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Energy Metabolism and Alginate Biosynthesis in *Pseudomonas aeruginosa*: Role of the Tricarboxylic Acid Cycle

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Infection with mucoid, alginate-producing strains of *Pseudomonas aeruginosa* is the leading cause of mortality among patients with cystic fibrosis. Alginate production by *P. aeruginosa* is not constitutive but is triggered by stresses such as starvation. The *algR2* (also termed *algQ*) gene has been previously identified as being necessary for mucoidy; an *algR2* mutant strain is unable to produce alginate when grown at 37°C. We show here that the levels of phosphorylated succinyl coenzyme A synthetase (Scs) and nucleoside diphosphate kinase (Ndk), which form a complex in *P. aeruginosa*, are reduced in the *algR2* mutant. We were able to correlate the lower level of phosphorylated Scs with a decrease in Scs activity. Western blots (immunoblots) also showed a decreased level of Ndk in the *algR2* mutant, but the presence of another kinase activity sensitive to Tween 20 provides the missing Ndk function. The effect of AlgR2 on tricarboxylic acid (TCA) cycle enzymes appears to be specific for Scs, since none of the other TCA cycle enzymes measured showed a significant decrease in activity. Furthermore, the ability of the *algR2* mutant to grow on TCA cycle intermediates, but not glucose, is impaired. These data indicate that AlgR2 is responsible for maintaining proper operation of the TCA cycle and energy metabolism.

Cystic fibrosis (CF) is the most common inheritable disease among the Caucasian population. The defect in CF occurs in the CF transmembrane conductance regulator, a chloride channel in the cell membrane. The production of a defective CF transmembrane conductance regulator results in abnormal ion transport and the clogging of the respiratory tract by a thick mucus (4). The CF lung is fertile ground for bacterial infections, particularly by *Staphylococcus aureus*, *Haemophilus influenzae*, and *Pseudomonas aeruginosa*. Although *S. aureus* and *H. influenzae* can be controlled through antibiotic therapy, *P. aeruginosa* is more difficult to control and eventually becomes dominant in the chronically infected CF lung, where it also undergoes genotypic transition to a mucoid, alginate-producing form. Alginate is a viscous exopolysaccharide consisting of D-mannuronic acids and L-guluronic acids (27). The role of alginate has recently been reviewed (26, 40), particularly with regard to its ability to prevent both opsonic (1) and nonopsonic (20) phagocytosis, thus protecting the cell from the host's immune response. Alginate is believed to prevent penetration of antibiotics inside the infecting cells and to aid the adherence of cells to the epithelial cells of the respiratory tract (27). Microcolonies of mucoid *P. aeruginosa* colonize the CF lung as a biofilm on the surface of the upper respiratory tract epithelium, which provides further protection against the dehydrating environment of the CF lung (3, 21). It has also been shown that mucoid *P. aeruginosa* cells are better able to adapt to and survive in a nutritionally poor environment than nonmucoid cells (45).

Several lines of evidence implicate the environment of the CF lung as a key factor in causing the conversion of *P. aeruginosa* from the nonmucoid to the mucoid phenotype. First is the prevalence of mucoid *P. aeruginosa* in the CF lung and its virtual absence in the healthy lung. Although approximately 90% of CF patients may be infected by mucoid *P. aeruginosa*, fewer than 2% of healthy lungs or lungs of bronchitis patients or patients suffering from other respiratory illnesses are colonized by mucoid cells. Second, infections of other sites such as the urinary tract and burns by *P. aeruginosa* rarely result in colonization by mucoid cells. Third, continued cultivation of mucoid clinical isolates outside the CF lung results in their rapid conversion to the nonmucoid form. Much effort has been devoted to identifying the environmental signals responsible for alginate production. Several studies have identified growth conditions which lead to the appearance of mucoid colonies. For example, growth on ethanol (8) or in chemostat under energy-limiting conditions (44) or, in general, growth under conditions of nutrient deprivation (43) has led to various fractions of the cells undergoing transition to mucoidy. A common theme emerging from these findings is that the energy status of the cell has an important role in the conversion to mucoidy (45). In addition to environmental factors, genetic changes such as mutations in the *muc* loci (7, 10, 13, 34) or the insertion of transposons in the region upstream of the *toxA* gene (41) result in the transition to mucoidy.

