in which the coding sequence of *mgo* was replaced by a *Spec<sup>r</sup>* gene (Table 1 and Fig. 2). Replacement of the *mgo* gene was unlikely to cause any polar effects on adjacent genes, since *mgo* does not appear to be part of an operon. The *mgo*::KO strain had the same reduced virulence phenotype observed for the AK4C9 mutant (Fig. 1). We also observed that in some exper-

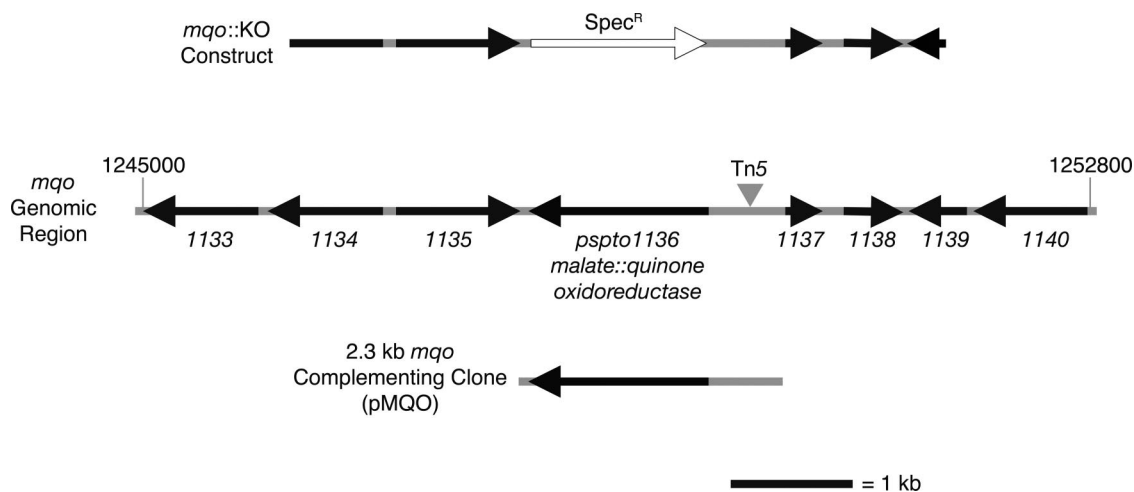


FIG. 2. Region of DC3000 genome containing the *mgo* gene. A map of the *P. syringae* pv. strain DC3000 genomic region containing the *mgo* coding sequence is shown. The italicized numbers (e.g., 1133) are abbreviations for the PSPTO-number for each gene. The Tn5 insertion location upstream of the *mgo* coding sequence is indicated. The *mgo*::KO construct shows the regions of the DC3000 genome flanking the *mgo* coding sequence that were cloned on either side of the *Spec<sup>r</sup>* gene into pJP5603. The genomic DNA included in the 2.3-kb *mgo* complementing clone (pMQO) is also shown. A size bar indicates the scale of the figure, and coordinates indicate the genomic position of the region in nucleotide bases (1245000 to 1252800).

