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NOTES

Xanthomonas campestris pv. campestris Possesses a Single Gluconeogenic Pathway That Is Required for Virulence†

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Disruption of *ppsA*, a key gene in gluconeogenesis, of *Xanthomonas campestris* pv. campestris resulted in the failure of the pathogen to grow in medium with pyruvate or C_4-dicarboxylates as the sole carbon source and a significant reduction in virulence, indicating that *X. campestris* pv. campestris possesses only the malic enzyme-PpsA route in gluconeogenesis, which is required for virulence.

The ability to acquire nutrients from the host is essential for a pathogen to establish an infection. Among those nutrients, carbon is one of the most basic elements. Bacteria may use gluconeogenesis (Fig. 1) to synthesize glucose from nonsugar C_2 or C_3 compounds or the intermediates of the tricarboxylic acid (TCA) cycle when there is not sufficient hexose in their niches (17, 24, 26, 27). The first step of gluconeogenesis in bacteria is the synthesis of phosphoenolpyruvate (PEP) through the phosphoenolpyruvate carboxykinase (PckA) route and/or the malic enzyme-phosphoenolpyruvate synthase (PpsA) route (13, 17, 24, 25, 26). In some bacteria, the function of PpsA to synthesize PEP can be performed instead by pyruvate phosphate dikinase (PPDK) (10, 26). The PckA route is a one-step reaction in which PEP is synthesized from oxaloacetate catalyzed by PckA, and the malic enzyme-PpsA (or PPDK) route consists of two reactions: the first reaction is the conversion of malate to pyruvate catalyzed by the malic enzyme MaeA or MaeB and the second reaction is the synthesis of PEP from pyruvate catalyzed by PpsA using pyruvate, H_2O, and ATP as substrates or by PPDK using pyruvate, phosphate, and ATP as substrates (24, 26). The PckA route and the malic enzyme-PpsA (or PPDK) route coexist in *Escherichia coli* (24), *Rhodopseudomonas palustris* (26), and *Sinorhizobium meliloti* (17). The synthesis of PEP from pyruvate is catalyzed by PpsA in *E. coli* and *R. palustris* and by PPDK in *S. meliloti* (17, 24, 26). *Campylobacter jejuni*, a bacterium that is unable to utilize glucose due to the absence of the key glycolytic enzyme 6-phosphofructokinase, possesses only the PckA route to synthesize PEP in gluconeogenesis (31). The gluconeogenic pathway has been shown to be required for virulence of the animal pathogen *Mycobacterium bovis* (5). The role of this pathway in the pathogenesis of a plant pathogen has not been reported.

The glyoxylate cycle is a truncated TCA cycle in which isocitrate is hydrolyzed to succinate and glyoxylate by isocitrate lyase (encoded by *aceA*, also named *icl*) and the glyoxylate is then condensed with acetyl coenzyme A to produce malate by malate synthase (encoded by *mls*). The primary function of the glyoxylate cycle is to permit C_2 compounds to be converted to glucose through the gluconeogenic pathway (21). It has been reported that the glyoxylate cycle plays an important role in the pathogenesis of a number of pathogens (16, 20, 23, 32, 33).

Xanthomonas campestris pv. campestris, a yellow-pigmented γ-proteobacterium, is the causal agent of the black rot disease of cruciferous crops worldwide, and it also infects the model plant *Arabidopsis thaliana*. It propagates and spreads in the apoplast of the host plant after infection (14, 29). *X. campestris* pv. campestris has been used as a model bacterium for studying microbe-plant interactions for over 2 decades, and a number of genes involved in pathogenicity have been identified (2, 4, 6, 9, 30). The whole genome of *X. campestris* pv. campestris strain ATCC 33913 was sequenced (8). Our group and collaborators have recently sequenced the whole genome of another *X. campestris* pv. campestris strain 8004 (our unpublished data). The genome annotation shows that homologs of all the appropriate genes for gluconeogenesis, except *pckA* and *maeA*, are present in both strains (8; our unpublished data).