The conversion of nonmucoid *P. aeruginosa* into the alginate-producing form is a complex developmental phenomenon. Several regulatory genes controlling alginate biosynthesis have been described (26, 27). These include *algR1* (*algR*) and *algB*, two genes homologous to the response regulator class of two-component systems; *algR3* (*algP*), a histone-like protein; *algU* (*algT*), which shows homology to a *Bacillus* sigma factor (25); and *algR2* (*algQ*). Studies of these genes have largely centered on their effects on the expression of the critical *algD* gene (7, 10, 17) encoding GDP-mannose dehydrogenase, as well as the *algC* gene encoding phosphomannose mutase (11, 51). Despite all of the studies on alginate regulation and biosynthesis, cellular biochemical changes (outside the synthesis of alginate) have not been well studied. Recently, an enzyme developmentally regulated in both prokaryotes and

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eukaryotes, nucleoside diphosphate (NDP) kinase (Ndk), has been purified from *P. aeruginosa* (18). It was found to copurify with succinyl coenzyme A (succinyl-CoA) synthetase (Scs), an enzyme of the tricarboxylic acid (TCA) cycle. We wanted to explore the possibility that these enzymes are regulated in alginate-producing cells, providing insight into how cellular metabolism functions in alginate-producing cells. In this report, we described the effects of a mutation in the *algR2* gene on the activity of Scs and Ndk and how this in turn affects operation of the TCA cycle and alginate synthesis.

MATERIALS AND METHODS

Bacterial strains and plasmids. *P. aeruginosa* 8830 is a stable alginate-producing strain (5). Strain 8830R2::Cm was derived from 8830 by inserting a chloramphenicol resistance gene cassette into a unique *Bgl*II site in the *algR2* coding region. For selection, chloramphenicol was included in *Pseudomonas* isolation agar (Difco) at 300 μg/ml. Plasmids pJK662 and pJK664 (17) contain *algR2* under its own promoter and under the *tac* promoter, respectively. Both pJK662 and pJK664 are derivatives of pMMB66EH (12). For selection of plasmid-harboring cells, carbenicillin was used at 300 μg/ml in Luria broth and 100 μg/ml in morpholine propanesulfonic acid (MOPS) minimal medium. For induction of the *tac* promoter, isopropylthiogalactopyranoside (IPTG) was included to a final concentration of 1 mM.

Autophosphorylation assay of succinyl-CoA synthetase and NDP kinase. To determine the levels of phosphorylated succinyl-CoA synthetase (Scs) and NDP kinase (Ndk), cells were grown for 18 h in Luria broth. The cells were disrupted, and proteins were autophosphorylated with [γ-^{32}P]ATP as described previously (18).

Western blotting (immunoblotting). To measure Ndk levels by Western blotting, 100-ml cultures were grown for 18 h in Luria broth. Cells were collected by centrifugation, washed with sterile 0.9% saline, resuspended in 50 mM Tris-HCl (pH 8.0), and disrupted by sonication. Protein samples were electrophoresed in a sodium dodecyl sulfate (SDS)–15% polyacrylamide gel and electroblotted onto a GeneScreen Plus membrane (NEN Research Products, Boston, Mass.). The membrane was incubated with antibody against *P. aeruginosa* Ndk (diluted 1:100). The blot was next reacted with ^{125}I-protein A (DuPont) to a final concentration of approximately 2.5×10^6 cpm/ml. Following washing, the image was developed by autoradiography. The radioactivity of the spots was quantitated by counting individual blot slices on a Pharmacia LKB Wallac Minigamma 1504 gamma counter.

Growth curves. Growth curves were obtained by growing the indicated strains for 16 to 18 h in Luria broth. This culture was used to inoculate 3.0 ml of MOPS minimal medium (32) with the carbon source at 20 mM and histidine at 50 μg/ml. The test tubes (13 by 100 mm) were shaken at 250 rpm at 37°C, and growth was monitored by measuring the A_{600} with a Bausch and Lomb Spectronic 20.

