Osmoprotectant-Dependent Expression of *plcH*, Encoding the Hemolytic Phospholipase C, Is Subject to Novel Catabolite Repression Control in *Pseudomonas aeruginosa* PAO1

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Expression of the hemolytic phospholipase C (PlcH) of *Pseudomonas aeruginosa* is induced under phosphate starvation conditions or in the presence of the osmoprotectants choline and glycine betaine. Because choline and glycine betaine may serve as carbon and energy sources in addition to conferring osmoprotection to *P. aeruginosa*, it seemed possible that induction of *plcH* is subject to catabolite repression control (CRC) by tricarboxylic cycle intermediates such as succinate. Total phospholipase (PLC) activity in osmoprotectant-induced cultures of *P. aeruginosa* PAO1 supplemented with 20 mM succinate was three- to fourfold lower than the levels in cultures supplemented with the non-catabolite-repressive substrate lactate. Analyses of osmoprotectant-dependent *plcH* expression in a derivative of strain PAO1 containing a *plcH::lacZ* operon fusion showed that (i) succinate prevented induction of *plcH* expression by osmoprotectants; and (ii) addition of succinate reduced or shut down further expression of *plcH* in osmoprotectant-induced bacteria, while cultures supplemented with lactate had little or no change in *plcH* expression. RNase protection analysis confirmed that repression of *plcH* occurs at the transcriptional level. However, a *P. aeruginosa* mutant decoupled in CRC exhibited a phenotype similar to that of the wild-type strain (PAO1) with respect to succinate-dependent repression of *plcH* expression. Osmoprotectant-induced total PLC activities, levels of expression of *plcH* measured with the same *plcH::lacZ* fusion, and levels of *plcH* transcription in a CRC-deficient strain reflected those seen in strain PAO1. This indicates that CRC of *plcH* functions by a distinct mechanism which differs from that regulating the glucose or mannitol catabolic pathway. A strain carrying a mutation in *vfr*, which encodes the *Escherichia coli* Crp homolog in *P. aeruginosa*, still exhibited a wild-type phenotype with respect to osmoprotectant-dependent expression and CRC of *plcH*. These data indicate that there is a novel CRC system that regulates the expression of *plcH* in *P. aeruginosa*.

Pseudomonas aeruginosa is an opportunistic pathogen of humans that causes high morbidity and mortality among such immunocompromised individuals as chemotherapy, burn, and transplant patients, as well as persons with cystic fibrosis (CF) (16, 37, 50, 51). Phospholipase C (PLC), one of the numerous virulence factors elaborated by *P. aeruginosa* (26), has been implicated as a significant virulence determinant in *P. aeruginosa* infections in animals and, more recently, in plants (2, 35, 37).

The precise role of PLC in the pathogenesis of *P. aeruginosa* infection remains unclear; however, PLC has been shown to induce a number of different effects on eukaryotic systems. In vitro, PLC increases the adherence of *P. aeruginosa* to respiratory epithelial cells (40, 41), causes aggregation of blood platelets (8), and produces mitogenic and cytotoxic effects on a number of different cell types (7, 18–20, 30). In vivo, PLC causes tissue necrosis and death (8).

PLC may impact virulence in a number of ways. It has been proposed that in the phosphatidylcholine-rich environment of the lungs of CF patients (5), the PLC degradation products of phosphatidylcholine, diacylglycerol, and phosphorylcholine (PC) play different roles in the disease process. Diacylglycerol may induce the abnormal release of inflammatory mediators and increase the host inflammatory response, thereby promoting tissue damage (11, 18, 23). PC can be converted to choline or glycine-betaine. These compounds serve as osmoprotectant

agents which shield bacteria against the effects of hyperosmolar environments (such as the lungs of CF patients) (9, 22, 46). Also, since *P. aeruginosa* is able to utilize choline and glycine betaine as sole sources of carbon, nitrogen, and energy, these compounds may serve as rich sources of nutrients (24, 46). Thus, PLC may also serve to liberate osmoprotective agents and nutrients for the growth and survival of *P. aeruginosa*.

