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Identification of C_4-Dicarboxylate Transport Systems in *Pseudomonas aeruginosa* PAO1[▽]†

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Pseudomonas aeruginosa **utilizes preferentially C_4-dicarboxylates such as malate, fumarate, and succinate as carbon and energy sources. We have identified and characterized two C_4-dicarboxylate transport (Dct) systems in *P. aeruginosa* PAO1. Inactivation of the *dctA* (PA1183) gene caused a growth defect of the strain in minimal media supplemented with succinate, fumarate or malate, indicating that DctA has a major role in Dct. However, residual growth of the *dctA* mutant in these media suggested the presence of additional C_4-dicarboxylate transporter(s). Tn*5* insertion mutagenesis of the Δ*dctA* mutant led to the identification of a second Dct system, i.e., the DctPQM transporter belonging to the tripartite ATP-independent periplasmic (TRAP) family of carriers. The Δ*dctA* Δ*dctPQM* double mutant showed no growth on malate and fumarate and residual growth on succinate, suggesting that DctA and DctPQM are the only malate and fumarate transporters, whereas additional transporters for succinate are present. Using *lacZ* reporter fusions, we showed that the expression of the *dctA* gene and the *dctPQM* operon was enhanced in early exponential growth phase and induced by C_4-dicarboxylates. Competition experiments demonstrated that the DctPQM carrier was more efficient than the DctA carrier for the utilization of succinate at micromolar concentrations, whereas DctA was the major transporter at millimolar concentrations. To conclude, this is the first time that the high- and low-affinity uptake systems for succinate DctA and DctPQM have been reported to function coordinately to transport C_4-dicarboxylates and that the alternative sigma factor RpoN and a DctB/DctD two-component system regulates simultaneously the *dctA* gene and the *dctPQM* operon.**

Pseudomonas aeruginosa is a versatile ubiquitous Gram-negative bacterium that has a phenomenal capacity to adapt to different environments and utilizes a wide variety of different organic molecules as carbon and energy sources (32). The 6.2-Mb genome of *P. aeruginosa* PAO1 contains a large number of genes for catabolism, nutrient transport, and metabolic regulation (46). *P. aeruginosa* preferentially utilizes tricarboxylic acid (TCA) cycle intermediates such as the C_4-dicarboxylates malate, fumarate and, in particular, succinate as carbon and energy sources (24, 28).

In various bacteria, carriers and sensors have been described to be involved in C_4-dicarboxylate utilization. In rhizobia, a C_4-dicarboxylic acid transport (Dct) system has been described in detail (58). It is composed of three genes clustered together: the *dctA* gene coding for a C_4-dicarboxylate transport protein, belonging to the dicarboxylate/cation symporter (DAACS) family (43), and the *dctB* and *dctD* genes coding for a two-component regulatory system, which responds to C_4-dicarboxylates and regulates *dctA* expression (6, 36, 58). In *Rhizobium leguminosarum* and *Sinorhizobium meliloti*, a functional DctA transporter is essential for symbiotic nitrogen fixation (7, 36). In the presence of C_4-dicarboxylates, the DctB membrane sensor is activated by autophosphorylation and then transfers a phosphate group to the response regulator DctD (12). Once DctD is activated, it binds to an upstream activator sequence (UAS) present in the *dctA* promoter region, enabling RNA polymerase with the sigma factor σ^{54} (RpoN) to transcribe the gene (21). In the absence of substrates, it has been proposed that the DctA and DctB proteins interact with each other in the cytoplasmic membrane, leading to inhibition of DctB autophosphorylation and consequently to low expression of the C_4-dicarboxylic acid transport system (12, 22, 23, 52). The Gram-positive bacteria *Bacillus subtilis* and *Corynebacterium glutamicum* contain a similar DctA transporter (2, 48). In *Escherichia coli*, C_4-dicarboxylates are utilized under aerobic and anaerobic growth conditions. During aerobic growth, DctA is the main C_4-dicarboxylate transporter, whereas a second carrier termed DcuA might further contribute to fumarate and succinate uptake (4, 14). During anaerobic growth, C_4-dicarboxylate transport is performed by the DcuA, DcuB, DcuC, and CitT carriers (13, 34, 42, 59). Furthermore, a quintuple mutant (*dctA*, *dcuA*, *dcuB*, *dcuC*, and *citT*), which is deficient in all known C_4-dicarboxylate transport functions, presents residual growth on succinate, indicating the presence of additional unidentified transporter(s) (18).

In the purple photosynthetic bacterium *Rhodobacter capsulatus*, the tripartite ATP-independent periplasmic (TRAP) carrier has been identified and characterized as a C_4-dicarboxylate transporter (8, 40). The TRAP transporter is encoded by three genes clustered together: the *dctP* gene coding for a C_4-dicarboxylate-binding protein and the *dctQ* and *dctM* genes coding for a C_4-dicarboxylate transporter (8). Furthermore, the *dctSR* operon, which is adjacent and divergent to the *dctPQM*

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TABLE 1. Strains and plasmids used in this study

Strain, bacteriophage, or plasmid	Genotype or relevant characteristics[a]	Source or reference
Strains		
E. coli		
DH5α	*recA1 endA1 hsdR17 supE44 thi-1 gyrA96 relA1* Δ(*lacZYA-argF*)*U169* [ϕ80d*lacZ*ΔM15]F$^-$ NaI^r	38
HB101	*proA2 hsdS20*(r_B^- m_B^-) *recA13 ara-14 lacYI galK2 rpsL20 supE44 xyl-5 mtl-1* F$^-$	38
S17-1/λ*pir*	*pro thi hsdR recA* chromosome::RP4-2 Tc::Mu Km::Tn*7*/λ*pir*; Tp^r Sm^r	27, 41
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17*6 *supE44 relA1 lac* [F′ *proAB lacI*^{q}ZΔM15::Tn*10*(53)]	Stratagene
P. aeruginosa		
PAO1	Wild type	17
PAO6358	PAO1 containing a 900-bp deletion in the *rpoN* locus	16
PAO6592	PAO1 containing a 1,296-bp deletion in the *dctA* locus	This study
PAO6705	PAO1 containing a 1,798-bp deletion in the PA5165 (*dctB*) locus	This study
PAO6706	PAO1 containing a 1,377-bp deletion in the PA5166 (*dctD*) locus	This study
PAO6707	PAO1 containing a double deletion in the PA5165 locus (1,798 bp) and in the PA5166 locus (1,377 bp)	This study
PAO6708	PAO1 containing a 2,899-bp deletion in the PA5167-PA5169 (*dctPQM*) operon	This study
PAO6709	PAO1 containing a double deletion in the *dctA* locus (1,296 bp) and in the *dctPQM* (PA5167-PA5169) operon (2,899 bp)	This study
PA6710	PAO6592 with PA5168::Tn*5*Gm; Gm^r	This study
PA6815	PAO1 with PA5168::Tn*5*Gm; Gm^r	This study
Bacteriophage		
E79*tv-2*	Temperate, transducing variant of E79	29
Plasmids		
pLM1	Tn*5*Gm delivery vector; Gm^r Ap^r	9
pMMB67HE	IncQ expression vector carrying an inducible *tac* promoter; Ap/Cb^r	11
pME3087	Suicide vector for allelic replacement; Tc^r; ColE1 replicon	51
pME6015	Cloning vector for translational *lacZ* fusions; Tc^r	39
pME6016	Cloning vector for transcriptional *lacZ* fusions; Tc^r	39
pME9506	Plasmid carrying a transcriptional *dctA-lacZ* fusion	This study
pME9507	Plasmid carrying a translational *dctA′-′lacZ* fusion	This study
pME9508	Plasmid carrying a translational *dctA′-′lacZ* fusion in which the *rpoN* putative box of the *dctA* promoter TGGCAC-N_5-CTGCA was replaced with TACCAC-N_5-CTTTA	This study
pME10031	Suicide construct used for deletion of the PA5165 gene; Tc^r	This study
pME10032	Suicide construct used for deletion of the PA5166 gene; Tc^r	This study
pME10033	Suicide construct used for deletion of the PA5167-PA5169 operon; Tc^r	This study
pME10034	Plasmid carrying a transcriptional *dctP-lacZ* fusion	This study
pME10035	Plasmid carrying a translational *dctP′-′lacZ* fusion	This study
pME10036	Plasmid carrying a translational *dctP′-′lacZ* fusion in which the *rpoN* putative box of the *dctPQM* promoter TGGCAC-N_5-TTGCT was replaced with TACCAC-N_5-TTTTT	This study
pME10037	pMMB67HE with *dctA*	This study
pME10038	pMMB67HE with PA5165	This study
pME10039	pMMB67HE with PA5166	This study
pME10040	pMMB67HE with PA5167-PA5169	This study
pME10041	Suicide construct used for deletion of the *dctA* gene; Tc^r	This study