Enzyme assays. Cells for enzyme assays were grown as 100-ml cultures for 18 h in Luria broth. Cells were collected by centrifugation, washed with sterile 0.9% NaCl, resuspended in 5 ml of 50 mM Tris-HCl (pH 7.6), and disrupted by sonication with a Branson model 450 Sonifier. Debris was removed by centrifugation at 15,000 rpm for 20 min, and extracts were used for enzyme assays. The TCA cycle enzymes were assayed according to published procedures. Aconitase (EC 4.2.1.3) activity was determined as described by Hanson and Cox (15). Fumarase (EC 4.2.1.2) was assayed by the method of Racker (36), using L-malate as the substrate. Isocitrate dehydrogenase (EC 1.1.1.42) and citrate synthetase (EC 4.1.3.7) were assayed as described by Jackson and Dawes (16), and succinate dehydrogenase (EC 1.3.99.1) was assayed as described by Veeger et al. (49). Succinyl-CoA synthetase (EC 6.2.1.6) was assayed as described by Bridger et al. (2), using the 45 to 65% $(NH_4)_2SO_4$ fraction of crude extract (18). NDP kinase (EC 2.7.4.6) was assayed by using crude extract as described by Kavanaugh-Black et al. (18). Where indicated, Tween 20 was included at a final concentration of 0.05%. The thin-layer chromatography plates were quantitated with an AMBIS Radioanalytic Imaging System (AMBIS Systems Inc., San Diego, Calif.). Substrates and reagents for enzyme assays were from Sigma Chemical Co. (St. Louis, Mo.). Protein concentration was determined using the Bio-Rad protein assay kit (Bio-Rad, San Diego, Calif.), using bovine serum albumin as a standard.

RESULTS

Construction of an *algR2* insertional mutant. For this study, we constructed an insertional mutant of the *algR2* gene. The *algR2* gene was first identified by virtue of its ability to complement a nonmucoid mutagen-induced strain, 8882 (17). We constructed an insertional mutant by cloning a chloramphenicol resistance gene from pCm7 (Pharmacia) into the unique *Bgl*II site in the coding region of *algR2*. The mutated *algR2* gene was cloned into pKTY320, a suicide vector unable to replicate in *Pseudomonas* species (19). The *algR2*::Cm cassette was introduced into the stable mucoid strain 8830 by triparental mating, and exconjugates were selected on *Pseudomonas* isolation agar (Difco) with chloramphenicol selection at 37°C. Nonmucoid colonies were obtained and scored for carbenicillin sensitivity. pKTY320 encodes carbenicillin resistance, and selecting for chloramphenicol resistance and carbenicillin sensitivity identifies products of double-crossover events. Insertional inactivation was demonstrated by Southern blotting of both 8830 and 8830R2::Cm genomic DNA, using a radiolabeled internal fragment of the *algR2* coding region as a probe (data not shown). The nonmucoid 8830R2::Cm was complemented to mucoidy by *algR2* in *trans* on plasmid pJK662 or pJK664.

Phosphorylation of Scs and Ndk in an *algR2* mutant background. A mutation in *algR2* renders the mucoid strain 8830 nonmucoid, demonstrating the requirement of functional AlgR2 for mucoidy at 37°C (17). The *algR2* mutation has also been shown to result in decreased expression of the *algD* promoter (6, 17). In *P. aeruginosa*, succinyl-CoA synthetase (Scs) and NDP kinase (Ndk) exist as a complex (18). Because both Ndk and Scs can be identified by their ability to autophosphorylate, we first sought to examine the effect of the *algR2* mutation on Ndk and Scs phosphorylation. $(NH_4)_2SO_4$ fractions of crude extract were incubated with [γ-^{32}P]ATP for a fixed time and subjected to SDS-polyacrylamide gel electrophoresis and autoradiography as described in Materials and Methods. Figure 1 shows the results of the phosphorylation assay. Compared with the parental strain 8830, the *algR2* knockout mutant clearly shows a decrease in autophosphorylation of both Scs and Ndk. Furthermore, when *algR2* is supplied to the *algR2* mutant in *trans* on pJK664, the levels of phosphorylation of both proteins return to parental levels. This result indicates that AlgR2 does have an important role in maintaining the levels of phosphorylated Scs and Ndk. The *algR2* mutation renders a mucoid cell nonmucoid at normal growth temperatures and also decreases the level of phosphorylated Scs and Ndk. When *algR2* is restored in *trans*, both alginate production and the levels of phosphorylated Scs and Ndk are returned to parental levels. This finding suggests a link

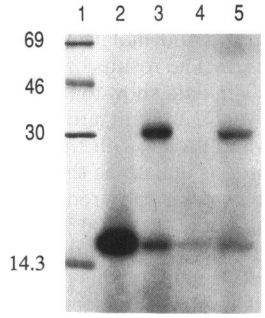

FIG. 1. Autophosphorylation of succinyl-CoA synthetase (Scs) and NDP kinase (Ndk) in an *algR2* mutant. Lanes: 1, molecular mass markers (sizes are shown in kilodaltons); 2, autophosphorylation of purified 16-kDa Ndk; 3 to 5, autophosphorylation of 33-kDa Scs and 16-kDa Ndk in partially purified 45 to 65% $(NH_4)_2SO_4$ fractions of 8830/pMMB66EH, 8830R2::Cm/pMMB66EH, and 8830R2::Cm/pJK664, respectively.

between alginate production and the levels of phosphorylated Scs and Ndk.