Using a molecular genetic approach, we have demonstrated that *X. campestris* pv. campestris has only the malic enzyme-PpsA route to synthesize PEP in gluconeogenesis and the gluconeogenic pathway is required for its full virulence. We further found that the glyoxylate cycle is not required for the multiplication in planta and virulence of the pathogen. We spec-

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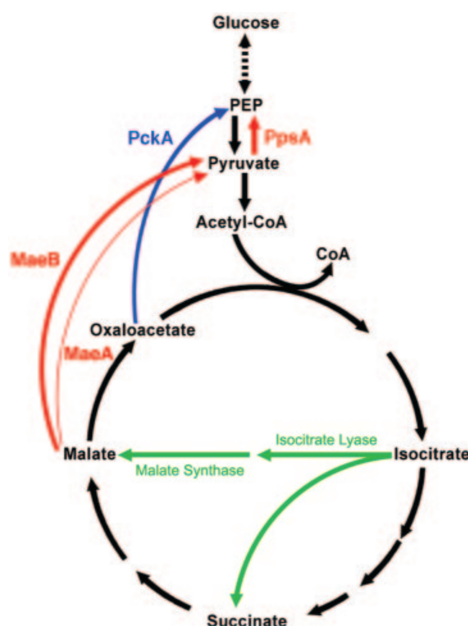

FIG. 1. Schematic drawing of the gluconeogenic pathway in microorganisms. The PckA route is shown as a blue arrow, the malic enzyme-PpsA route as red arrows, and the glyoxylate cycle as green arrows (modified from reference 24). CoA, coenzyme A.

ulate that *X. campestris* pv. campestris uses C_4-dicarboxylates rather than C_2 compounds as the primary carbon source through gluconeogenesis via the malic enzyme-PpsA route in planta.

The malic enzyme-PpsA route is the only route to synthesize PEP in gluconeogenesis of *X. campestris* pv. campestris. There exist two routes to synthesize PEP in gluconeogenesis in bacteria: the PckA route and the malic enzyme-PpsA route. The PckA route has been generally considered to be the main pathway of gluconeogenesis (19, 22). However, the genome annotation data show that there is no homolog of *pckA* in *X. campestris* pv. campestris (8; our unpublished data). To investigate whether the PckA route is functionally absent in *X. campestris* pv. campestris, *ppsA* was disrupted by using a method described previously (34). A 542-bp internal fragment of the *ppsA* coding region (from positions 12 to 553) was amplified by using chromosomal DNA of *X. campestris* pv. campestris wild-type strain 8004 (6) as the template and ppsAMF-ppsAMR mutation primer set (Table 1). After confirmation by sequencing, the amplified DNA fragment was cloned into the suicide plasmid pK18mob (28) to create recombinant plasmid pK1880 (Table 2). The plasmid pK1880 was introduced from *E. coli* JM109 (35) into *X. campestris* pv. campestris strain 8004 by triparental conjugation using pRK2073 as the helper plasmid (18). Transconjugants were screened on NYG medium (5 g of peptone, 3 g of yeast extract, and 20 g of glycerol per liter) (7) supplemented with rifampin and kanamycin to the final concentrations of 50 μg/ml and 25 μg/ml, and the obtained transconjugants with a mutation in the *ppsA* gene were confirmed by PCR using the chromosomal DNA of the transconjugants as the template and the p18conF-ppsAconR confirmation primer set (Table 1). The expected PCR product was further confirmed by sequencing. The resulting *ppsA* mutant was designated NK1880 (Table 2).

For complementation of the *ppsA* mutant, the wild-type *ppsA* gene was amplified from strain 8004 by PCR using primer set ppsAF-ppsAR (Table 1) and cloned into pLAFR6 (15). The obtained recombinant plasmid pXC1880 (Table 2) was introduced into NK1880 by triparental conjugation. NK1880 carrying pXC1880 was screened on NYG supplemented with rifampin (50 μg/ml), kanamycin (25 μg/ml), and tetracycline (5 μg/ml) and confirmed by PCR with a specific primer set, ppsAF-ppsAR (Table 1). The resulting strain was designated CNK1880 (Table 2).