[a] Nal^r, nalidixic acid resistance; Gm^r, gentamicin resistance; Tc^r, tetracycline resistance; Ap^r, ampicillin resistance; Sm^r, streptomycin resistance; Cb^r, carbenicillin resistance; Tp^r, trimethoprim resistance.

operon, encodes a two-component regulatory system controlling the expression of the *dctPQM* operon (15). Similarly, studies on fumarate respiration in *Wolinella succinogenes* showed that the C_4-dicarboxylate transport was catalyzed by a DctPQM transporter (50). In *Pseudomonas chlororaphis* strain O6, the expression of *dctA* is activated by succinate (31) and a *dctA* mutant does not grow on succinate or fumarate but can grow on malate (30, 53, 58).

In the present study, we report the identification and characterization of two Dct systems involved in C_4-dicarboxylate uptake in *P. aeruginosa* PAO1. DctA was found to be the major carrier at high succinate concentrations, whereas the DctPQM transporter, belonging to the TRAP family of carriers, was efficient at low succinate concentrations. In addition, we demonstrate that both DctPQM and DctA are positively regulated by σ^{54} (RpoN), DctA, and the DctB/DctD two-component system.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains and plasmids used in the present study are listed in Table 1 and oligonucleotides are listed in Table S1 in the supplemental material. Growth and β-galactosidase experiments were performed in nutrient yeast broth (NYB) (45) or in basalt salt medium (BSM) (44) supplemented with different carbon sources (succinate, fumarate, malate, oxaloacetate, citrate, glucose, or mannitol) to a final concentration of 40 mM or with succinate to a final concentration of 5 μM. Growth was performed in 50-ml Erlenmeyer flasks filled with 20 ml of medium, with shaking at 180 rpm and at 37°C. Nutrient agar (NA) was used as a solid medium. When required, antibiotics were added to these media at the following concentrations: 100 μg of ampicillin/ml, 25 μg of tetracycline/ml, and 10 μg of gentamicin (Gm)/ml for *E. coli* and 300 μg of carbenicillin/ml, 50 μg of gentamicin/ml, and 125 μg of tetracycline/ml for *P. aeruginosa*.

Constructions of plasmids and gene replacement mutants. DNA cloning and plasmid preparation were performed according to standard methods (38). Large-scale preparations were performed using JETstar 2.0 (Genomed). Restriction and DNA-modifying enzymes were used according to the instructions of the manufacturers. Transformation of *E. coli* DH5α (for cloning) and *E. coli* XL1-Blue (for mutagenesis) and *P. aeruginosa* was carried out by electroporation (33).

A translational *dctA'-'lacZ* fusion was constructed by amplifying a 397-bp PCR fragment with primers Dct1 and Dct2b. This fragment was digested with EcoRI and PstI and cloned into the corresponding sites of pME6015. In the resulting plasmid pME9507 the promoter region of the *dctA* gene and the sequence encoding the first seven amino acids of *dctA* were fused to the *'lacZ* reporter. A transcriptional *dctA-lacZ* fusion was constructed using the primer pair Dct1 and Dct3x to amplify a 248-bp fragment containing the *dctA* promoter region. The PCR fragment was digested with EcoRI and PstI and ligated into the corresponding sites of pME6016, resulting in pME9506.

A translational *dctP'-'lacZ* fusion (pME10035) was constructed by inserting a 334-bp fragment, carrying the proximal part of the PA5167 gene, into the *'lacZ* gene of pME6015 previously digested EcoRI-BamHI. The 334-bp fragment was obtained by PCR using the EcoRI-tagged primer TRAP-trsc1 and the BamHI-tagged primer TRAP-trnl2 to amplify the PAO1 genome carrying the promoter region of the *dctPQM* (PA5167-PA5169) operon, the Shine-Dalgarno sequence, and the first three codons of the PA5167 gene. A transcriptional *dctP-lacZ* fusion was constructed by using the primer pair TRAP-trsc1 and TRAP-trsc2 to amplify a 171-bp fragment containing the *dctP* promoter region. The PCR fragment was digested with EcoRI and PstI and ligated into the corresponding sites of pME6016, resulting in pME10034.

The mutations in the RpoN box sequence T(GG→AC)CACAGCCTCT (GC→TT)A and T(GG→AC)CACAGGGCTT(GC→TT)T of the *dctA* and *dctP* promoter were introduced into pME9507 and pME10035, respectively, according to the QuikChange site-directed mutagenesis protocol with the mutagenesis primer pairs SDM1-SDM2 and SDMtrap1-SDMtrap2, respectively. The parental DNA template was digested with DpnI, and the mutated plasmid was transformed into *E. coli* XL1-Blue, generating pME9508 and pME10036.

For the inactivation of the *dctA* gene in the *P. aeruginosa* PAO1 chromosome, a 597-bp fragment containing the upstream region and the first two codons of *dctA* and a 600-bp fragment containing the *dctA* terminator were amplified by PCR using the primer pairs Mut1/Mut2 and Mut3/Mut4, respectively. These products were digested with EcoRI-BglII and BglII-HindIII, respectively, and cloned into the corresponding sites of the suicide vector pME3087, yielding plasmid pME10041. Plasmid pME10041, carried by *E. coli* DH5α, was then introduced into *P. aeruginosa* PAO1 by triparental mating, using the helper strain *E. coli* HB101(pRK2013). Merodiploids were resolved as previously described (57). The resulting strain, PAO6592, carried an in-frame Δ*dctA* mutation.