Activities of Scs and Ndk in an *algR2* mutant background. Given the differences in the level of phosphorylation of Scs and Ndk in the *algR2* knockout mutant, we wanted to examine how the level of phosphorylation affected enzyme activity. Succinyl-CoA synthetase specific activities were assayed as described in Materials and Methods and found to be 2.6, 0.2, and 1.9 U for strains 8830/pMMB66EH, 8830R2::Cm/pMMB66EH, and 8830R2::Cm/pJK664, respectively. The *algR2* knockout mutant shows a large decrease in Scs activity. Furthermore, the drop in activity seen in the *algR2* mutant strain is significantly restored when *algR2* is supplied in *trans* on pJK664. The decrease in enzyme activity parallels the decrease in the level of autophosphorylation described in Fig. 1.

To study Ndk activity, cell extracts of 8830/pMMB66EH, 8830R2::Cm/pMMB66EH, and 8830R2::Cm/pJK662 were prepared and assayed for Ndk activity as described in Materials and Methods. The assay system used directly measures the amount of [γ-^{32}P]GTP, [γ-^{32}P]CTP, or [γ-^{32}P]UTP formed from [γ-^{32}P]ATP and GDP, CDP, or UDP. The substrate and products are separated by thin-layer chromatography and quantified. Despite the results of Fig. 1 showing a decrease in the level of phosphorylation of Ndk in the *algR2* mutant, no significant difference in Ndk activity was found between the *algR2* mutant 8830R2::Cm and its parent 8830 (data not shown). Thus, although we do observe a decrease in the level of phosphorylated Ndk in an *algR2* mutant, and the parental level of phosphorylated Ndk is restored upon supplying *algR2* in *trans* (Fig. 1), we cannot correlate this with a change in Ndk activity.

A possible reason for the lack of a decrease of Ndk activity is interference from another kinase. In *Escherichia coli*, pyruvate kinase can substitute for Ndk in the synthesis of nucleoside triphosphates (NTPs) (39). We further explored this possibility by repeating the Ndk assay in the presence of 0.05% Tween 20, since we found that Tween 20 inhibited the interfering activity without having any effect on Ndk. The results are shown in Fig. 2. In the presence or absence of 0.05% Tween 20, 8830 crude extract was able to produce [γ-^{32}P]UTP, [γ-^{32}P]CTP, or [γ-^{32}P]GTP from [γ-^{32}P]ATP and the corresponding NDP (Fig. 2, lanes 1 to 4). However, 8830R2::Cm crude extract was able to produce [γ-^{32}P]UTP, [γ-^{32}P]CTP, or [γ-^{32}P]GTP from [γ-^{32}P]ATP only in the absence of Tween 20 (lane 6). In the presence of Tween 20, there was very little phosphate