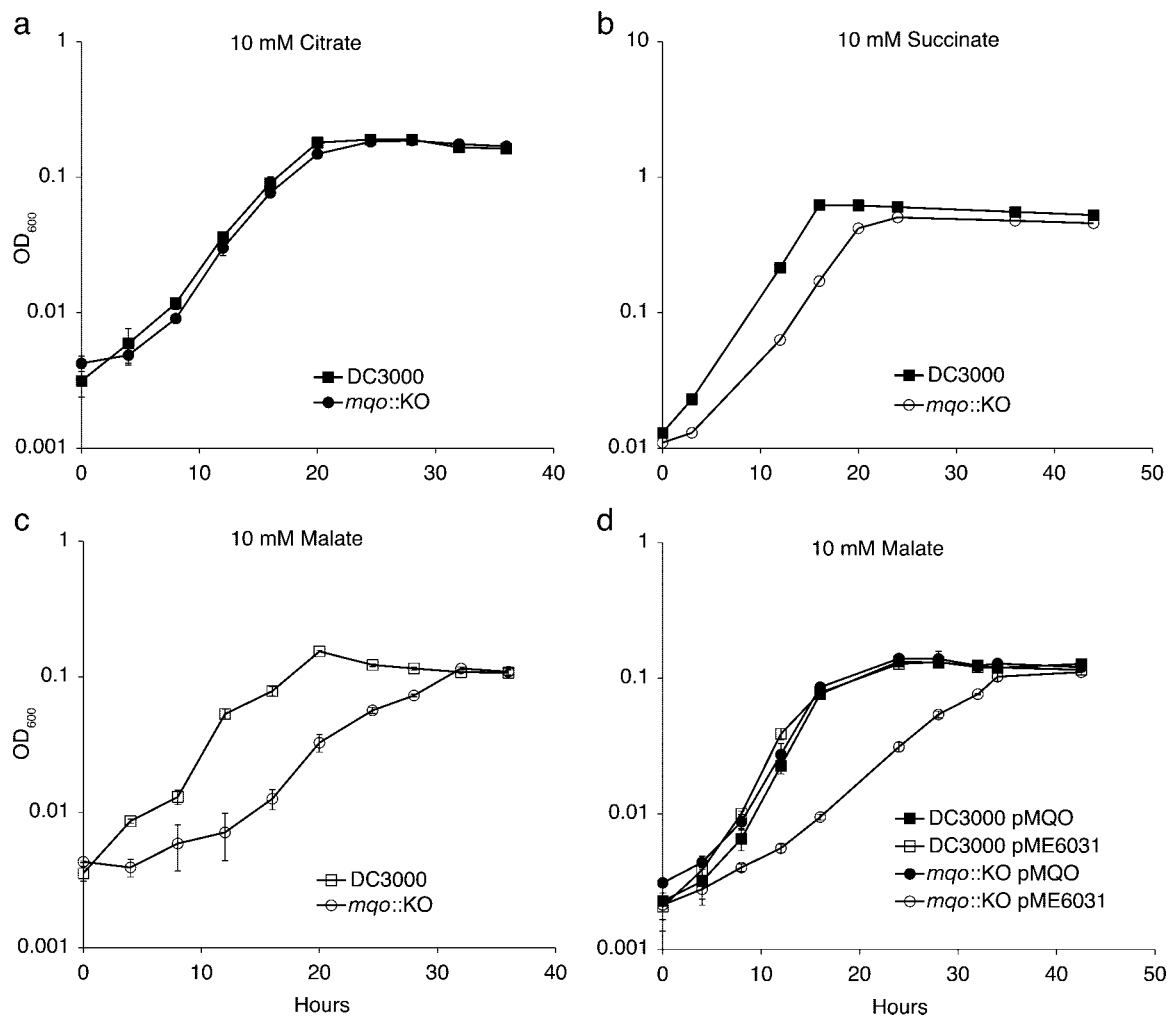


FIG. 3. Growth of wild-type DC3000 and *mgo::KO* in culture. Wild-type DC3000 and the *mgo::KO* mutant were grown in HS media with a 10 mM concentration of the indicated carbon source: citrate (a), succinate (b), and malate (c and d). Similar results were obtained for at least two additional independent experiments. In panels a, c, and d, three replicate cultures were used for each growth curve, and the error bars represent the standard deviation of the mean. In panel b, each time point represents a single sample.

iments *mgo::KO* reached wild-type levels of growth by 4 days postinoculation (Fig. 1b). Interestingly, disease symptoms never approached the severity of those caused by wild-type DC3000, even in experiments in which the *mgo::KO* bacteria grew to the same level as wild-type (Fig. 1c and d). Virulence of the *mgo::KO* mutant could be restored to wild-type levels, both at the level of in planta growth (Fig. 1b) and disease symptom production (data not shown) by introduction of a complementing clone containing the *mgo* coding region and upstream regulatory sequences (pMQO; Fig. 2), indicating that the *mgo* gene is required for DC3000 virulence.