The abilities of *X. campestris* pv. campestris strains to utilize different carbon sources were determined by measuring the growth rates of the strains in noncarbohydrate minimal medium (NCM) [modified from MMX minimal medium (7), containing 2.0 g/liter $(NH_4)_2SO_4$, 4.0 g/liter K_2HPO_4, 6.0 g/liter KH_2PO_4, and 0.2 g/liter $MgSO_4 \cdot 7H_2O$] supplemented with different carbon sources (glucose, sucrose, malate, succinate, fumarate, and pyruvate at 0.5% [wt/vol] and acetate at 0.15% [wt/vol]).

The wild-type strain 8004 was able to grow in the NCM

TABLE 1. Primer sets used in this work

Primer set	Sequences (5′ to 3′)[a]	Product length (bp)
For mutagenesis		
ppsAMF-ppsAMR	TATCCTGTGGTTGCATGAGCTACGC/GGAACACATCTTCGTGCTTGAAGCC	542
mlsMF-mlsMR	TGCCACCGCCGCTTCCGCCC/CACCGCGGCCGCCAACGTGC	439
aceAMF-aceAMR	TCCCTGCCCGAGGAGACG/TGGTGCAACTGATCGGCGCG	423
For mutant confirmation		
P18conF-ppsAconR	GCCGATTCATTAATGCAGCTGGCAC/CGTCGAACGCCTTGATCCGG	3,086
P18conF-mlsconR	GCCGATTCATTAATGCAGCTGGCAC/TGGTGCAACTGATCGGCGCG	2,411
P18conF-aceAconR	GCCGATTCATTAATGCAGCTGGCAC/TGGTGCAACTGATCGGCGCG	2,468
For complementation (ppsAF-ppsAR)	ACT<u>GGATCC</u>TTTCAGCGGTGATACCGGTTCC/CCC<u>AAGCTT</u>GGCGGCCGCTCCCGCAGCCGCC	2,626

[a] Added restriction sites are underlined.

TABLE 2. Bacterial strains and plasmids used in this work

Strain or plasmid	Relevant characteristics	Reference or source
E. coli JM109	*recA1 endA1 gyrA96 thi supE44 relA1* Δ(*lac-proAB*)/F′ [*traD36 lacI*^{q} *lacZ*ΔM15]	35
X. campestris pv. campestris		
8004	Wild type, Rif^r	6
NK1880	As 8004, but *ppsA*::pK18mob, Rif^r Kan^r	This work
NK0216	As 8004, but *mls*::pK18mob, Rif^r Kan^r	This work
NK0217	As 8004, but *aceA*::pK18mob, Rif^r Kan^r	This work
CNK1880	1880nk harboring pXC1880, Rif^r Kan^r Tc^r	This work
Plasmids		
pLAFR6	Broad-host-range cloning vector, Tc^r	15
pRK2073	Helper plasmid, Tra$^+$, Mob$^+$, CoLE1, Spc^r	18
pK18mob	Suicide plasmid in *X. campestris* pv. campestris, Kan^r	28
pK1880	pK18mob containing a 542-bp internal fragment of *ppsA* gene, Kan^r	This work
pK0216	pK18mob containing a 439-bp internal fragment of *mls* gene, Kan^r	This work
pK0217	pK18mob containing a 423-bp internal fragment of *aceA* gene, Kan^r	This work
pXC1880	pLAFR6 containing entire *ppsA* gene, Tc^r	This work

medium supplemented with each of the following carbohydrates as the sole carbon source: glucose, sucrose, malate, succinate, fumarate, pyruvate, and acetate (Fig. 2). The *ppsA* mutant grew normally in NCM medium supplemented with glucose or sucrose but was unable to grow in NCM medium supplemented with malate, succinate, fumarate, pyruvate, or acetate as the sole carbon source (Fig. 2). Furthermore, the complemented strain CNK1880 behaved like the wild-type strain (Fig. 2). These results demonstrated that the PckA route is functionally absent and the malic enzyme-PpsA route is the only route to synthesize PEP from C_4-dicarboxylate in gluconeogenesis in *X. campestris* pv. campestris.