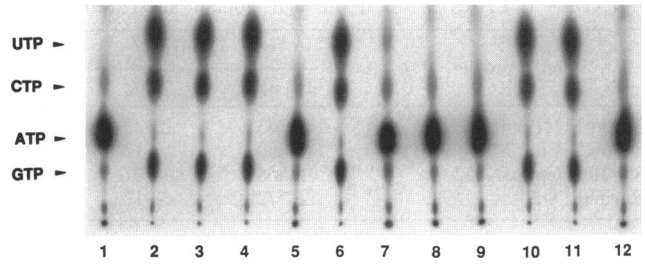

FIG. 2. Ndk activity in *algR2*$^+$ and *algR2* cells in the absence and presence of Tween 20. Ndk activity was measured in crude extracts from strains 8830 and 8830R2::Cm in the presence and absence of 0.05% Tween 20 to inhibit interfering kinases. [γ-^{32}P]ATP (1 μCi, 10 μCi/μl, 3,000 Ci/nmol) was used as the phosphate donor, and a mixture of GDP, CDP, and UDP (1 μM each, final concentration) was used as the recipient of the terminal phosphate. Lanes: 1, 8830 crude extract with [γ-^{32}P] ATP; 2, 8830 crude extract with [γ-^{32}P] ATP plus GDP, CDP, and UDP; 3, 8830 crude extract with [γ-^{32}P] ATP plus GDP, CDP, UDP, and 0.05% Tween 20; 4, 8830 crude extract with [γ-^{32}P] ATP plus GDP, CDP, UDP, and antibody against Ndk (α-Ndk); 5, 8830R2::Cm crude extract with [γ-^{32}P] ATP; 6, 8830R2::Cm crude extract with [γ-^{32}P]ATP plus GDP, CDP, and UDP; 7, 8830R2::Cm crude extract with [γ-^{32}P]ATP plus GDP, CDP, UDP, and 0.05% Tween 20; 8, 8830R2::Cm crude extract with [γ-^{32}P]ATP plus GDP, CDP, UDP, 0.05% Tween 20, and α-Ndk; 9, purified Ndk with [γ-^{32}P]ATP; 10, purified Ndk with [γ-^{32}P]ATP plus GDP, CDP, and UDP; 11, purified Ndk with [γ-^{32}P]ATP plus GDP, CDP, UDP, and 0.05% Tween 20; 12, purified Ndk with [γ-^{32}P]ATP plus GDP, CDP, UDP, and α-Ndk.

transfer from [γ-^{32}P]ATP to CDP, GDP, or UDP in the 8830R2::Cm extract (lane 7), as contrasted with the extract from 8830 (lane 3). Since Ndk activity is not inhibited by Tween 20 (lane 11), it is clear that the *algR2* mutant is highly deficient in Ndk activity. Thus, Ndk and one or more other kinases sensitive to Tween 20 together provide the bulk of the cellular NDP kinase activity in *P. aeruginosa*.

To further probe the effect of AlgR2 on Ndk levels, we used Western blot analysis to determine if there was a change in the amount of Ndk in the *algR2* mutant. Figure 3 and Table 1 show the result of the Western blot analysis. It is clear from the data that the levels of Ndk are drastically reduced in the *algR2* mutant, and the parental levels are restored upon supplying *algR2* in *trans*, in agreement with the phosphorylation data in Fig. 1. The drop in Ndk levels as shown by Western blotting is

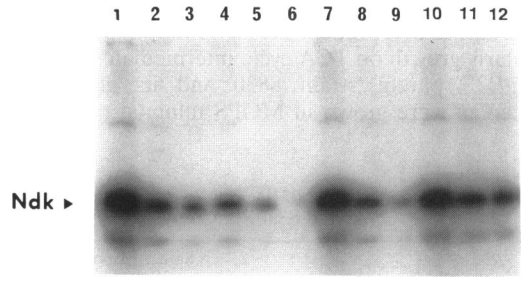

FIG. 3. Ndk levels in an *algR2* mutant. Crude extracts of 8830/pMMB66EH, 8830R2::Cm/pMMB66EH, and 8830R2::Cm/pJK662 were subjected to Western blotting with antibodies to *P. aeruginosa* Ndk. Lanes: 1 to 3, 100, 10, and 1 μg of crude extract from 8830/pMMB66EH; 4 to 6, 100, 10, and 1 μg of crude extract from 8830R2::Cm/pMMB66EH; 7 to 9, 100, 10, and 1 μg of crude extract from 8830R2::Cm/pJK662; lanes 10 to 12, 100, 10, and 1 ng of purified Ndk from *P. aeruginosa*.

TABLE 1. Levels of Ndk in the *algR2* mutant and parent strain

Strain or protein	cpm[a]		
	100 μg	10 μg	1 μg
8830/pMMB66EH	20,363	2,422	238
8830R2::Cm/pMMB66EH	3,000	512	52
8830R2::Cm/pJK662	15,668	1,701	211
Purified Ndk[b]	25,772	3,012	800

[a] Amount of radioactivity present in the Ndk bands as shown in Fig. 3.
[b] Purified Ndk lanes contain 100, 10, and 1 ng of protein.

reflected in the reduced level of phosphorylated Ndk (Fig. 1) and is in good agreement with the Ndk activity data of Fig. 2, which showed that an interfering kinase could provide the Ndk activity in absence of a functional Ndk enzyme.