To examine the possibility that reduced virulence of the *mgo::KO* mutant could be due to a defect in suppression or evasion of SA-dependent defenses in the host, we inoculated SA-deficient *sid2-2* (49) plants with the *mgo::KO* mutant. Growth of the *mgo::KO* mutant was not restored to wild-type levels 48 h after inoculation onto *sid2-2* plants, indicating that *mgo* is not involved in suppression of SA-dependent defenses.

Thus, it is likely that the reduced virulence of the *mgo* mutant is due to a defect in some other aspect of pathogenesis.

**Mgo is required for wild-type growth in media containing dicarboxylates.** Because the Mgo enzyme is a component of the TCA cycle, it is possible that the *mgo* mutants are impaired for growth on specific carbon sources. To investigate this, we assayed the growth of the *mgo::KO* mutant in culture using various carbon sources. Growth of wild-type DC3000 and the *mgo::KO* mutant was indistinguishable in rich media (KB; data not shown). Several types of minimal media have been developed for studies on plant-pathogenic bacteria in an effort to develop culture conditions that are similar to those encountered in the plant apoplast. We chose to work with the defined minimal HS medium, since both coronatine biosynthesis genes and *hrp/hrc* pathogenesis genes are induced in this minimal medium (43). The *mgo::KO* strain was indistinguishable from wild-type bacteria when grown in HS minimal medium supplemented with either sucrose, citrate, or oxaloacetate as the only

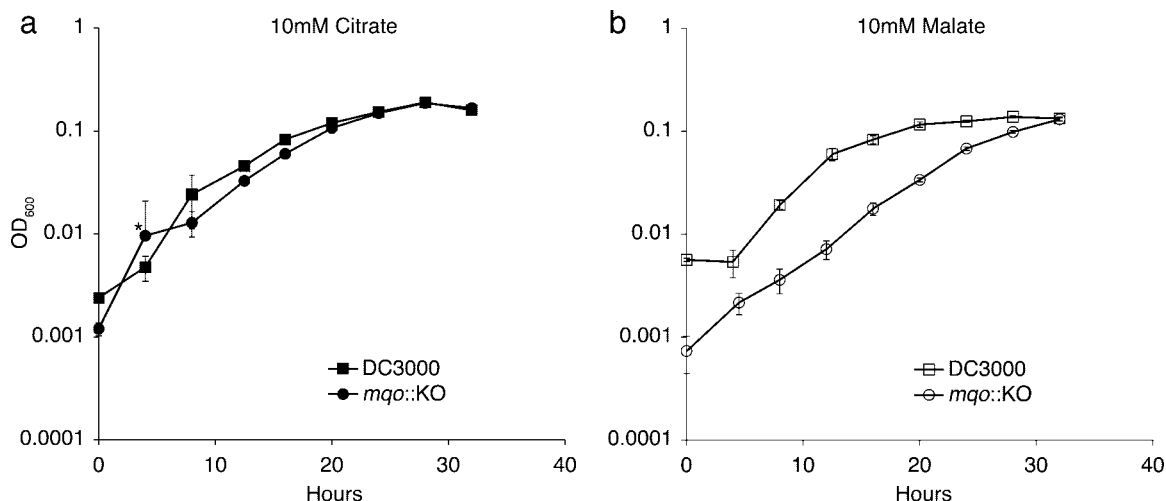


FIG. 4. Growth of wild-type DC3000 and the *mgo::KO* mutant after subculturing. Wild-type DC3000 and the *mgo::KO* mutant were grown in HS media with a 10 mM concentration of citrate or malate, and at mid-log (OD<sub>600</sub> of 0.07 to 0.09) approximately  $8 \times 10^7$  cells from each culture were used to inoculate a fresh (a) citrate or (b) malate culture. The graphs show the growth of the strains after subculturing. Three replicate cultures were used for each growth curve, and the error bars represent standard deviation of the mean. An asterisk at the 4-h data point for the *mgo::KO* growing in citrate indicates that the error bar below the mean for this time point is not shown (standard deviation = 0.011).