Of the 213 bacterial strains (eubacteria and archaea) with the whole genomes sequenced so far, 90 strains possess a single route for the synthesis of PEP in gluconeogenesis (36 possess the genes encoding the PckA route, and 54 possess the genes encoding the malic enzyme-PpsA route), 84 possess the genes encoding both the PckA and the malic enzyme-PpsA routes, and 39 have neither the *pckA* homolog nor the *ppsA* (or the *ppd* kinase gene) homolog (see Table S1 in the supplemental material). Theoretically, possession of both the PckA route and the malic enzyme-PpsA route for synthesizing PEP in gluconeogenesis may confer the bacterium advantages in acquiring carbon nutrients to survive in more diversified environments. For example, in *E. coli*, which possesses both the PckA route and the malic enzyme-PpsA route for the synthesis of PEP in gluconeogenesis, disruption of one of the two routes alone does not affect the normal function of gluconeogenesis of this bacterium (24). The limited genome data seem to show the trend that most free-living bacteria, including dominantly the saprophytic and opportunistic pathogens, possess both the PckA and the malic enzyme-PpsA routes, while the semiparasitic pathogens tend to have a single route, either PckA or malic enzyme-PpsA. Interestingly, *Buchnera* spp., the endocellular symbionts, and *Mycoplasma* spp., the obligate pathogens, have no homologous genes for PEP synthesis in gluconeogenesis (see Table S1 in the supplemental material). Thus, the pathway variation in gluconeogenesis seems to correlate with the living niches of the different bacteria to ensure the acquisition of sufficient carbon source.

The gluconeogenic pathway is required for virulence and in planta growth of *X. campestris* pv. campestris. Gluconeogenesis has been shown or implied to be required for virulence of a number of animal pathogens such as *Salmonella enterica* serovar Typhimurium (1), *M. bovis* (5, 19), *Mycobacterium tuberculosis* (23), and *Candida albicans* (20). To investigate whether the virulence of the pathogen *X. campestris* pv. campestris is affected by a mutation in *ppsA*, the virulence of *ppsA* mutant NK1880 was tested by inoculating the mutant onto its host plant Chinese radish (*Raphanus sativus* L. cv. radiculus Pers.) grown in a greenhouse with a day/night cycle of 12 h/12 h illumination by fluorescent lamps at a temperature of 25 to 28°C. Two leaves per plant at the stage of four full-expanded leaves were inoculated by the leaf-clipping method (9) with bacterial cells grown in NYG medium at 28°C with shaking at 200 rpm for 15 h at a concentration of 10^8 cells/ml. Fifty leaves were inoculated for each strain in one assay that was repeated three times. After being kept at 100% humidity for 24 h, the inoculated plants were maintained in the growth conditions described above. Lesion lengths were measured 10 days postinoculation, and the data were statistically analyzed. As analyzed by *t* test, the mean length of the lesion caused by *ppsA* mutant NK1880 was significantly shorter than that of the lesion caused by the wild-type strain at a P of 0.01 (*t* test) (Fig. 3). Meanwhile, the lesion lengths of the complemented strain CNK1880 and the wild-type strain were not significantly different at a P of 0.05 (Fig. 3). To verify whether the reduced virulence was correlated to the reduced growth rate of the pathogen, we investigated the growth of bacteria in planta. A group of five inoculated radish leaves was homogenized in sterilized water and plated on NYG medium supplemented with rifampin (50 μg/ml) (for the wild-type strain), rifampin (50 μg/ml) plus kanamycin (25 μg/ml) (for the mutant strain), or rifampin (50 μg/ml) and kanamycin (25 μg/ml) plus tetracycline (5 μg/ml) (for the complemented strain). Bacterial CFU were counted after incubation at 28°C for 3 days. During the observation period, the in planta multiplication level of the *ppsA* mutant was significantly lower than that of the wild-type strain at each of the test points ($P = 0.01$), and the number of CFU of the *ppsA* mutant in the infected leaves was about