Activity of other TCA cycle enzymes in an *algR2* mutant. Succinyl-CoA synthetase is one of the TCA cycle enzymes. It catalyzes the concomitant hydrolysis of succinyl-CoA and production of ATP from ADP and P_i. The TCA cycle is one of the most important metabolic pathways in aerobic organisms, and its anabolic and catabolic functions have been well documented. We have shown that Scs activity is reduced in an *algR2* mutant. An interesting question is, how general is AlgR2's control over TCA cycle enzymes, and does AlgR2 regulate any other or perhaps all TCA cycle enzymes? To address this question, we set out to assay all of the TCA cycle enzymes in *P. aeruginosa* 8830 and its *algR2* derivative 8830R2::Cm. The results of the assays are shown in Table 2. We had difficulty in obtaining reliable measurements for malate dehydrogenase and isocitrate dehydrogenase because of high levels of NADH oxidase in *P. aeruginosa* and thus do not report values for these enzymes. For the remaining enzymes, there is little difference between their activities in the *algR2* mutant and its parent. This finding suggests that *algR2*'s effect on the TCA cycle is limited to succinyl-CoA synthetase. The TCA cycle enzymes of *P. aeruginosa* are not subject to catabolite repression by glucose (46). It may be significant that the reaction catalyzed by succinyl-CoA synthetase is the only reaction of the TCA cycle that directly produces ATP by substrate-level phosphorylation. Regulating the level of Scs may serve as an important mechanism in providing energy and substrates for cellular functions and growth.

The *algR2* mutation slows growth on TCA cycle intermediates. AlgR2 is known to be important for alginate synthesis (17). If the role of AlgR2 in regulating succinyl-CoA synthetase activity is physiologically significant in terms of supplying energy and substrates for cellular functions and growth, it is important to determine if the *algR2* mutation affects growth, particularly growth on TCA cycle intermediates. To study this, the *algR2*$^+$ parent strain 8830 and its *algR2* derivative 8830R2::Cm were grown in MOPS minimal medium supplemented with different carbon sources. The TCA cycle intermediates succinate, citrate, and malate were chosen, along with glucose for comparison. The results of the growth experiments are shown in Fig. 4. The data show that with glucose as the sole carbon source, the *algR2* mutant grows nearly as well as its parent. However, when grown with the TCA cycle intermediates citrate, succinate, or malate as the sole source of carbon, the *algR2* mutant grew at a greatly reduced rate. Furthermore, the growth pattern of the parental strain is restored when *algR2* is supplied in *trans*. Taken with the data on decreased succinyl-CoA synthetase activity, this finding indicates that the loss of AlgR2 results in markedly decreased activity of a TCA cycle enzyme and that this defect is severe enough to slow the growth of cells using TCA cycle intermediates as their sole source of carbon. When growing on TCA cycle intermediates, *P. aeruginosa* strongly represses synthesis of enzymes for carbohydrate uptake and utilization (22). Thus, the TCA cycle intermediates must be oxidized by the TCA cycle to produce energy because the enzymes of the Entner-Doudoroff pathway and the oxidative portion of the pentose phosphate pathway are to a large extent unavailable to oxidize any hexoses formed from the TCA cycle compounds (46, 47). The TCA cycle is the major pathway available for oxidation of these carbon sources and production of energy. This illustrates the special and important role of the TCA cycle in *P. aeruginosa* (46, 47), particularly with regard to the role of Scs and Ndk, which as a complex allow efficient energy transduction from the operation of the TCA cycle to the generation of various NTPs for the maintenance of appropriate intracellular energy pools.

TABLE 2. Activities of TCA cycle enzymes in 8830 and 8830R2::Cm

Enzyme	Sp act (μmol of product formed/min/mg of protein	
	8830	8830R2:Cm
Aconitase	525 ± 15	475 ± 5
Fumarase	1,276 ± 43	1,008 ± 36
Succinate dehydrogenase	94 ± 14	90 ± 6
Isocitrate dehydrogenase	430 ± 10	490 ± 10
Citrate synthetase	160 ± 20	220 ± 60

DISCUSSION

A critical question in studying the production of alginate by *P. aeruginosa* is defining events which cause conversion of cells from the typical nonmucoid form to the alginate-producing mucoid form. *P. aeruginosa* contains the genetic capacity to produce alginate, but only in rare cases do the alginate genes become activated, leading to the production of alginate. One such case is *P. aeruginosa* infection of the CF lung. Several studies have identified environmental factors which can either cause production of alginate or lead to activation of the *algD* or *algC* promoter. Particularly interesting are studies of *P. aeruginosa* grown in chemostat cultures with energy-rich or energy-poor sources of carbon or nitrogen (44, 45). It was observed that growth on energy-rich carbon sources such as gluconate failed to lead to transition to mucoidy, whereas cultures using an energy-poor carbon source such as acetate did give rise to mucoid subpopulations. A similar correlation was found for the nitrogen source used. Growth on phosphorylcholine or nitrate, both energy-poor nitrogen sources, led to the appearance of mucoid variants, while growth on glutamate, an energy-rich nitrogen source, did not produce mucoid colonies. Growth on other poor carbon and energy sources such as acetamide (43), or long-term growth in the rat lung (50), has also been shown to give rise to mucoid cells. A central theme that has emerged from these studies is that stressed cells, slowly growing in nutritionally poor environments, are prone to undergo transition to mucoidy. It has been suggested that a unifying hypothesis for these findings is the role of energy metabolism in alginate production (45). It was demonstrated that limiting the growth of *P. aeruginosa* by using *N,N'*-dicyclohexylcarbodiimide (DCCD) or gramicidin to inhibit energy metabolism resulted in the appearance of mucoid colonies. Combining DCCD treatment with phosphate limitation resulted in conversion of up to 55% of the colonies to the