carbon source (Fig. 3a, data not shown). Thus, *mgo::KO* does not have a general growth defect in rich medium or in minimal medium with sucrose, citrate, or oxaloacetate. However, when grown in minimal medium containing malate or succinate as the only carbon source, *mgo::KO* grew at a slower rate than did wild-type bacteria (Fig. 3b, c, and d). The slower growth rate was more pronounced on malate than on succinate. When cultured in minimal medium with malate as the sole carbon source, wild-type bacteria grew with an approximate doubling time of 3.5 h, while the *mgo::KO* strain grew with a doubling time of ~5.5 h. The slow-growth phenotype of the *mgo::KO* mutant in malate was restored to wild-type levels by introduction of the pMQO complementing clone (Fig. 3d). The growth defect of the mutant in culture was similar to what we observed in planta, in that there was slower growth than with the wild type initially but that the number of bacteria eventually reached wild-type levels in many experiments.

For the pseudomonads *P. aeruginosa* and *P. citronellolis* the

Mqo enzyme is required for the glyoxylate pathway, for utilization of ethanol, acetate, or linear terpenes as a carbon source (13, 25). In contrast, we found that DC3000 did not grow in culture when acetate was the only available carbon source (data not shown), indicating that the glyoxylate pathway is not likely to be an important carbon utilization pathway for DC3000.

The *mgo::KO* mutant is still able to use dicarboxylates such as malate as carbon sources; however, its growth rate is slower than that of wild-type bacteria when relying solely on these compounds. One explanation for this slower growth rate is that the *mgo::KO* mutant needs time to induce alternative pathways for dicarboxylate utilization, but once these pathways are active, the mutant can begin to grow. For instance, DC3000 is predicted to have a NAD<sup>+</sup>-dependent malic enzyme (ME; PSPTO\_3924), which has 64% amino acid identity and 79% similarity to the NAD<sup>+</sup>-dependent malic enzyme from *E. coli*. Malic enzyme can convert malate to pyruvate, possibly bypass-

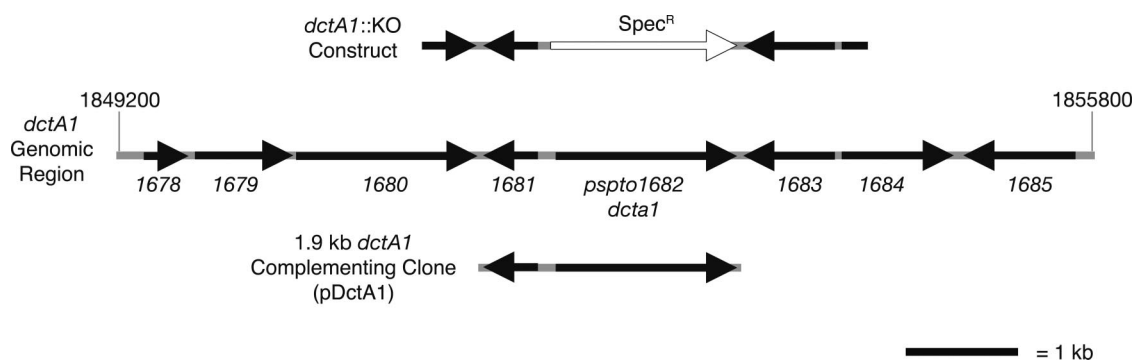


FIG. 5. Region of DC3000 genome containing the *dctA1* gene. The DC3000 genomic region containing the *dctA1* (PSPTO\_1682) coding sequence is shown. The italicized numbers (e.g., 1678) are abbreviations for the PSPTO number for each gene. The regions flanking *dctA1* used to create the *dctA1::KO* construct are indicated. A representation of the genomic sequence used in the *dctA1* complementing clone (pDctA1) is also shown. A size bar indicates the scale of the figure, and coordinates indicate the genomic position of the region in nucleotide bases (1849200 to 1855800).