P. aeruginosa carries two genes encoding PLC activity, *plcH* and *plcN* (33, 34). The genes and their products are homologous, exhibiting 40% identity over the length of the two peptide sequences, but also exhibit some notable differences (36). One PLC, PlcH, is hemolytic, is an acidic protein, and is active on PC and sphingomyelin. The other, PlcN, does not lyse sheep or human erythrocytes, is basic, and acts on phosphatidylserine but not sphingomyelin (46).

The expression of *plcH* and *plcN* is induced by either a limiting phosphate concentration or an osmoprotectant, choline or glycine betaine. Both *plcH* and *plcN* are induced when *P. aeruginosa* is grown under inorganic phosphate (P_i)-limiting conditions (46). PhoB, a two-component response regulator, positively controls the P_i starvation-dependent expression of these genes (1, 12, 13, 36, 44, 46). Production of both PLCs is also induced by their substrate products, PC, choline, and glycine betaine (27, 44, 45). Expression of *plcH* by these compounds is PhoB and phosphate starvation independent, while induction of *plcN* by osmoprotectants requires PhoB and P_i -limiting conditions (44–46). *plcH* is expressed from either P_i starvation- or osmoprotectant-dependent promoters; *plcN* expression uses only a single transcriptional start site (46).

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One of the regulatory mechanisms controlling carbon metabolism is catabolite repression control (CRC) (6, 28). *P. aeruginosa* preferentially utilizes succinate and other tricarboxylic acid (TCA) cycle intermediates over other substrates, such as glucose, mannitol, histidine, and acetamide (6, 28). For example, when *P. aeruginosa* is grown in medium containing succinate and another carbon source, such as glucose, succinate will be utilized first and the formation of the enzymes of glucose be metabolized (6, 28). Certain other substrates do not exert catabolite repression, including pyruvate, lactate, and alanine. Growth of *P. aeruginosa* in the presence of glucose and one of these substrates does not repress induction of the glucose-catabolic enzymes.

Since *P. aeruginosa* utilizes choline and glycine betaine as sole carbon and energy sources (24, 25), it seemed possible that osmoprotectant-dependent formation of PLC is subject to CRC. In this report, we demonstrate that full induction of *plcH* by choline requires several hours when *P. aeruginosa* is grown in a medium containing succinate as the carbon source (45); however, if a non-catabolite-repressing substrate for *P. aeruginosa*, such as lactate, is substituted for succinate, induction occurs within 30 min (39). Also, choline transport and acid phosphatase, an enzyme involved in the conversion of PC to choline, have been reported to be subject to CRC (42). Preliminary reports have indicated that production of phospholipase C in strains carrying a mutation in the *crc* gene, which is required for CRC, is affected (3, 4). Thus, it was of interest to more fully examine whether phospholipase C expression is affected by CRC in *P. aeruginosa*.

The results presented here demonstrate that CRC directly regulates osmoprotectant-dependent expression of *plcH*. This regulation appears to be distinct from the CRC in *P. aeruginosa* that regulates the expression of the carbohydrate-catabolic pathways for glucose and mannitol. We also report that another regulatory-related factor, the *Escherichia coli* catabolite repressor protein homolog Vfr, neither plays a role in CRC in *P. aeruginosa* nor affects osmoprotectant-dependent *plcH* expression.

MATERIALS AND METHODS

Strains and plasmids. Bacterial strains and plasmids used in this study are presented in Table 1.

Growth of bacteria. All bacteria were maintained at -70°C in brain heart infusion (BHI) medium (Difco Laboratories, Detroit, Mich.) supplemented with 50% glycerol. Bacteria were recovered from frozen stocks by growth on BHI plates. When required, antibiotics were used at the following concentrations for the two organisms: for *P. aeruginosa*, tetracycline was used at 100 $\mu\text{g}/\text{ml}$, while for *E. coli*, tetracycline was used at 25 $\mu\text{g}/\text{ml}$, ampicillin was used at 100 $\mu\text{g}/\text{ml}$, and kanamycin was used at 50 $\mu\text{g}/\text{ml}$.