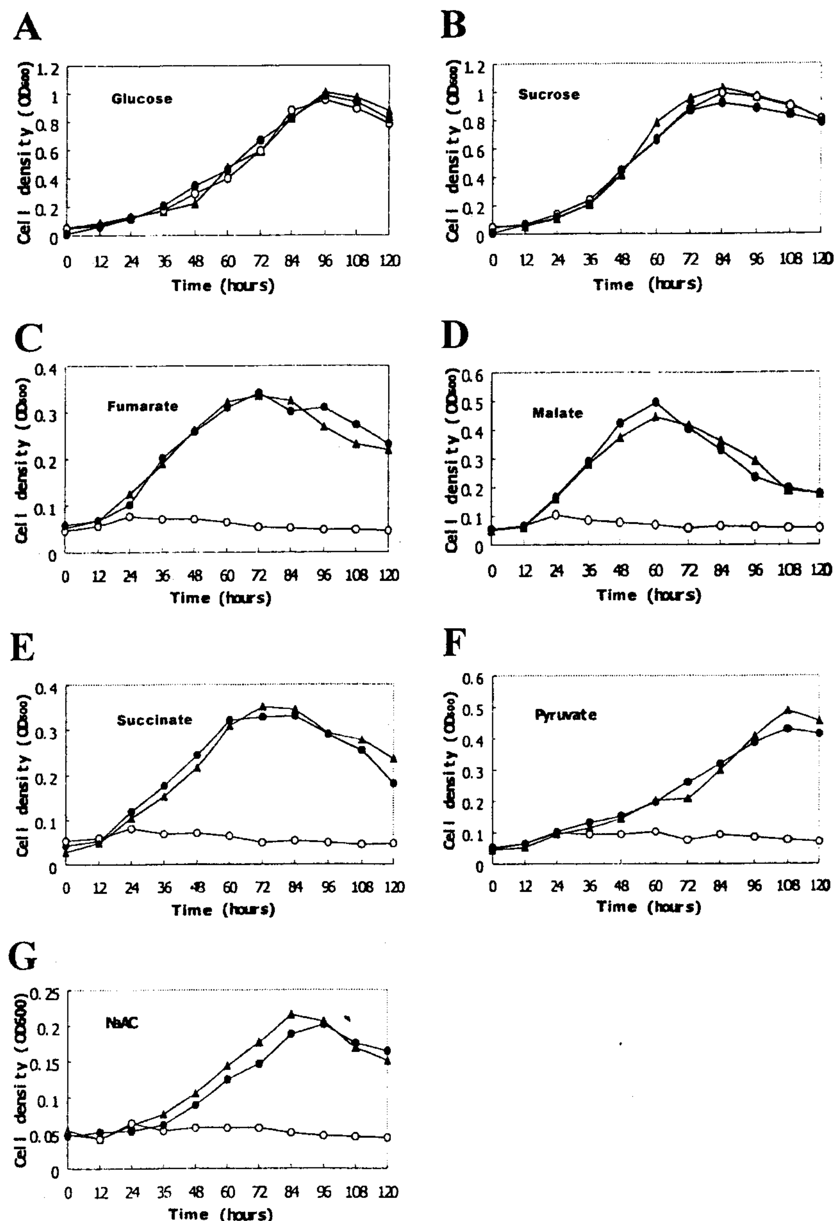

FIG. 2. Growth of *X. campestris* pv. campestris strains (▲, wild-type strain 8004; ○, *ppsA* mutant NK1880; ●, *ppsA*-complemented strain CNK1880) in media with different carbon sources. The NCM medium was supplemented with glucose (A), sucrose (B), fumarate (C), malate (D), succinate (E), or pyruvate (F) at 0.5% or sodium acetate (NaAC) at 0.15% (G). Data presented were from a representative experiment.