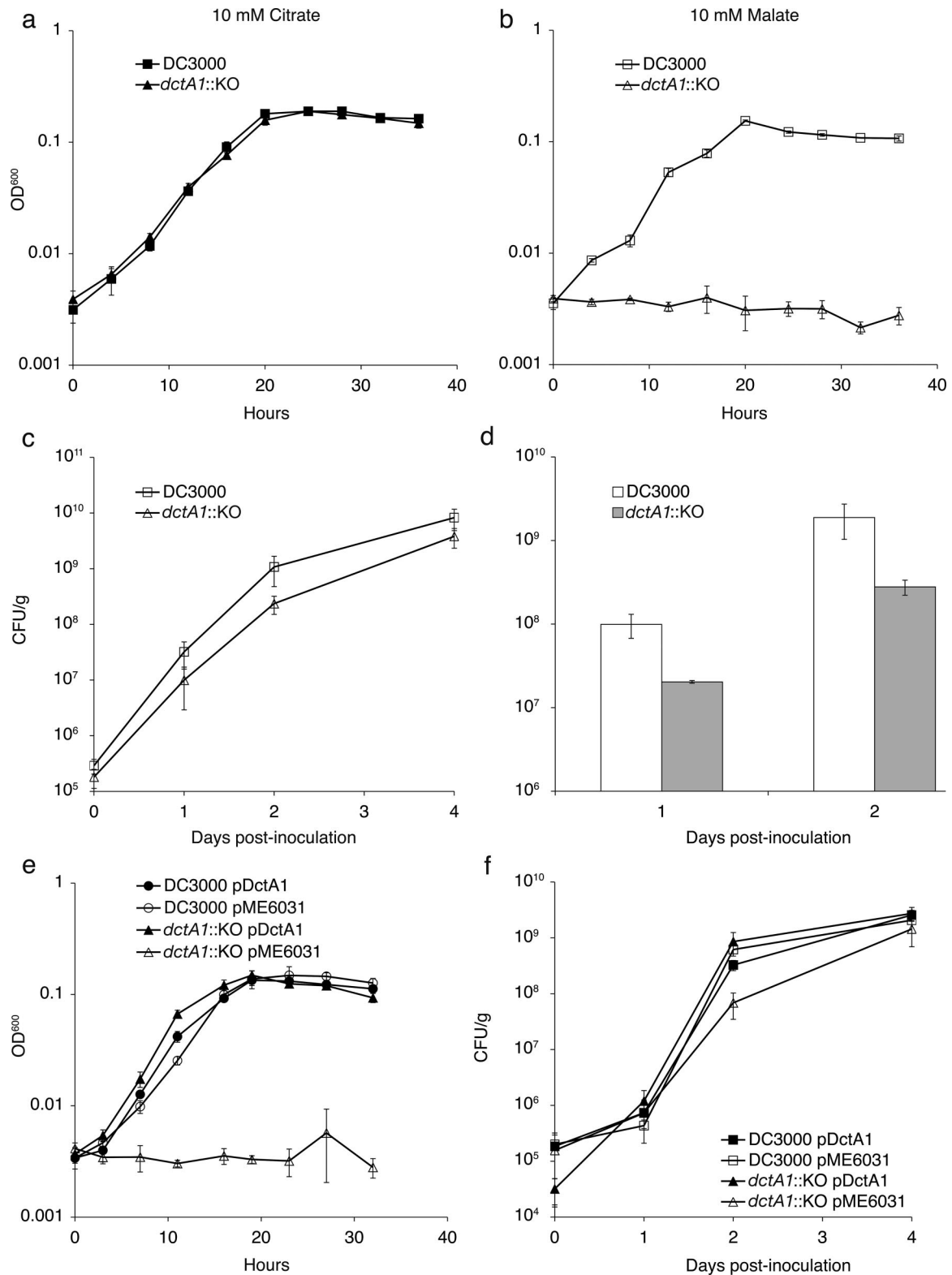


FIG. 6. Growth of the DC3000 *dctA1::KO* mutant in minimal media cultures and in planta. The *dctA1::KO* mutant and wild-type strains were grown in citrate (a) or malate (b). Three replicate cultures were used for each growth curve, and the error bars represent standard deviation of the mean. Similar results were obtained for at least two additional independent experiments. Two representative growth curves show growth of the *dctA1::KO* mutant in *A. thaliana*. (c) Seven samples were used for each time point, and error bars represent the standard errors of the mean. Student *t* tests with unpaired equal variation and two-tailed distribution gave *P* values of 0.24 for wild type and *dctA1::KO* at day 1 and 0.19 at day 2. Each strain contained the empty vector pME6031. (d) Three samples were used for each time point, error bars represent the standard error of the mean, and Student *t* tests with unpaired equal variation and two-tailed distribution gave *P* values of 0.07 for day 1 and 0.13 for day 2. Similar

ing the need for Mqo. Thus, in the absence of functional Mqo, DC3000 may need time to induce this alternative malate/dicarboxylate utilization gene. Alternatively, the *mgo::KO* mutant may simply be less efficient than wild-type bacteria at producing the energy it needs for growth on malate or succinate. For example, the growth rate of the *mgo* mutant on dicarboxylates may be reduced due to the reduction in the number of pathways available to convert malate to oxaloacetate and complete the TCA cycle. If the first scenario were true, and *mgo::KO* exhibits an initial lag in growth as it needs to induce alternative malate utilization genes, we would expect that *mgo::KO* bacteria from a mid-log-phase culture would grow at a wild-type rate when subcultured into fresh malate medium. If, however, *mgo::KO* is simply less efficient when growing on malate, the bacteria would still exhibit a slower growth rate than the wild type when introduced into a new malate culture. When we took mid-log-phase bacteria from cultures grown in HS with malate and used them to reinoculate fresh HS with malate cultures, we found that the *mgo::KO* bacteria still had a slower growth rate compared to wild-type bacteria. Thus, the slow-growth phenotype of the *mgo::KO* mutant is most likely due to less efficient utilization of malate (Fig. 4).