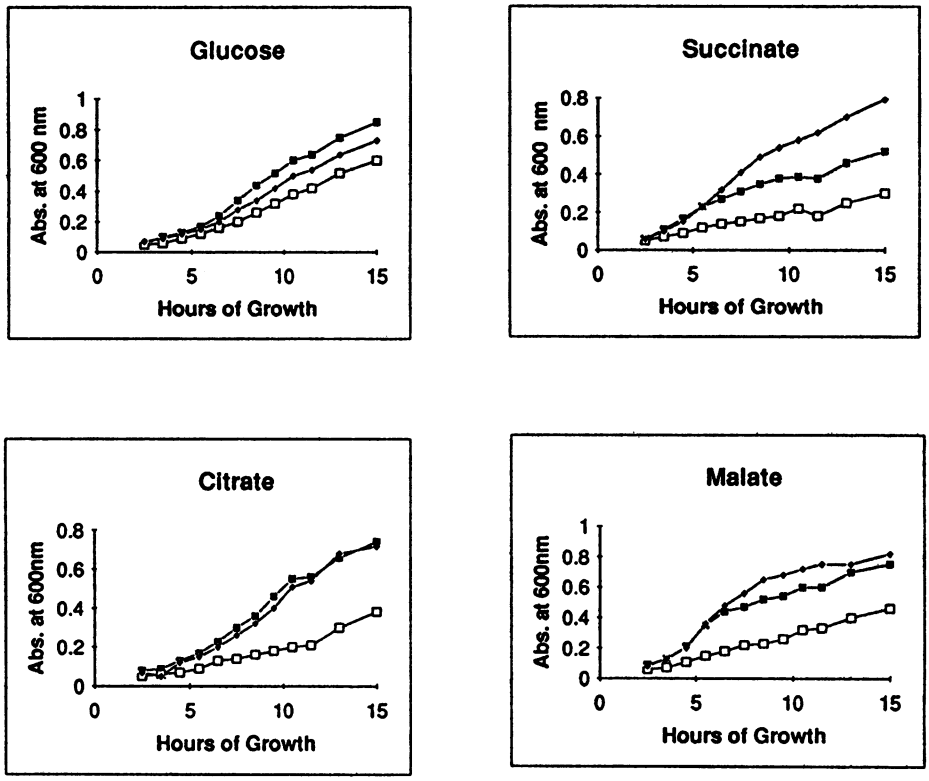

FIG. 4. Growth of strains 8830/pMMB66EH, 8830R2::Cm/pMMB66EH, and 8830R2::Cm/pJK662 on TCA cycle intermediates as the sole source of carbon. Closed boxes, 8830/pMMB66EH; open boxes, 8830R2::Cm/pMMB66EH; closed diamonds, 8830R2::Cm/pJK662.

mucoid phenotype. These data directly link energy metabolism with alginate production.