For measurement of PLC and β -galactosidase activities and for RNA extractions, bacteria were grown in a modified version of the HEPES-buffered mineral salts of Hancock in which 20 mM sodium lactate replaced 24 mM sodium succinate as the carbon source (15). For measurement of glucose-6-phosphate dehydrogenase activities, bacteria were grown in a 20 mM lactate and phosphate-buffered mineral salts solution which has been previously described (39). All carbon sources except choline and glycine betaine were added to a final concentration of 20 mM. Choline was added at 0.2% (vol/vol); glycine betaine was added at 0.1%.

Enzyme assays. Measurement of PLC activity followed a procedure, described by Kurioka and Matsuda (21) but with modifications described by Berka et al. (2), utilizing the colorimetric reagent *p*-nitrophenylphosphorylcholine (NPPC) (Sigma Chemical Co., St. Louis, Mo.). PLC activities are presented in units of nanomoles of NPPC cleaved per minute per A_{600} unit of the culture. Measurement of β -galactosidase activity followed the procedure of Miller as previously described (44). Assays of glucose-6-phosphate dehydrogenase activity were performed as described previously (39). The protein contents of extracts were measured with a bicinchoninic acid protein assay reagent kit (Pierce Chemicals, Rockford, Ill.).

DNA manipulations. DNA manipulations followed procedures described by Sambrook et al. (43). Restriction enzyme digestions were performed as recommended by the manufacturers (Bethesda Research Labs, Rockville, Md., and Promega Corp., Madison, Wis.).

TABLE 1.

Strain or plasmid	Description	Reference(s) or source
Strains		
<i>P. aeruginosa</i>		
PAO1	Prototroph; <i>chl</i> -3	17
PAO8023	<i>crc::pSUP203</i> ; Cb ^r	P. V. Phibbs, Jr., Greenville, N.C.
PAO8024	Δ <i>crc</i>	P. V. Phibbs, Jr.
PAO9002	<i>vfr::gm</i> ; Gm ^r	S. West, Milwaukee, Wis.
PAO1::pAES110	<i>plcH::pAES110</i>	This study
PAO8024::pAES110	<i>plcH::pAES110</i>	This study
<i>E. coli</i> DH5 α		
	F ⁻ <i>endA1 recA1 hsdR17</i> (r_K^- m_K^+) <i>supE44</i> λ^- <i>thi-1 gyrA96 relA1</i> Φ 80 <i>lacZ</i> M15 Δ (<i>lacZ</i> YA- <i>argF</i>) _{U169} ; Amp ^r	14
Plasmids		
pRK2013	Mob ⁺ Tra ⁺ ; Km ^r	45
pAES110	<i>pSUP203::plcH::lacZ</i> Y	This study
pVDS100	<i>pGEM7Z-5'</i> BamHI- <i>StuI plcH</i>	44, 45

Construction of *plcH::lacZ* operon fusion strains of *P. aeruginosa*. *P. aeruginosa* *plcH::lacZ* operon fusion strains were constructed by filter mating the recipient *P. aeruginosa* strain with *E. coli* DH5 α (pAES110) and *E. coli* HB101(pRK2103) on nonselective BHI medium. Cells were then resuspended in BHI broth and plated on BHI medium supplemented with 100 μg of tetracycline per ml. Tc^r colonies were purified and tested for insertion of the *plcH::lacZ* fusion by plating them on HEPES medium containing 33 μg of 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal; Diagnostic Chemicals Ltd., Oxford, Conn.) supplemented with 0.2% choline. Colonies with a blue appearance were selected, as they contained the fusion construct inserted into the chromosome.

RNAse protection analyses. Total RNA was isolated by the acid lysis-hot phenol method described by Von Gabain et al. (47) and then DNase I treated. Total RNA (10 μg) was hybridized with a ^{32}P -labeled runoff transcript prepared from *Dra*I-digested pVDS100 by using the Gemini II core runoff transcript kit of Promega and T7 polymerase as recommended by the manufacturer. Hybridization mixtures were then digested with RNases A and T1, and the protected RNA fragment was purified and resolved by polyacrylamide gel electrophoresis.