For inactivation of the PA5165 (*dctB*) locus, a 515-bp fragment overlapping the PA5165 upstream region and the first three codons and a 521-bp fragment overlapping the last codons of PA5165 and the downstream region were amplified by PCR using primer pairs p5165.1/p5165.2 and p5165.3/p5165.4, respectively. These products were digested with EcoRI-BglII and BglII-HindIII, respectively, and cloned into pME3087, yielding plasmid pME10031. Plasmid pME10031 was then used as described above to produce strain PAO6705 (Δ*dctB*).

A ΔPA5166 (Δ*dctD*) mutant of PAO1, constructed by amplifying a 506-bp fragment overlapping the ATG start codon of PA5166, and a 509-bp fragment overlapping the TGA stop codon of PA5166 were amplified by PCR using the primer pairs p5166.1/p5166.2 and p5166.3/p5166.4, respectively. These products were digested with EcoRI-BglII and BglII-HindIII, respectively, and cloned into pME3087, yielding plasmid pME10032. Plasmid pME10032 was then introduced into *P. aeruginosa* PAO1 as described above; after excision of the integrated plasmid, strain PAO6706 (Δ*dctD*) was obtained.

Analogous procedures were used to generate a *P. aeruginosa* PAO1 mutant (PAO6708) deleted in the PA5167-PA5169 (*dctPQM*) operon by using pME10033, a plasmid derived from the suicide vector pME3087, which had been digested with EcoRI-HindIII for insertion of a PCR fragment. The following primer pairs were used to create this fragment: pTRAP.1/pTRAP.2 (digested EcoRI-BglII) and pTRAP.3/pTRAP.4 (digested BglII-HindIII).

A double mutant (PAO6707) with the PA5165 *dctB* and the PA5166 *dctD* genes deleted and a double mutant (PAO6709) with the PA5167-PA5169 (*dctPQM*) operon and *dctA* gene deleted were obtained as follows. Plasmid pME10031 and plasmid pME10033 were crossed into PAO6706 and PAO6592, respectively, as described above, yielding strains PA6707 (PAO1 Δ*dctBD*) and PA6709 (PAO1 Δ*dctPQM* Δ*dctA*).

In all of the mutants described here, the deletions were confirmed by PCR, and the PCR fragments were checked by sequencing.

The deletions in strains PAO6592, PAO6705, PAO6706, and PAO6709 were complemented with fragments carrying, respectively, the *dctA*, *dctB*, *dctD*, and *dctPQM* genes. The fragments had been amplified by PCR with primer pairs Cd.1-Cd.2, C65.1-C65.2, C66.1-C66.2, and Ct.1-Ct.2, respectively, and subcloned into pMMB67HE under the control of an inducible *tac* promoter (resulting in plasmids pME10037 to pME10040). All plasmids were verified by sequencing.

Strain PAO6815 (*dctQ*::Tn*5*Gm) was obtained by transduction with phage E79*tv-2* (9). The phage preparations used for transduction were obtained as described previously (9). As a donor, strain PAO6710 was used, and the recipient PAO1 was infected with the phage, selecting for gentamicin resistance (50 μg/ml). Transductants were purified several times on selective medium and screened by PCR with the primers pTRAP.1 and tnpRL17-1.

β-Galactosidase assays. These were performed as described previously (26), with *P. aeruginosa* strains grown in BSM medium containing a unique carbon source (fumarate, succinate, malate, oxaloacetate, citrate, glucose, or mannitol) or in rich NYB medium. The data are mean values of three independent samples ± the standard deviations.

Transposon mutagenesis. About 5,000 random Tn*5*Gm insertions in strain PAO6592 (Δ*dctA*) were generated with plasmid pLM1 (Table 1), selecting for gentamicin resistance on NA amended with NYB. The growth of each mutant candidate was tested first on plates containing BSM minimal medium supplemented with 40 mM succinate. Then, replica plating onto BSM glucose (40 mM) and onto NA plates revealed auxotrophs which were discarded. Candidates that grew more slowly than strain PA6592 in the presence of succinate as the sole carbon source were retested in liquid media (BSM succinate, BSM glucose, and NYB). Genomic DNA of confirmed candidates was extracted by using the Wizard Genomic DNA purification kit (Promega), restricted with BamHI, self-ligated, and introduced into *E. coli* S17-1/λ*pir* by electroporation, with selection for gentamicin resistance. After isolation of the plasmid containing Tn*5*Gm and flanking host sequences, the transposon insertion site was determined by nucleotide sequencing with the transposon-specific primer tnpRL17-1 (9) and was localized on the PAO1 chromosome (55) using BLASTN analysis (1).

Competition experiments. A 20-ml culture in BSM containing 40 mM or 5 μM succinate was inoculated with a 50:50 mixture of exponential-phase cultures of the strains PAO6815 (*dctQ*::Tn*5*Gm) and PAO1 (wild-type), PAO6592 (Δ*dctA*) and PAO1 (wild type [WT]), or PAO6845 and PAO6592; the inoculum consisted of 100 CFU/ml. Serial dilutions were plated onto NA, NA-gentamicin, and BSM succinate plates to confirm the inoculum at t_0. After overnight growth, 100 cells of the BSM–40 mM succinate cultures (*dctQ*::Tn*5*Gm versus WT, Δ*dctA* versus WT, and *dctQ*::Tn*5*Gm versus Δ*dctA* strains) or 100 cells of the BSM 5 μM succinate cultures (*dctQ*::Tn*5*Gm versus WT, Δ*dctA* versus WT and *dctQ*::Tn*5*Gm versus Δ*dctA* strains) were reinoculated into fresh 20-ml cultures of BSM containing 40 mM or 5 μM succinate, respectively. Growth of the strains was determined at 3 and 5 h after reinoculation by appropriate serial dilutions and plating onto NA, NA-gentamicin, and BSM succinate plates, as described above. Strain PAO6592 was recognized by its poor growth on succinate and PAO6815 by its gentamicin resistance (Gm^r). The competitive index (CI) was defined as the mutant/WT or mutant/mutant ratio divided by the corresponding ratio in the inoculum (10, 25, 47). CI values are the means of three independent experiments ± the standard deviation. Each CI value was analyzed with a Student *t* test using as null hypothesis that the mean index was not significantly different from 1.0 ($P = 0.05$) (25).

RESULTS

Identification of the C_4-dicarboxylate transporter DctA in *P. aeruginosa* PAO1. In the *P. aeruginosa* PAO1 genome, the gene PA1183 is annotated as *dctA* and consists of a 1,311-bp open reading frame encoding a predicted C_4-dicarboxylate transport protein of 436 amino acid residues (55). *P. aeruginosa* PAO1 DctA has 74% amino acid sequence identity with *Escherichia coli* DctA (NP_417985.1), 79% amino acid sequence identity with *P. chlororaphis* 06 DctA (AAO60164.1), 55% amino acid sequence identity with *R. leguminosarum* DctA (YP-002282214), and 54% amino acid sequence identity with *S. meliloti* 1021 DctA (NP_438063).