**DctA1 is required for growth in malate cultures and for normal virulence on *A. thaliana*.** Given that *mgo* is required for both virulence of DC3000, and wild-type growth in culture with dicarboxylates such as malate and succinate, we reasoned that DC3000 may use dicarboxylates as carbon sources when growing in the plant apoplast. Thus, we predicted that a DC3000 mutant that cannot take up dicarboxylates would also show impaired growth in planta. *Rhizobiaceae* utilize dicarboxylates provided by their plant hosts as carbon sources during their symbiotic relationship with legume plants (32), and in *S. meliloti* the DctA transporter is required for this (9, 48). DC3000 has two potential orthologs of the *S. meliloti* *dctA* gene, *dctA1* (PSPTO\_1682) and *dctA2* (PSPTO\_4063). The DC3000 *dctA1* gene encodes a protein with 60% identity and 78% similarity to *S. meliloti* DctA, while DC3000 *dctA2* encodes a protein with 50% identity and 75% similarity to *S. meliloti* DctA. We were unable to identify DC3000 orthologs of the *dctB* and *dctD* genes in the vicinity of *dctA1*. These genes encode the two-component regulators that control expression of the DctA transporter in *Rhizobiaceae* and are found adjacent to *dctA* (41, 47). Transcript levels of the DC3000 *dctA1* gene were increased within 60 min after the bacteria were transferred to minimal medium containing malate, compared to when they were grown on sucrose or rich media (data not shown). In contrast, the *dctA2* gene was expressed at a very low level (data not shown) in minimal medium containing malate. Thus, we reasoned that DctA1 may be involved in dicarboxylate uptake. We generated a *dctA1* marker replacement mutant, replacing

the *dctA1* gene with a *Spec<sup>r</sup>* gene. This strain, *dctA1::KO*, was unlikely to have any polar effects on adjacent genes, since *dctA1* does not appear to be part of an operon (Fig. 5). The strain was used to determine whether *dctA1* is required for growth on dicarboxylates in culture and/or for growth in *A. thaliana*.