RESULTS

Succinate reduces PLC activities in choline- and glycine betaine-induced cultures of strain PAO1. In phosphate-replete medium supplemented with the osmoprotectant choline or glycine betaine, *plcH*, but not *plcN*, is expressed (44). Previously, it was observed that in medium containing succinate, choline-dependent induction of *plcH* expression took several hours (44, 45). In contrast, when the carbon source was lactate, induction of *plcH* expression by choline took less than 30 min (39). To examine if osmoprotectant-dependent formation of PLC activity is repressed by the TCA cycle intermediate succinate, strain PAO1 was grown for 6 h (to late log phase) in a HEPES-buffered minimal medium supplemented with 0.2% choline and either 20 mM succinate or 20 mM lactate. Total PLC activities of the cultures were then compared (Fig. 1A). Supernatants from cultures of succinate-grown, choline-induced bacteria contained exhibited approximately fourfold-lower PLC activity than those from cultures that were supplemented with lactate. When 0.1% glycine betaine was substituted for choline in these experiments, the lactate-grown culture produced threefold more PLC activity than the succinate-grown culture (Fig. 1B). These results demonstrate that osmopro-

FIG. 1. Repression of PLC formation by succinate. Cells of strain PAO1 from an overnight culture grown in HEPES medium were diluted to an optical density at 600 nm (OD_{600}) of 0.05 in fresh HEPES medium supplemented with 0.2% choline (A) or 0.1% glycine-betaine (B) and either 20 mM lactate or 20 mM succinate. The cultures were incubated at 37°C for 6 h, and the total PLC activity in each culture supernatant was assayed as described in Materials and Methods. PLC activity is presented as nanomoles of NPPC cleaved per minute per OD_{600} unit of the respective cultures.

tectant-dependent formation of PlcH is repressed by succinate present in the medium.

Repression of osmoprotectant-dependent *plcH* expression in strain PAO1 by succinate and other TCA cycle intermediates. Measurement of PLC activity determines the accumulation of this enzyme at the end of culture growth; it does not provide a means of examining *plcH* expression during growth of the culture. To further characterize succinate-dependent repression of *plcH* in strain PAO1, a *plcH-lacZYA* fusion was constructed in strain PAO1 by insertion of pAES110 into the chromosome (see Materials and Methods). pAES110 carries *lacZYA*, and its intact translational signals are under the control of the *plcH* promoter, allowing *plcH* expression to be followed by measurement of β -galactosidase activity. Since PLC activity is difficult to assay in the early log phase of growth of *P. aeruginosa*, the presence of the fusion provided a means of easily and accurately measuring *plcH* expression under these growth conditions. This allowed *plcH* expression to be examined over the entire course of bacterial growth, not just at an end point as in the previous experiment.

Succinate-dependent repression of *plcH* induction was examined in cultures of *P. aeruginosa* PAO1::pAES110 in HEPES-buffered minimal medium supplemented with 0.2% choline. To one culture was added an additional 20 mM succinate; the other was supplemented with the nonrepressive substrate lactate. At measured time points, β -galactosidase activities in the respective cultures were determined. The results of a representative experiment are presented in Fig. 2A. Lactate-exposed bacteria exhibited an immediate increase in β -galactosidase activity that continued over the course of the experiment. In

contrast, the β -galactosidase activity exhibited by the culture supplemented with succinate remained at a noninduced level until 4 h after addition of choline. At this time, when the succinate in the culture presumably had become depleted, β -galactosidase levels increased until they reached those of the lactate-exposed culture. When 0.1% glycine betaine was substituted for choline (Fig. 2B), the succinate-exposed bacteria again exhibited little or no β -galactosidase activity until several hours after induction of the culture with glycine betaine. Again, the lactate-grown culture exhibited an immediate increase in β -galactosidase activity within 45 min that was more rapid than that observed in the choline-induced culture. The culture supplemented with succinate exhibited noninduced levels of β -galactosidase activity.