100-fold less than that of the wild-type strain from day 4 to day 10 after inoculation (Fig. 3C). These results demonstrated that an intact gluconeogenic pathway is required for full virulence and the reduced virulence is probably related to the reduced bacterial numbers of the mutant in planta.

The glyoxylate cycle is not required for virulence and in planta growth of *X. campestris* pv. campestris. The involvement of the glyoxylate cycle in microbial pathogenesis has been reported for both animal and plant pathogens (16, 20, 23, 32, 33). To investigate whether or not the glyoxylate cycle is required for the virulence of *X. campestris* pv. campestris, *aceA* and *mls*, genes encoding the glyoxylate cycle enzymes isocitrate lyase and malate synthase, respectively, were disrupted by using the same method for inactivating *ppsA*. The 439-bp fragment of the *aceA* coding region (from positions 6 to 444) and the 423-bp fragment of the *mls* coding region (from positions 9 to 431) were generated by PCR with specific primers (Table 1). The resulting *aceA* and *mls* mutants, NK0217 and NK0216 (Table 2), could grow normally in NCM medium supplemented with each of the following carbohydrates at a concentration of 0.5% (wt/vol) as the sole carbon source: glucose, sucrose, glycerol, succinate, fumarate, malate, and pyruvate (data not shown); however, they were unable to grow in NCM medium supplemented with acetate (0.15%) as the sole carbon source, in contrast to the wild-type strain (Fig. 4), indicating that the glyoxylate cycle had been specifically blocked in the