To confirm that *dctA* is involved in C_4-dicarboxylic acid transport in *P. aeruginosa* PAO1, we constructed a *dctA* deletion mutant (PAO6592) and tested its growth in BSM mini-

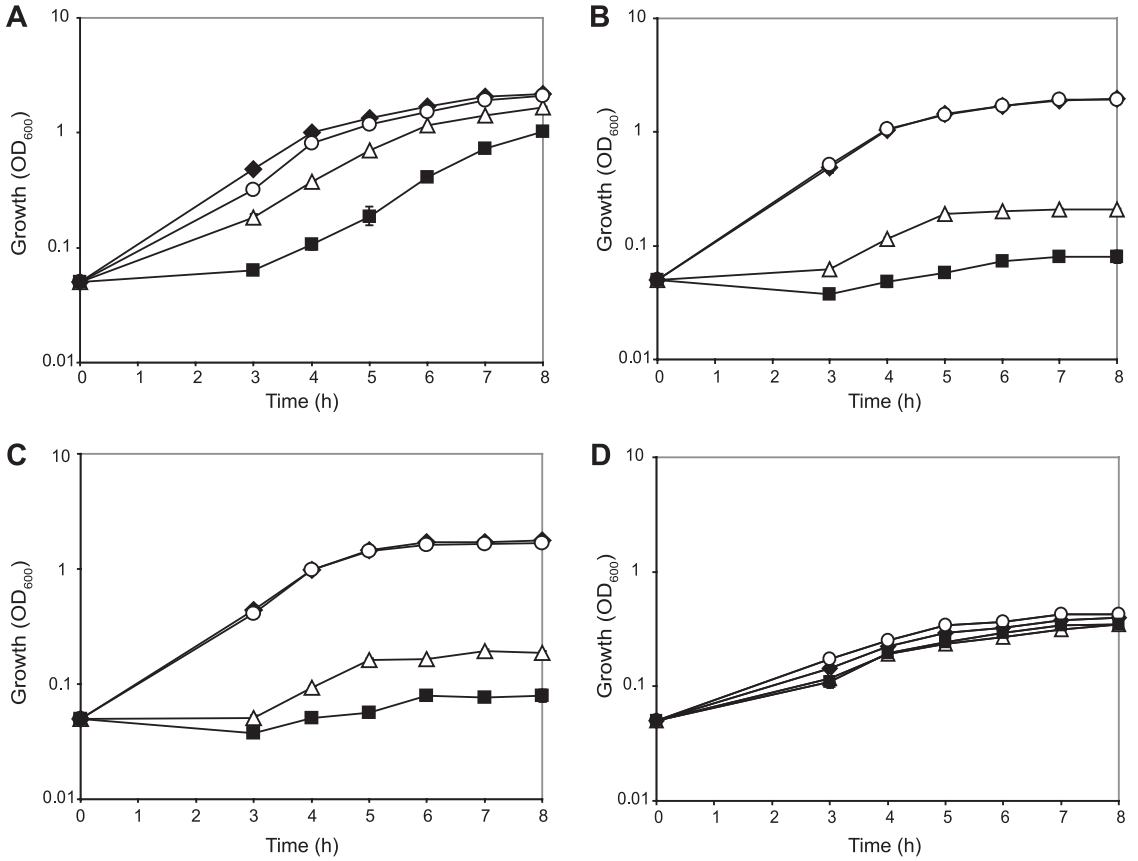

FIG. 1. Growth properties of the wild-type PAO1 and of mutant strains in minimal medium supplemented with C_4-dicarboxylates. Growth curves of the wild-type PAO1 (◆), the Δ*dctA* mutant PAO6592 (△), the Δ*dctPQM* mutant PAO6708 (○), and the Δ*dctA* Δ*dctPQM* mutant PAO6709 (■) in BSM minimal medium containing succinate (A), fumarate (B), malate (C), or oxaloacetate (D) as the unique carbon source (40 mM) are shown. Each value is the average of three different cultures ± the standard deviation. In some instances, the standard deviation bars are smaller than the symbols used.

mum medium supplemented with 40 mM the TCA cycle intermediates succinate, fumarate, malate, or oxaloacetate as the sole carbon source. The growth rate of the mutant on succinate was reduced ~2-fold compared to the wild-type growth rate (Fig. 1A). Furthermore, growth of PAO6592 was severely impaired on fumarate and malate but similar to that of the wild-type strain on oxaloacetate (Fig. 1B, C, and D). In contrast, in rhizobia and in *P. chlororaphis* O6, the growth of a *dctA* mutant is strongly impaired on succinate (6, 31, 36). Unlike *P. aeruginosa* PAO6592, the *dctA* mutant of *P. chlororaphis* O6 is still able to grow on malate (31). The complementing plasmid pME10037, carrying *dctA* under the control of the inducible *tac* promoter, fully restored the growth of the mutant PAO6592 in succinate, fumarate and malate media (see Fig. S1 in the supplemental material). These data indicate that DctA is specifically involved in the utilization of C_4-dicarboxylates, i.e., succinate, fumarate, and malate, but not in the utilization of oxaloacetate. The residual growth of the *dctA* mutant in the succinate, fumarate, and malate media suggests the presence of additional C_4-dicarboxylate transport system(s).

Search for a second C_4-dicarboxylate transporter in a PAO1 Δ*dctA* background. To identify new C_4-dicarboxylate transport system(s) in *P. aeruginosa* PAO1, we carried out transposon mutagenesis in the Δ*dctA* mutant PAO6592. Approximately 5,000 Tn*5* insertion mutants were generated. Of the three mutants displaying a severe growth defect on minimal medium supplemented with succinate, one was further studied. Its Tn*5* insertion was mapped to the PA5168 gene. This gene is proposed to be a probable dicarboxylate transporter gene showing 47% similarity to the *dctQ* gene product of *R. capsulatus* (55). Furthermore, the PA5168 gene is annotated as being part of the PA5167-PA5169 operon, encoding a TRAP-type C_4-dicarboxylate transport system. The flanking genes PA5167 and PA5169 are proposed, in the *Pseudomonas* database, to encode a probable periplasmic C_4-dicarboxylate-binding protein with 67% similarity to the *dctP* gene product of *R. capsulatus* and to encode a probable C_4-dicarboxylate-transporter with 72% similarity to the *dctM* gene product of *R. capsulatus*, respectively (55).