The repressive effects of other TCA cycle intermediates and nonrepressive substrates were also examined. Levels of β -galactosidase were assayed as described above, except citrate and acetate were substituted for succinate. These substrates serve to catabolite repress the formation of the same enzymes and pathways as succinate (6, 28, 31, 32). As with succinate, these compounds repressed the level of β -galactosidase to the same

FIG. 2. Repression of osmoprotectant-dependent induction of *plcH* by succinate in strain PAO1::pAES110. Shown are the results of a representative experiment comparing the respective levels of β -galactosidase activity in choline- (A) and glycine betaine- (B) induced cultures of *P. aeruginosa* that had been supplemented with 20 mM lactate (■) or 20 mM succinate (●). Bacteria incubated overnight in HEPES medium were diluted to an optical density at 600 nm (OD_{600}) of 0.05 in fresh HEPES medium supplemented with osmoprotectant and either 20 mM lactate or 20 mM succinate. At timed intervals, the OD_{600} and β -galactosidase activity of each culture were measured.

degree as succinate. In contrast, when the nonrepressive carbon sources pyruvate and alanine were used, β -galactosidase activities reflected those found in the lactate-exposed cultures of strain PAO1 (data not shown).

It was previously reported that choline transport is subject to CRC by succinate (42). It was possible that the above observations resulted from the repression of choline uptake, which prevented bacterial accumulation of choline, which is required for induction of *plcH* under phosphate-replete conditions. To determine if CRC either affected *plcH* directly or prevented accumulation of the inducer choline, succinate-dependent inhibition of *plcH* expression was examined. If CRC affects choline uptake alone, β -galactosidase activities produced from the *plcH::lacZYA* fusion would not be immediately affected by addition of succinate. On the other hand, if *plcH* expression was directly affected by CRC, then the addition of succinate would cause an immediate drop in β -galactosidase activity. A choline-induced culture of strain PAO1::pAES110 was split, either 20 mM lactate or 20 mM succinate was added to each aliquot, and β -galactosidase levels were assayed over the growth of the cultures. The results are presented in Fig. 3A. Succinate-exposed bacteria exhibited a loss of β -galactosidase activity. However, the reduction in β -galactosidase activity did not reflect complete proportionality with the growth rate of the culture. This suggested that *plcH* expression was not completely repressed. β -Galactosidase activities in the lactate-exposed bacteria remained at relatively constant levels.

The same experiment was repeated with 0.1% glycine betaine as the inducing compound instead of choline (Fig. 3B). Again, addition of succinate shut down further glycine betaine-dependent formation of β -galactosidase from the *plcH::lacZ* chromosomal fusion and resulted in a dilution of β -galactosidase activity due to growth of the culture. These results are an indirect demonstration that succinate affects *plcH* expression rather than choline transport.

Repression of choline-dependent expression of *plcH* by succinate occurs at the level of transcription. To determine if *plcH* transcription is directly affected by CRC, amounts of *plcH* mRNA in both succinate- and lactate-exposed, choline-induced cultures of strain PAO1 were compared. Bacteria were grown in HEPES medium plus choline and subsequently divided into separate aliquots to which either 20 mM lactate or 20 mM succinate was added. Total RNA was isolated at measured intervals before and after separation of the aliquots and addition of the carbon sources. RNase protection analysis of the samples of RNA was then performed with a runoff transcript specific for the 5' end of *plcH*. As is seen in Fig. 4, the *plcH* transcript could not be detected in the succinate-exposed culture until 4 h after the cultures were split. After an initial drop in detectable transcript levels, lactate-exposed bacteria exhibited levels of *plcH* mRNA close to those observed prior to division of the cultures. These results indicate that CRC represses *plcH* expression at the level of transcription.

Phosphate starvation-dependent expression of *plcH* (and *plcN*) is unaffected by succinate. Both *plcH* and *plcN*, the gene encoding the nonhemolytic PLC, are subject to phosphate starvation-dependent induction (36, 44, 46). The effect of CRC on phosphate starvation-dependent expression of *plcH* was examined. Strain PAO1::pAES110 was grown in phosphate starvation HEPES medium supplemented with either 20 mM lactate or 20 mM succinate (44, 46) (see Materials and Methods). The β -Galactosidase activities in aliquots of the two cultures were then assayed at measured intervals. No significant differences were seen in the β -galactosidase levels of the lactate- and succinate-grown cultures (mean activity $\pm$ standard deviation, 224 ± 15 versus 296 ± 22 Miller units, respectively, after 3 h of