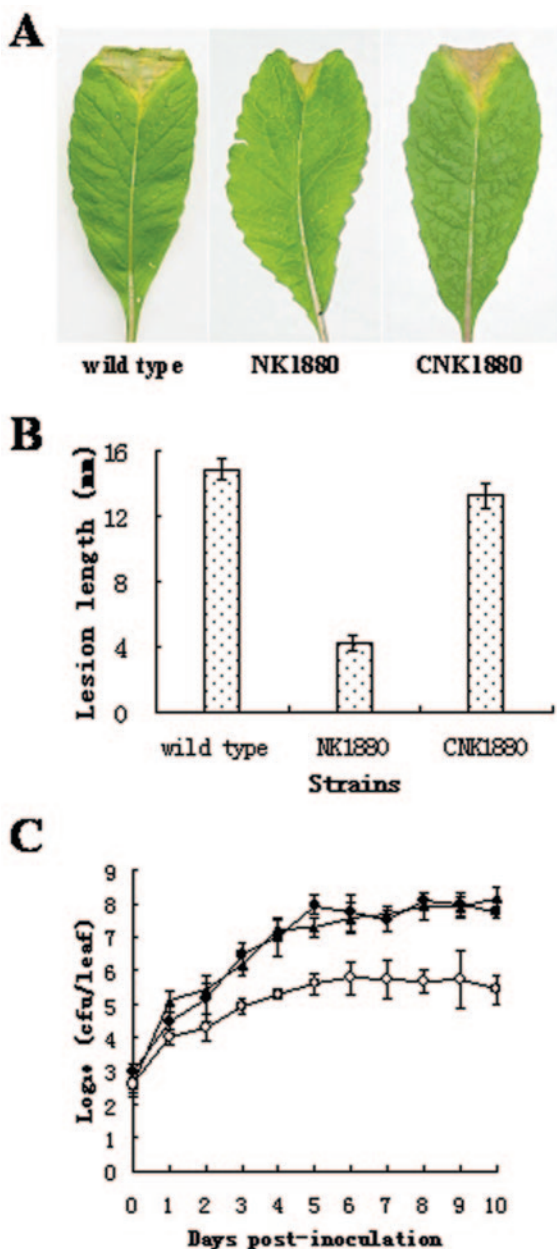

FIG. 3. *ppsA* is required for virulence and in planta growth of *X. campestris* pv. campestris. (A) Black rot disease symptoms caused by *X. campestris* pv. campestris strains on inoculated leaves of Chinese radish. Photographs were taken at day 10 postinoculation. (B) Average lesion lengths caused by *X. campestris* pv. campestris strains. Values are the means ± standard deviations (SD) from three repeats, using two leaves each from 25 plants. (C) Growth of bacteria in inoculated leaves. Data are the means ± SD from three repeats. ▲, wild-type strain 8004; ○, *ppsA* mutant NK1880; ●, *ppsA*-complemented strain CNK1880.

mutants. Upon inoculation onto the plants by the leaf-clipping method at a bacterial concentration of 10^8 cells/ml, both mutants showed full virulence on Chinese radish and grew as well as the wild-type strain (Fig. 5), demonstrating that the glyoxylate cycle is not required for virulence and in planta multiplication of *X. campestris* pv. campestris.

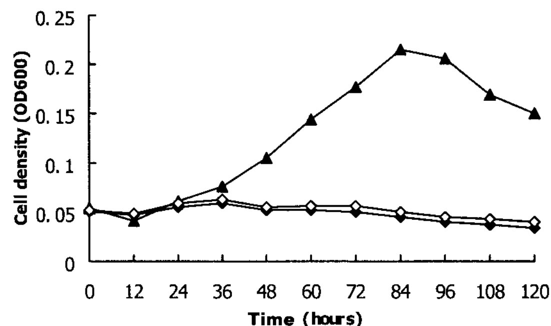

FIG. 4. The *mls* mutant and *aceA* mutant are unable to grow on minimal medium supplemented with acetate as the sole carbon source. ▲, wild-type strain 8004; ◇, *mls* mutant NK0216; ◆, *aceA* mutant NK0217. OD600, optical density at 600 nm.

C_4-dicarboxylates could be the principal carbon sources for *X. campestris* pv. campestris during infection. *X. campestris* pv. campestris propagates and spreads in the apoplast of the host plant after infection (14, 29). Thus, the ability to acquire nutrients from the apoplast is critically important for it to cause disease. However, the nutritional requirements of *X. campestris* pv. campestris during infection and the molecular mechanism by which it acquires nutrients from the apoplast are still unclear. The observation that disruption of the gluconeogenic pathway resulted in significant reductions both in multiplication in planta and virulence of *X. campestris* pv. campestris suggested that the apoplast is lacking hexose but rich in gluconeogenic substrates (C_2 or C_3 compounds or the intermediates of the TCA cycle), and the gluconeogenic pathway is the only route to utilize these carbon sources to maintain the carbon and energy supplies for normal growth of *X. campestris* pv. campestris during infection. Furthermore, disruption of the glyoxylate cycle (mutation in *aceA* or *mls*) of *X. campestris* pv. campestris resulted in failure to grow in medium with C_2 compounds as the sole carbon source (Fig. 4) but did not affect its virulence and growth in planta (Fig. 5), indicating that C_2 compounds are not the major carbon source for *X. campestris* pv. campestris in planta. Combining these results with the fact that the wild-type strain grew well on medium with C_4-dicarboxylic acids (Fig. 2) but poorly on medium with citrate (data not shown) as the sole carbon source, we suppose that C_4-dicarboxylates could be the principal carbon sources for *X. campestris* pv. campestris during infection. This hypothesis is consistent with the findings with *Rhizobium* spp., that C_4-dicarboxylic acids are the principal carbon sources for the bacteroid within nodules (3, 11, 12) but in contrast to the reports that nonsugar C_2 compounds are the major carbon sources for *M. tuberculosis* and *C. albicans* during the infection of macrophage (20, 23). This information suggests that the carbon diet of a pathogen inside the host is correlated to the composition of the available carbon source in the infection site. From an evolutionary point of view, the nutritional requirements of a pathogen during infection and the molecular mechanism by which this pathogen acquires nutrients from the host may be the results of coevolution of the pathogens with their hosts.