To demonstrate that the product of the PA5167-PA5169 (*dctPQM*) operon is involved in C_4-dicarboxylate transport in *P. aeruginosa* PAO1, we constructed the deletion mutants PAO6708 (Δ*dctPQM*) and PAO6709 (Δ*dctPQM* Δ*dctA*) and tested their growth rates in BSM supplemented with succinate, fumarate, malate, or oxaloacetate as the sole carbon source. The growth rate of the Δ*dctPQM* mutant on succinate, fumarate, malate, and oxaloacetate was similar to that of the wild-type strain (Fig. 1). The Δ*dctPQM* Δ*dctA* double mutant had an

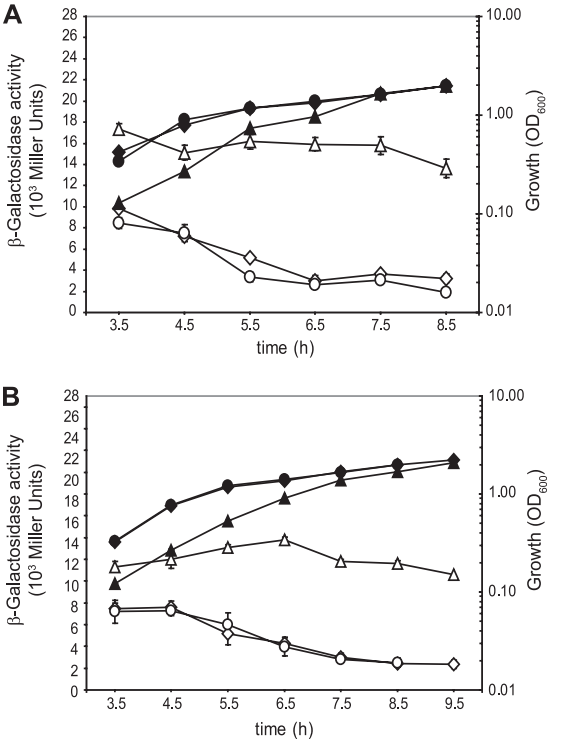

FIG. 2. Cell population density-dependent β-galactosidase expression (open symbols) of a *dctA′-′lacZ* fusion (pME9507) (A) and a *dctP′-′lacZ* fusion (pME10035) (B) in wild-type PAO1 (diamonds), Δ*dctA* mutant PAO6592 (triangles), and Δ*dctPQM* mutant PAO6708 (circles). Strains were cultivated in BSM amended with 40 mM succinate. Cell growth was monitored by measuring the optical density at 600 nm (OD_{600}) (solid symbols). Each value is the average of three different cultures ± the standard deviation.

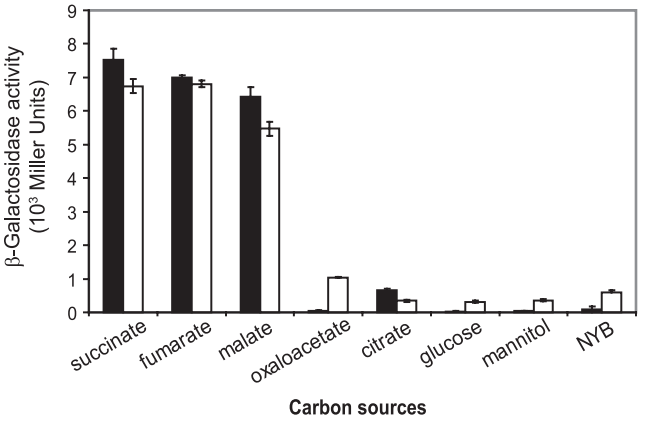

FIG. 3. β-Galactosidase activity of a *dctA′-′lacZ* (■) and a *dctP′-′lacZ* (□) fusion carried by plasmid pME9507 and pME10035, respectively, in wild-type PAO1 in BSM minimal medium amended with succinate, fumarate, malate, oxaloacetate, citrate, glucose, or mannitol or in rich NYB medium. Strains were cultivated to an OD_{600} of ~0.8, and each value is the average of three different cultures ± the standard deviation.

8-fold-lower growth rate compared to the wild-type strain on succinate and could not grow on fumarate and malate (Fig. 1A to C) but grew normally on oxaloacetate (Fig. 1D). The complementing plasmid pME10040, carrying the *dctPQM* operon under the control of the inducible *tac* promoter, restored the growth of the Δ*dctPQM* Δ*dctA* mutant to the level of the Δ*dctA* mutant on succinate (see Fig. S1 in the supplemental material). Taken together, these results indicate that the *dctPQM* operon and the *dctA* gene specify major systems required for the utilization of C_4-dicarboxylates, i.e., succinate, fumarate, and malate, but not for the utilization of oxaloacetate.

***dctA* and *dctPQM* expression depends on the growth phase and carbon sources.** The expression of the *dctA* gene and the *dctPQM* operon in *P. aeruginosa* PAO1 was monitored in succinate minimal medium by using translational *dctA′-′lacZ* and *dctP′-′lacZ* fusions (carried by plasmids pME9507 and pME10035, respectively). The expression of both reporter fusions was maximal in early exponential growth phase and declined with cell density to reach a plateau in the stationary growth phase (Fig. 2). As a control, a similar expression pattern for *dctA* was observed with the use of a chromosomal *dctA′-′lacZ* fusion (data not shown).

The expression of the *dctA* gene and the *dctPQM* operon was induced by the C_4-dicarboxylates succinate, fumarate, and malate but was low in the presence of oxaloacetate, citrate, glucose, mannitol, or NYB, as revealed by using translational *dctA′-′lacZ* and *dctP′-′lacZ* fusions (Fig. 3).

The Δ*dctA* mutant outcompetes the *dctQ*::Tn*5* mutant at a low succinate concentration. To investigate the specific functions of the DctA and DctPQM transporters, we performed growth competition experiments in minimal medium supplemented with either 5 μM or 40 mM succinate. In 5 μM succinate, as expected, the wild-type PAO1 had a competitive advantage over both the *dctQ*::Tn*5* PAO6815 and the Δ*dctA* PAO6592 mutants after 3 and 5 h of reincubation (Fig. 4A and B). Interestingly, under these conditions, the PAO1Δ*dctA* mutant showed a competitive advantage over the PAO1*dctQ*::Tn*5* mutant after 3 and 5 h, demonstrating that the DctPQM transporter is more efficient than the DctA transporter for utilization of succinate in the micromolar range.

A similar experiment was performed in minimal medium supplemented with 40 mM succinate. Again, the wild-type PAO1 had a competitive advantage over the *dctQ*::Tn*5* and the Δ*dctA* mutants strains after 3 and 5 h of reincubation (Fig. 4C and D). However, now the *dctQ*::Tn*5* mutant outcompeted the Δ*dctA* mutant, indicating that the DctA transporter is more efficient than the DctPQM transporter for utilization of succinate in the millimolar range.