In this report, we have shown the effect of a mutation in the *algR2* gene on an enzyme of the TCA cycle, succinyl-CoA synthetase. Other TCA cycle enzymes tested did not show any significant alteration of activity in the *algR2* mutant. AlgR2 is required for mucoidy; a mutation in *algR2* renders the cell unable to produce alginate. In an *algR2* mutant background, the level of autophosphorylated Scs and the activity of succinyl-CoA synthetase are drastically reduced. Succinyl-CoA synthetase is known to form a phosphorylated intermediate in the course of its reaction. The *E. coli* enzyme is autophosphorylated on a histidine residue (His-246) of the α subunit; replacement of this histidine with an aspartate residue led to the elimination of activity and inability to autophosphorylate (24). Thus, it is not entirely unexpected to find a close relationship between the level of phosphorylation and enzyme activity, as reported here. Furthermore, this decrease in activity is sufficient to significantly slow the growth of cells using a TCA cycle intermediate as the sole carbon source, a situation in which the TCA cycle is the major catabolic pathway for oxidation of the carbon source and production of energy. The reaction catalyzed by succinyl-CoA synthetase is the only step of the TCA cycle that directly produces ATP by substrate-level phosphorylation. This demonstrates the role of AlgR2 in maintaining proper activity of the TCA cycle through succinyl-CoA synthetase. The role of the TCA cycle in terminal oxidation of nutrients and generation of energy and reducing equivalents has long been known. Although transition to mucoidy appears to occur in response to energy deprivation, it is important to note that alginate biosynthesis itself places strong energy demands on the cell. The production of GDP-mannose from mannose-1-phosphate, required for both alginate and lipopolysaccharide biosynthesis (23), requires GTP. Considering that many alginate-producing strains of *P. aeruginosa* convert over 50% of their available carbon source into alginate (29), a considerable amount of energy must be available to produce alginate. Thus, in an environment which is likely nutrient poor, *P. aeruginosa* would need a mechanism for efficient operation of catabolic and anabolic pathways to supply energy and substrates for growth and alginate synthesis.

We were also able to show a decreased level of NDP kinase, a key enzyme which controls the NTP and dNTP levels. Ndk from several sources, including *Myxococcus xanthus* (30, 31), *E. coli* (37), and humans (14), has been studied. Ndk can transfer the terminal phosphate from any NTP or dNTP to any other NDP or dNDP. A phosphoenzyme is an intermediate of the reaction and is believed to be a phosphohistidine. However, the *M. xanthus* enzyme is phosphorylated at two residues, His-117 and an unidentified serine residue (30). As with other Ndks, autophosphorylation of the histidine residue is believed to be essential for enzyme activity. Severely reduced enzyme activity was also demonstrated in the presence of Tween 20, used to inhibit an interfering kinase. AlgR2 has been previously reported to be a protein kinase capable of undergoing autophosphorylation (38). This conclusion was based on the observation that a protein of a size similar to AlgR2 (16 to 18 kDa) was autophosphorylated in *algR2*$^+$ cells but missing in *algR2* cells. The data presented here suggest that AlgR2 itself is not a kinase, but rather that it regulates a kinase of similar size (Ndk).

The results described here are reminiscent of the control of the TCA cycle during sporulation in *Bacillus subtilis*. When starved for nutrients, *B. subtilis* differentiates into spores which

protect genetic material until an environment more favorable for growth returns. Sporulation can also be induced with inhibitors of purine synthesis to starve cells for guanine nucleotides. Guanine nucleotide deprivation leads to an increase of intracellular acetyl-CoA, which causes the induction of α-ketoglutarate dehydrogenase (48). The increased levels of α-ketoglutarate dehydrogenase lower the levels of two TCA cycle intermediates (α-ketoglutarate and L-malate) which inhibit aconitase and citrate synthetase, resulting in higher activities of these enzymes as well (9, 48). The activities of other TCA cycle enzymes, such as fumarase (35) and succinate dehydrogenase (28), are also increased during sporulation. The recent characterization of a *uspA* (universal stress and starvation protein) mutant in *E. coli* (33) is noteworthy in this respect. The synthesis of UspA is strongly induced when the growth rate of the cell falls below the maximal rate supported by the media, regardless of the cause of growth inhibition. A mutant in *uspA* exhibits abnormal utilization of carbon. Carbon which normally enters the TCA cycle is instead catabolized to acetate and released into the media. This finding suggests that the role of UspA is to direct the flow of carbon in central metabolism of *E. coli* during stress (33). Although neither *E. coli* nor *P. aeruginosa* sporulates upon starvation as does *B. subtilis*, all of these bacteria do alter gene expression as part of an active process of starvation survival. It appears that proper operation of the TCA cycle and flow of carbon into the TCA cycle to generate succinate for energy and α-ketoglutarate for biosynthetic processes is an important part of the cellular response to stress (33, 42). It should be noted that we studied the role of *algR2* in strain 8830, which has been mutagenized to allow for stable alginate production. It will be interesting to study Ndk and Scs regulation under energy stress conditions in wild-type *P. aeruginosa*. It will be important to determine the mechanism by which AlgR2 regulates the level of Scs and Ndk, as well as to study the regulation of *algR2* itself. Construction of null mutants in *scs* and *ndk* will allow us to directly identify a link between TCA cycle operation and alginate biosynthesis. Such experiments are currently under way in our laboratory.

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