FIG. 3. Reduction of *plcH* expression by addition of succinate to osmoprotectant-induced strain PAO1::pAES110. Shown are the results of a representative experiment comparing the levels of β -galactosidase activity in choline-induced (A) and glycine-betaine-induced (B) cultures of *P. aeruginosa* PAO1::pAES110 to which 20 mM lactate (■) or 20 mM succinate (●) had been added (arrows). Bacteria that had been incubated overnight in HEPES medium were diluted to an optical density at 600 nm (OD_{600}) of 0.05 in fresh HEPES medium containing osmoprotectant and grown until full induction of *plcH* had occurred, as measured by β -galactosidase activity. At that point, the cultures were split and either lactate or succinate was added to the aliquoted samples to a final concentration of 20 mM. At timed intervals, the OD_{600} and β -galactosidase activity of each culture were determined as described in Materials and Methods.

growth). This indicates that CRC does not affect phosphate starvation-dependent expression of *plcH*.

Total PLC activities of lactate- or succinate-supplemented cultures of strain PAO1 were then compared. Since phosphate-dependent *plcH* expression is unaffected by CRC, the measurement of total PLC provided a means of determining whether CRC affected *plcN* expression. Bacteria were again incubated for 3 h in phosphate starvation HEPES medium supplemented with lactate or succinate. No significant differences were observed in the total PLC activities of phosphate starvation-grown cultures supplemented with lactate (37 ± 4 PLC units) and those supplemented with succinate (34 ± 3 PLC units), indicating that CRC does not affect expression of *plcN*.

The CRC-deficient strain PAO8024 (*crc*) is not decoupled with respect to succinate-dependent repression of *plcH* expression. Previously, mutants deficient in CRC have been isolated and characterized (49). These mutants fail to exhibit Krebs cycle intermediate-dependent repression of the synthesis of the enzymes of a number of different catabolic pathways. To de-

FIG. 4. RNase protection analysis of succinate-dependent repression of *plcH* expression in choline-induced cultures of strain PAO1. Bacteria incubated overnight were diluted to an optical density at 600 nm (OD_{600}) of 0.05 in fresh HEPES medium containing 0.2% choline and incubated 2 h (until the OD_{600} approximated 0.1). Total RNA was extracted from two aliquots (I_1 and I_2) 30 min apart, and the culture was split to separate flasks containing 20 mM lactate (L) or 20 mM succinate (S). RNA was then extracted from each culture 45 (1), 90 (2), 180 (3), and 240 (4) min after splitting, hybridized with a runoff transcript (Probe) complementary to the 5' end of *plcH*, RNase treated, and resolved. The protected *plcH* fragment is marked by the arrows. An RNase-treated probe was included as a control.

termine whether *plcH* expression is controlled by this same regulatory mechanism, the CRC-deficient strains PAO8023 and PAO8024 were examined for CRC of *plcH*. Total PLC activities of lactate- or succinate-exposed, choline-induced cultures of the CRC-deficient mutants PAO8023 and PAO8024 were compared. Succinate-exposed cultures of both strains exhibited over a 10-fold reduction in total PLC activity compared to those grown in lactate (8.9 ± 0.1 versus 0.8 ± 0.2 for PAO8023, and 5.2 ± 0.5 versus 0.5 ± 0.1 for strain PAO8024). *plcH* expression was then examined to determine if it was similarly repressed in the CRC-deficient mutants. The *plcH::lacZYA* construct pAES110 was introduced into strain PAO8024, and β -galactosidase activities of choline-induced cultures of strain PAO8024::pAES110 supplemented with either lactate or succinate were compared. As seen with results obtained with the wild-type strain PAO1::pAES110, succinate still repressed choline-dependent induction of *plcH* (Fig. 5A). Addition of succinate also inhibited further *plcH* expression in a choline-induced culture of strain PAO8024::pAES110, causing a reduction in β -galactosidase activity similar to that seen with strain PAO1::pAES110 (Fig. 5B). Finally, transcription of *plcH* in a choline-induced culture of strain PAO8024 was determined to be repressed by addition of succinate, but not lactate, when amounts *plcH* transcript were compared by RNase protection analysis (Fig. 6). Similar results were obtained when glycine betaine was substituted for choline in these experiments (data not shown). These results indicate that CRC of *plcH* expression appears to function by a mechanism distinct from that which represses the formation of the enzymes of carbohydrate metabolism. It should be noted that both strains PAO8024 and PAO8023 exhibited a CRC-deficient phenotype with respect to the formation of carbohydrate-catabolic enzymes such as glucose-6-phosphate dehydrogenase (data not shown).