The expression of *dctA* and *dctPQM* is negatively regulated by DctA and appears not to be regulated by DctPQM. Previous work on rhizobia and *E. coli* has shown that the expression of *dctA* is increased in a Δ*dctA* mutant, suggesting that DctA controls its own synthesis (4, 20, 35, 55). To test the autoregulation of DctA and the possible regulation of the *dctPQM* operon by DctA in *P. aeruginosa* PAO1, we measured the expression of a *dctA′-′lacZ* and a *dctP′-′lacZ* translational fusion in the wild-type PAO1 and in the Δ*dctA* mutant PAO6592 growing in BSM amended with 40 mM succinate. The expression of both reporter constructs was increased 2.5-fold in the Δ*dctA* mutant compared to the wild-type (Fig. 2). In contrast, both constructs gave similar β-galactosidase activities in the wild-type PAO1 and in the Δ*dctPQM* mutant PAO6708, indi-

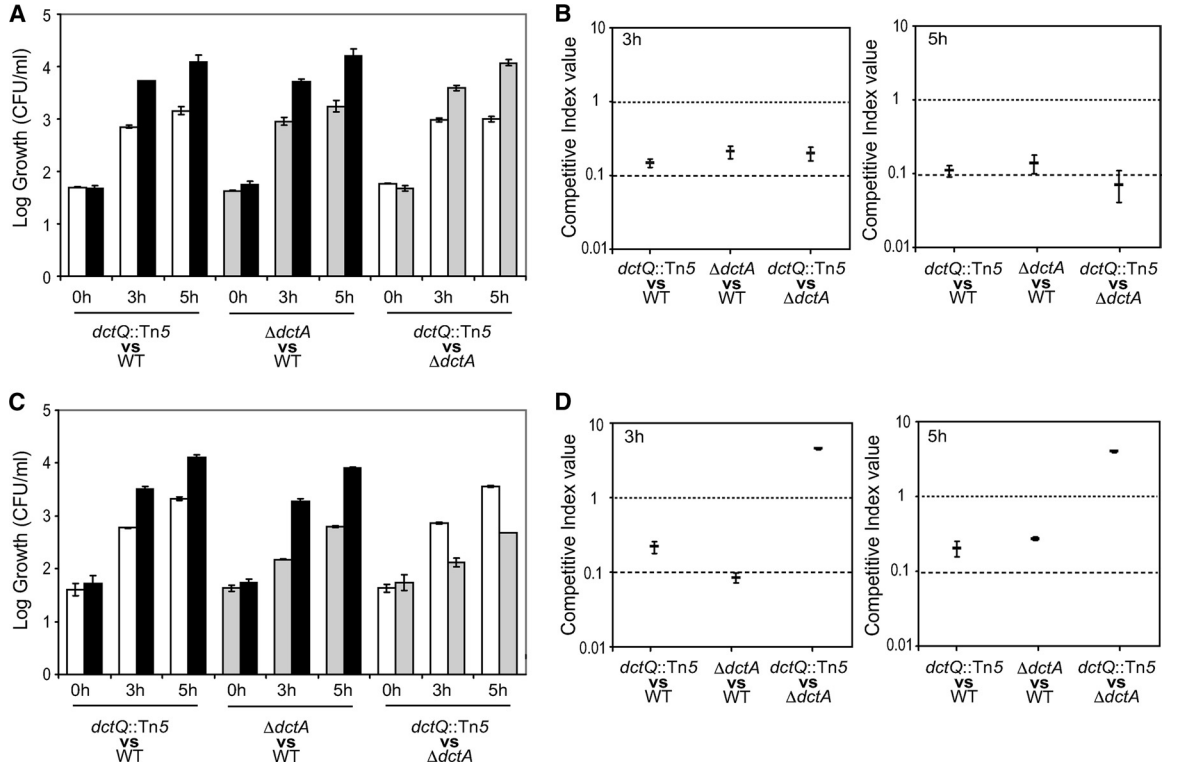

FIG. 4. Competitive abilities of wild-type PAO1 and Δ*dctA* and *dctQ*::Tn*5* mutant strains. Cultures were generated from mixed inoculations of wild-type PAO1 (■) and *dctQ*::Tn*5* mutant PAO6815 (□), wild-type PAO1 and Δ*dctA* mutant PAO6592 (▨), and *dctQ*::Tn*5* and Δ*dctA* mutants. The cultures were grown in BSM medium supplemented with 5 μM (A and B) or 40 mM (C and D) succinate, and each population size was recorded after 0 h of cell growth or after 3 or 5 h of cell growth after reinoculation (see Materials and Methods) (A and C). Competition index values are generated for each competition experiment in 5 μM (B) or 40 mM (D) succinate and defined as the mutant/wild-type or mutant/mutant ratio within the different time points, divided by the initial ratio (0 h) in the inoculum (see Materials and Methods). The competitive index values are mean values of three different cultures. Error bars represent the standard deviation.

cating that DctPQM does not control its own synthesis nor that of DctA in this condition (Fig. 2). The same result was obtained with transcriptional reporter fusions (see Fig. S2 in the supplemental material).

Complementation of the Δ*dctA* mutant PAO6592 with the plasmid pME10037, carrying the *dctA* gene, restored the β-galactosidase activity of the *dctA′-′lacZ* and *dctP′-′lacZ* fusions to the wild-type level (see Fig. S3 in the supplemental material).

The expression of *dctA* and *dctPQM* is positively regulated by RpoN. Previous studies in rhizobia and *P. chlororaphis* have demonstrated that the transcription of the C_4-dicarboxylate transporter *dctA* is activated by the alternative sigma factor RpoN (30, 37). RpoN binds to promoters with the consensus sequence (T**GG**CAC-N_5-TT**GC**W) at −24/−12 upstream of the transcription start site (3). In the *dctA* and *dctP* promoter regions, highly conserved RpoN binding sites, T**GG**CACAGC CTCT**GC**A and T**GG**CACAGGGCTT**GC**T, respectively, were found. The expression of the translational *dctA′-′lacZ* and *dctP′-′lacZ* fusions was tested in the wild-type PAO1 and in the Δ*rpoN* mutant PAO6358 growing in NYB. Rich medium was used in this experiment because of the growth defect of the Δ*rpoN* mutant in minimal medium supplemented with succinate, fumarate, or malate (Fig. 5A). In the Δ*rpoN* mutant, *dctA* and *dctPQM* expression was abolished (Fig. 5B and C). To confirm regulation by RpoN, we constructed *dctA′-′lacZ* and *dctP′-′lacZ* fusions carrying mutations in the RpoN-binding site (pME9508 and pME10036, respectively) and tested them in the PAO1 wild-type strain growing in minimal medium with succinate. As expected, the expression of both *dctA* and *dctP* mutated in the RpoN box was lost (Fig. 5B and C), showing that RpoN initiates the transcription of *dctA* and *dctPQM*.

Identification of a two-component system regulating C_4-dicarboxylate utilization in *P. aeruginosa* PAO1. Promoters activated by the alternative sigma factor RpoN require another transcriptional regulatory protein for their activation (49). In the case of C_4-dicarboxylates, RpoN requires the two-component system DctB/DctD for *dctA* regulation in rhizobia and DctS/DctR for *dctPQM* regulation in *R. capsulatus*. Therefore, to identify the two-component system(s) in *P. aeruginosa* PAO1 regulating *dctA* and *dctPQM*, we searched for *dctB*/*dctD*-like two-component systems in the *Pseudomonas* database (55). Three *dctB*/*dctD* candidate genes were identified: PA1336/PA1335 with 48 and 67% nucleotide sequence identity, PA5165/PA5166 with 49 and 67% nucleotide sequence identity, and PA5512/PA5511 with 51 and 69% nucleotide sequence identity to *S. meliloti dctB*/*dctD*, respectively (55), whereas no significant homology with *R. capsulatus dctS*/*dctR* was observed. Interestingly, the candidate PA5165/PA5166 is located upstream of the *dctPQM* operon. To determine which