The *dctA1::KO* strain grew similar to wild-type bacteria in HS supplemented with citrate but failed to grow in HSS medium containing only malate (Fig. 6a and b), demonstrating that DctA1 is likely to be the primary transporter used for uptake of malate when DC3000 is growing in minimal media. When the *dctA1::KO* strain was used to infect plants, the mutant strain reproducibly exhibited a small growth defect. Growth of the mutant was fivefold lower than that of the wild type on day 1 and/or day 2 after inoculation (Fig. 6c and d). Both the lack of growth of *dctA1::KO* in minimal medium with malate cultures and the reduced growth of *dctA1::KO* in planta could be restored to wild-type levels of growth by introduction of the pDctA1 complementing clone (Fig. 6e and f). This demonstrates that the DctA1 transporter is required for normal growth in planta, and suggests that dicarboxylates (e.g., malate) are an important carbon source for DC3000 at early stages of pathogenesis. Because the growth defect of the *dctA1::KO* is relatively small, we wondered whether one or more additional dicarboxylate transporters might also be involved in malate uptake and growth of DC3000 in planta. We constructed a mutant, *dctA2::pJP5603*, in which the gene encoding the second potential DctA transporter in DC3000, *dctA2*, was disrupted. We also constructed a double knockout of *dctA1* and *dctA2*. The *dctA2* mutant exhibited wild-type growth in *A. thaliana*, and the *dctA1::KO* *dctA2::pJP5603* double mutant strain did not show any further reduction in virulence over the *dctA1::KO* single mutant (data not shown), indicating that *dctA2* does not compensate for lack of *dctA1* in planta.

## DISCUSSION

We identified a novel DC3000 virulence gene, *mgo*, which unexpectedly encodes an enzyme that is part of a central metabolic pathway, the TCA cycle. To investigate the role of *mgo* in promoting virulence, we explored the impact of the *mgo* mutation on several aspects of host-pathogen interactions. We first explored whether the *mgo* mutant was impaired in type III secretion. The AK4C9 (*mgo::Tn5*) mutant elicited a normal HR on tobacco (data not shown), indicating that type III secretion is not impaired in this mutant. Thus, *mgo* is not required for *hrp/hrc* gene function. In addition, expression of *mgo* is not coregulated with type III secretion genes (10). Further, *mgo* is not directly regulated by the HrpL RNA polymerase sigma factor, which governs expression of the *hrp/hrc*

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results were obtained for at least 3 additional independent experiments. (e and f) The growth of wild-type and the *dctA1::KO* mutant carrying the pDctA1 complementing clone is shown. Panel e shows growth of these strains in minimal media with 10 mM malate, and panel f shows growth of the strains in *A. thaliana*. In panel e, three replicate cultures were used for each growth curve, and error bars represent the standard deviations. Similar results were obtained for two independent experiments. In panel f, six samples were used for each time point (except for day 1 for strain *dctA1::KO/pME6031*, which had five samples), error bars represent the standard error of the mean, and Student *t* tests with unpaired equal variation and two-tailed distribution gave *P* values of 0.01 for day 2 between DC3000/pME6031 and *dctA1::KO/pME6031*.



genes, since there is no HrpL binding site (*hrp* box) upstream of the *mgo* coding sequence (10, 14).

We next looked at whether *mgo* was involved in suppressing host defenses. Several DC3000 virulence factors, including TTSS effector proteins and coronatine, promote virulence by suppressing SA-mediated defenses. Our finding that growth of the *mgo::KO* mutant was not restored to wild-type levels on *sid2-2* plants indicates that *mgo* is not involved in suppression of SA-dependent defenses (data not shown). These data, combined with the finding that *mgo* mutants are not impaired for TTSS, suggests that Mgo is required for a novel aspect of virulence. We hypothesize that Mgo is required for virulence because DC3000 utilizes this enzyme to grow efficiently on dicarboxylates, a carbon source present in the plant apoplast (30, 40).