The reduced virulence of the *ppsA* mutant may be due to its inability to convert C_4-dicarboxylic acids to glucose for growth in planta. The gluconeogenic pathway may be required for

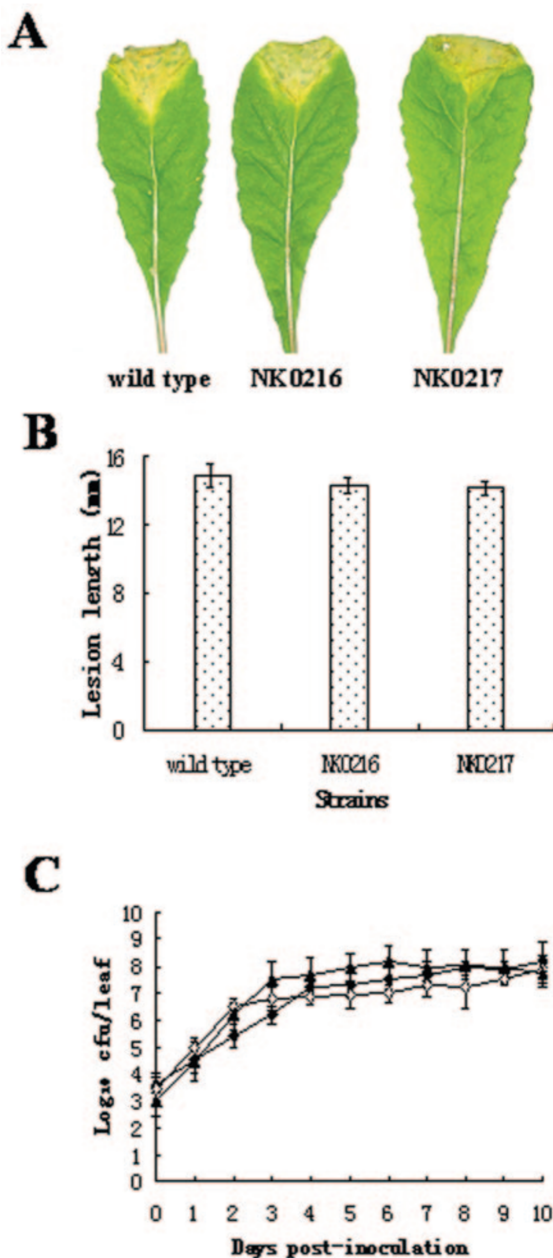

FIG. 5. *mls* and *aceA* are not required for virulence and in planta growth of *X. campestris* pv. campestris. (A) Black rot disease symptoms caused by *X. campestris* pv. campestris strains on inoculated leaves of Chinese radish. Photographs were taken at day 10 postinoculation. (B) Average lesion lengths caused by *X. campestris* pv. campestris strains. Values are the means ± standard deviations (SD) from three repeats, each with 50 leaves. (C) Growth of bacteria in inoculated leaves. Data are the means ± SD from three repeats. ▲, wild-type strain 8004; ◇, *mls* mutant NK0216; ◆, *aceA* mutant NK0217.

fitness of *X. campestris* pv. campestris in planta and unlikely for the production of virulence factor(s). Although metabolic pathways are generally not considered to be virulence factors, the elucidation of the mechanism to acquire and metabolize nutrients during infection is critically important for fully understanding the pathogenesis of a pathogen.

Nucleotide and amino acid sequence accession numbers. The sequences of *ppsA*, *aceA*, and *mls* have been submitted to the GenBank database under accession numbers AY618213, AY724680, and AY724681, respectively.

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