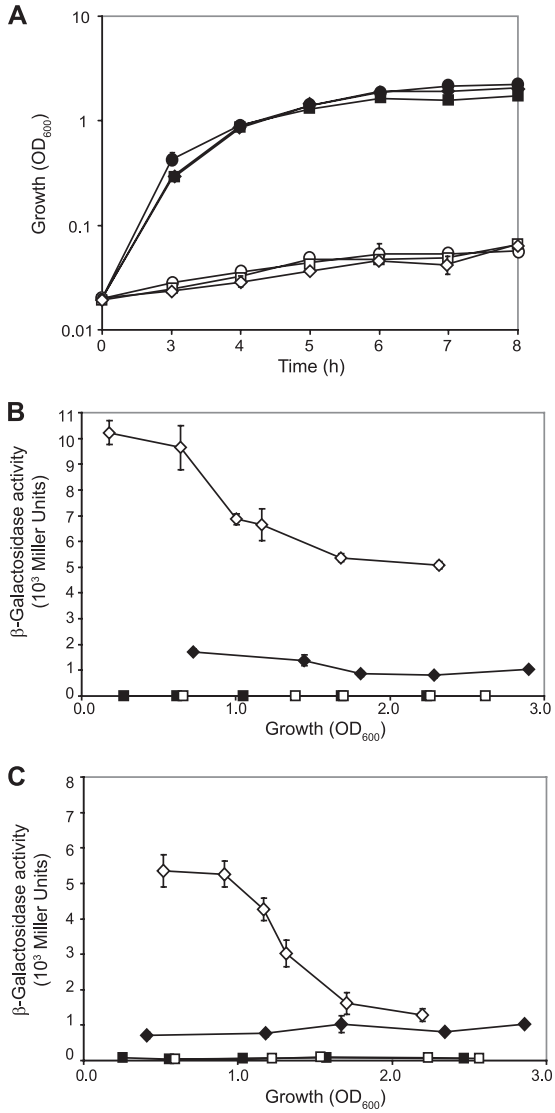

FIG. 5. Regulation of *dctA* and *dctPQM* gene expression by RpoN. (A) Growth of wild-type PAO1 (filled symbols) and the Δ*rpoN* mutant PAO6358 (open symbols) in BSM minimal medium amended with succinate (diamonds), fumarate (circles), or malate (squares) as aunique carbon source (40 mM). (B) Cell density-dependent β-galactosidase expression of a *dctA′-′lacZ* translational fusion (pME9507) in the wild-type PAO1 (◆) and the Δ*rpoN* mutant PAO6358 (■) grown in NYB medium and in PAO1 wild-type (◇) in BSM medium amended with 40 mM succinate. Values of a *dctA′-′lacZ* translational fusion mutated in the RpoN-box (pME9508) are given for the PAO1 wild-type (□) grown in BSM medium amended with 40 mM succinate. (C) Cell density-dependent β-galactosidase expression of a *dctP′-′lacZ* translational fusion (pME10035) in the wild-type PAO1 (◆) and the Δ*rpoN* mutant (■) grown in NYB medium and in the wild-type PAO1 (◇) in BSM medium amended with 40 mM succinate. Values of a *dctP′-′lacZ* translational fusion mutated in the RpoN-box (pME10036) are given for the wild-type PAO1 (□) grown in BSM medium amended with 40 mM succinate. Each value is the average of three different cultures ± the standard deviation. In some instances, the standard deviation bars are smaller than the symbols used.

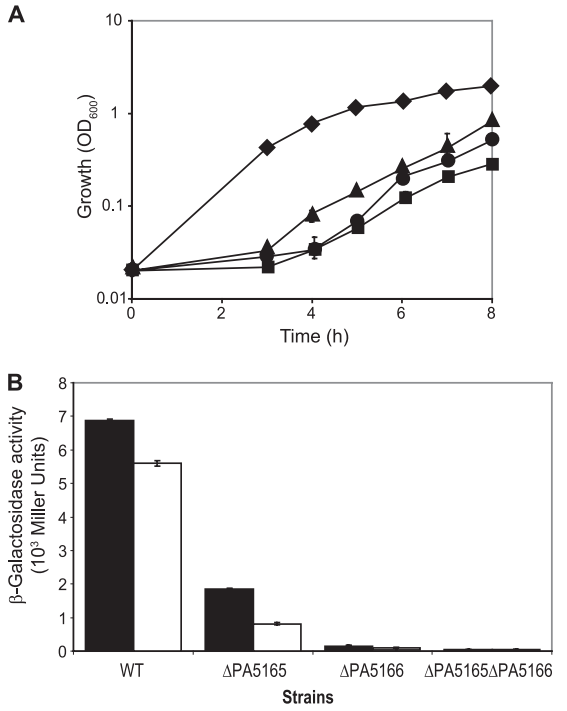

FIG. 6. Regulation of succinate uptake by PA5165-PA5166 (DctB-DctD). (A) Growth properties of the wild-type PAO1 (diamonds), the ΔPA5165 mutant PAO6705 (triangles), the ΔPA5166 mutant PAO6706 (circles), and the ΔPA5165 ΔPA5166 mutant PAO6707 (squares) in BSM medium containing 40 mM succinate. Each value is the average of three different cultures ± the standard deviation. (B) β-Galactosidase activities of a *dctA′-′lacZ* (■) and *dctP′-′lacZ* fusion (□) (pME9507 and pME10035, respectively) in wild-type PAO1, ΔPA5165 mutant (PAO6705), ΔPA5166 mutant (PAO6706), and ΔPA5165 ΔPA5166 mutant (PAO6707). Cultures were grown aerobically to an OD_{600} of ~0.8 in BSM medium containing 40 mM succinate. Each value is the average of three different cultures ± the standard deviation. In some instances, the standard deviation bars are smaller than the symbols used.

of the three candidate gene pairs might be relevant for C_4-dicarboxylate uptake in PAO1, we constructed deletion mutants and tested them for their ability to grow on minimal medium supplemented with succinate. The ΔPA5165 ΔPA5166 double mutant (PAO6707) and the ΔPA5165 (PAO6705) and ΔPA5166 (PAO6706) single mutants were the only mutants showing a growth defect on succinate compared to the wild-type PAO1 (Fig. 6A). On fumarate and malate the same growth defects as on succinate were observed (data not shown). These results indicate that PA5165-PA5166 is involved in C_4-dicarboxylate utilization. However, residual growth of the ΔPA5165 ΔPA5166 mutant suggests the existence of additional systems regulating C_4-dicarboxylate utilization (Fig. 6A).

The expression of *dctA* and *dctPQM* is activated by the two-component system PA5165-PA5166. To examine further the regulatory role of the predicted two-component system PA5165-PA5166, we tested the expression of the translational *dctA′-′lacZ* and *dctP′-′lacZ* fusions in the wild-type PAO1, the ΔPA5165 and the ΔPA5166 single mutants and the ΔPA5165 ΔPA5166 double mutant. The expression of both reporter fusions was strongly reduced in the ΔPA5165 and the ΔPA5166 single mutants and abolished in the ΔPA5165 ΔPA5166 double mutant (Fig. 6B). The Δ*dctB* PAO6705 and Δ*dctD* PAO6707 mutants were complemented with the plasmids pME10038 and pME10039, respectively, restoring the growth (see Fig. S1 in the supplemental material) and β-galactosidase activity of the

dctA′-′lacZ and *dctP′-′lacZ* fusions (see Fig. S3 in the supplemental material) to the wild-type level. We conclude from these data that PA5165-PA5166 is a DctB/DctD-like two-component system activating the expression of both the *dctA* gene and the *dctPQM* operon.