Consistent with this hypothesis, the *mgo::KO* bacteria exhibited growth defects in culture when provided with dicarboxylates (e.g., malate and succinate). It is important to note that the growth of the *mgo::KO* mutant was not impaired on rich medium or on minimal medium containing other carbon sources (e.g., sucrose or citrate), indicating that this mutant does not have a general metabolic or growth defect. This also suggests that one or more dicarboxylates may be important carbon sources during growth in the plant. Various studies have indicated that dicarboxylates such as malate are available in both the *A. thaliana* and tomato plant apoplast (28, 30). However, sucrose is also available in the apoplast (21, 39). Sucrose is the primary transport sugar in many plants and has been proposed to be a carbon source for apoplast-dwelling pathogens (6, 21). A study investigating which DC3000 metabolic pathways were active in tomato apoplast extracts indicated that, despite the availability of sucrose, sucrose utilization pathways were not induced (40). Thus, DC3000 may preferentially utilize dicarboxylates over other sugars available in the plant apoplast. Other studies have also suggested that TCA cycle intermediates are preferred carbon sources for DC3000, as indicated by the increased growth rate on TCA cycle intermediates (19).

One intriguing characteristic of the *mgo* mutants is that their growth defect in the plant is more pronounced at early time points, with the number of bacteria often reaching wild-type levels by 4 days postinoculation (Fig. 1b). However, the disease symptoms exhibited by plants infected with the *mgo* mutants never reach the severity of those caused by wild-type bacteria (Fig. 1c and d). The observation that wild-type levels of growth at later stages in infection does not result in normal symptom production raises the possibility that the number of bacteria must reach a certain threshold early during the infection for disease symptoms to develop normally. It is unclear why the in planta growth of *mgo* mutants eventually reaches wild-type levels in many experiments. One possibility is that other carbon sources become available later in infection, allowing DC3000 to grow without relying on dicarboxylates.

We have also shown that the *dctA1* gene, which encodes a dicarboxylate transporter, is required for growth of DC3000 in medium containing malate. In contrast, the *dctA1::KO* mutant exhibited only slightly reduced growth on *A. thaliana* compared to wild-type bacteria. There are several possible explanations for why the *dctA1::KO* does not have a more severe phenotype in planta. One possibility is that, while DctA1 is the primary

transporter for dicarboxylates in culture, there may be other transporters that are active when the bacteria are growing in plant tissue. Several other potential dicarboxylate transporters are encoded by the DC3000 genome, including two potential tellurite-resistance/dicarboxylate transporter transporters (PSPTO\_4613 and PSPTO\_3541) (17) and a tripartate ATP-independent periplasmic transporter consisting of three subunits encoded by the PSPTO\_1049, PSPTO\_1050, and PSPTO\_1051 genes (22). One or more of these other transporters may be active in planta, but do not function during growth in minimal medium. Another possibility is that DC3000 uses dicarboxylates at early stages of colonization but then switches to one or more other carbon sources as they become available later in infection. Finally, we cannot rule out the possibility that *mgo* is required for other as-yet-unknown functions that contribute to pathogenesis, in addition to its role in the TCA cycle. For example, *mgo* could be responsible for generating or detecting a signal regulating carbon source utilization and/or expression of other DC3000 virulence factors.

The genomes of several pathogenic and nonpathogenic *Pseudomonas* species, including *P. syringae* B728a, *P. syringae* pv. phaseolicola, *P. putida* KT2440, and *P. fluorescens* Pf-5, are also predicted to encode one or more *mgo* genes, suggesting that *mgo* may have important functions in other pathogenic and nonpathogenic bacteria. Future studies on the regulation and function of *mgo* will provide insight regarding the role of this novel virulence factor during pathogenesis and will further our understanding of the strategies used by DC3000 and other pathogens for nutrient acquisition and adaptation to growth in host tissue.

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