DISCUSSION

P. aeruginosa utilizes preferentially TCA cycle intermediates such as malate, fumarate, and succinate as carbon and energy sources (24, 28). However, their uptake in *Pseudomonas* had not been studied and, for this reason, we decided to investigate C_4-dicarboxylate utilization in this species.

Using a genetic approach, we discovered that a low-affinity system (DctA) and a high-affinity system (DctPQM) together account for most of C_4-dicarboxylate transport in strain PAO1. *P. aeruginosa* is the first organism investigated that uses such a dual strategy. Even though growth of a Δ*dctPQM* Δ*dctA* double mutant was dramatically reduced on succinate, residual growth was still observed, suggesting that succinate transport is catalyzed by multiple carriers in *P. aeruginosa* PAO1, whereas fumarate and malate transport are only catalyzed by the DctA and DctPQM carriers. In the PAO1 genome, two additional *dctPQM*-like operons (PA0884-PA0886 and PA3779-PA3781) are predicted (55), and whether they act as succinate transporters remains to be determined.

The expression of *dctA* and *dctPQM* was growth phase dependent, being maximal in the early exponential growth phase and induced by succinate, malate, and fumarate (Fig. 2 and 3). The *dctA* pattern of expression in strain PAO1 is in contrast to the expression of the *E.coli dctA* gene, which is enhanced in the stationary phase (31). As in rhizobia and *P. chlororaphis* (30, 46), the alternative sigma factor RpoN activates the expression of the *dctA* gene and of the *dctPQM* operon (Fig. 5) and, as in rhizobia and *E. coli* (4, 35, 36, 52, 53), DctA controls negatively its own synthesis, as well as the synthesis of DctPQM. Previous work on *Rhizobium* and *E. coli* has proposed that in the absence of substrates, the DctA and DctB proteins interact with each other in the cytoplasmic membrane, leading to inhibition of the autophosphorylation of DctB and consequently to low expression of the C_4-dicarboxylic acid transport system (58, 20, 56, 35, 54). Therefore, we propose that in the absence of DctA, DctB would be in a permanently active state in *P. aeruginosa*. The fact that the expression of a *dctA′-′lacZ* and of the *dctPQM* operon is constitutively activated in a *dctA* mutant is in favor of this model. Interestingly, in 40 mM succinate, DctPQM does not seem to regulate its own synthesis nor that of DctA, suggesting that DctPQM may not interact with DctB in this condition. To decipher the regulation of the Dct system in *P. aeruginosa* PAO1, we searched for a two-component system sensing C_4-dicarboxylates and responding by activating the expression of *dctA* and *dctPQM*. We found that a ΔPA5165 ΔPA5166 mutant showed a growth defect on succinate and that the expression of *dctA* and *dctPQM* was abolished in this mutant, indicating that PA5165-PA5166 is involved in C_4-dicarboxylate utilization in PAO1 (Fig. 6).

Since the PA5165-PA5166 system regulates the expression of *dctA*, belonging to the DAACS family, and that of *dctPQM*, belonging to the TRAP family, we next addressed the question whether the two-component system PA5165-PA5166 is similar to the two-component system DctB/DctD of rhizobia and *E. coli* or to the two-component system DctS/DctR of *R. capsulatus*. The sensor-regulator pair DctB/DctD belongs to the NtrB/NtrC family, whereas the DctS/DctR system belongs to the FixL/FixJ family (19). The sensor kinase proteins of the NtrB family consist of a PAS periplasmic sensing domain at the N terminus, a histidine kinase A domain, and a histidine kinase-like ATPase domain at the C terminus. The response regulator proteins of the NtrC family are characterized by a CheY-homologous receiver domain in the N-terminus region, an AAA ATPase domain and an FIS helix-turn-helix motif in the C-terminus region. The sensor kinase proteins of the FixL family contain two transmembrane segments in the N-terminus region, a PAS domain, PAC motifs, a histidine kinase A domain, and a histidine kinase-like ATPase domain in the C terminus. The response regulator proteins of the FixJ family consist of a CheY-homologous receiver domain in the N-terminus region and a LuxR helix-turn-helix motif in the C-terminus region (5). The PA5165/PA5166 amino acid sequence was analyzed by using the SMART program (http://smart.embl.de/). PA5165 is predicted to contain transmembrane segments, a histidine kinase A domain, and a histidine kinase-like ATPase domain at the C terminus, whereas PA5166 consists of a CheY-homologous receiver domain at the N terminus, an AAA ATPase domain, and a FIS helix-turn-helix motif at the C terminus (see Fig. S4 and S5 in the supplemental material). Additionally, PA5165/PA5166 show sequence similarity to DctB/DctD of *S. meliloti* and NtrB/NtrC of *P. aeruginosa* and no significant sequence homology to DctR/DctS of *R. capsulatus* (seeFig. S4 and S5 in the supplemental material). We conclude that PA5165/PA5166 is a DctB/DctD two-component system.

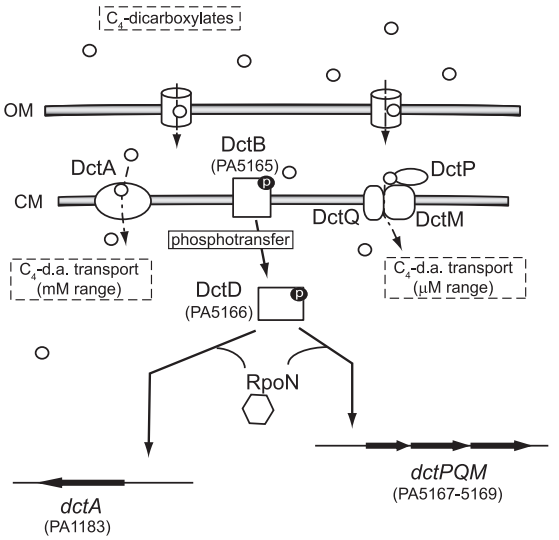

FIG. 7. Model for C_4-dicarboxylate transport in *P. aeruginosa* PAO1. C_4-dycarboxylates (C_4-d.a.) trigger the activation of the DctB/DctD two-component system (PA5165/PA5166) enabling RNA polymerase with the RpoN sigma factor to transcribe the *dctA* gene (PA1183) and the *dctPQM* operon (PA5167-PA5169). DctA is the major transporter for utilization of succinate in the mM range, whereas DctPQM transporter is more effective in the μM range. →, Positive effect.

In conclusion, the DctA and DctPQM carriers function coordinately for C_4-dicarboxylate uptake and *dctA* and *dctPQM* expression is regulated by the same two-component system DctB/DctD (Fig. 7).

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