CRC of *plcH* expression in *P. aeruginosa* is not regulated by the *E. coli* Crp homolog, Vfr. Since it appeared that carbon source-dependent regulation of *plcH* is mediated by a mechanism distinct from that which mediates CRC of the catabolic-pathway enzymes, the role of the regulatory factor Vfr was examined. Vfr is homologous to the Crp protein of *E. coli*, the protein involved in CRC in that organism (48). *vfr* has been found to restore a wild-type phenotype to a Crp-deficient strain of *E. coli* (48). Also, Vfr has been implicated in the regulation of PLC formation (48).

It was possible that Vfr was the regulatory factor mediating CRC of *plcH*. Total PLC activities of choline-induced cultures of strains PAO1 and PAO9002 that had been supplemented with either lactate or succinate were compared to examine this possibility. Strain PAO9002 contains a gentamycin resistance cassette inserted into *vfr* gene. The lactate-exposed cultures of the two strains exhibited similar levels of total PLC activity (10.2 ± 0.7 for PAO1 and 9.8 ± 0.6 for PAO9002), indicating that Vfr is not required for *plcH* expression. In the succinate-exposed cultures, the *vfr* mutant again exhibited a reduced level of total PLC activity like that of strain PAO1 (2.6 ± 0.2 for PAO1 versus 3.1 ± 0.1 for PAO9002, respectively). To further confirm that CRC of *plcH* was not subject to regulation by Vfr, pAES110 was introduced into strain PAO9002 and β -galactosidase activities of HEPES-grown, choline-induced cultures supplemented with either lactate or succinate were compared. β -Galactosidase activities of strain PAO9002::pAES110 were repressed by succinate to the same degree as those of the wild-type strain. Also, no significant differences in the levels of β -galactosidase in the lactate-grown, choline-in-

A

B

FIG. 5. Analysis of CRC of *plcH* expression in the *crc* mutant strain PAO8024. Shown are the results of representative studies on the effect of succinate on levels of β -galactosidase activity in choline-induced cultures of *P. aeruginosa* PAO8024::pAES110. Experimental conditions were the same as described in the legends to Fig. 2 and 3. (A) Effect on choline-dependent *plcH* induction in strain PAO8024::pAES110 (as measured by β -galactosidase levels) by 20 mM lactate (■) or 20 mM succinate (●). (B) Effect of addition of 20 mM lactate (■) or 20 mM succinate (●) on choline-induced *plcH* expression in strain PAO8024::pAES110. The arrow indicates the point of lactate or succinate addition. Succinate or lactate was added at the 45-min time point.

FIG. 6. RNase protection analysis of succinate-dependent repression of *plcH* expression in choline-induced cultures of the CRC-deficient strain PAO8024. Shown are the results of an experiment involving comparison of *plcH* transcript levels in choline-induced cultures of strain PAO8024 supplemented with 20 mM lactate or 20 mM succinate. Bacteria incubated overnight were diluted to an optical density at 600 nm (OD_{600}) of 0.05 in fresh HEPES medium containing 0.2% choline and incubated 2 h (until the OD_{600} approximated 0.1). RNA was extracted from two aliquots (I_1 and I_2) 30 min apart, and the culture was split to separate flasks containing 20 mM lactate (L) or 20 mM succinate (S). RNA was extracted from each culture 45 (1), 90 (2), and 180 (3), and 240 (4) min after splitting, hybridized with a probe complementary to the 5' end of *plcH*, RNase treated, and resolved by denaturing polyacrylamide gel electrophoresis. The *plcH* fragment is marked by the arrows. An RNase-treated probe was also included as a control.

duced cultures of strains PAO1::pAES110 and PAO9002::pAES110 were observed (data not shown). These data demonstrate that Vfr does not appear to play a role in CRC of *plcH*.

DISCUSSION

CRC is a regulatory mechanism that modulates the pathways of carbon source utilization in response to the availability of carbon substrates. Over 70% of the surfactant of the lung consists of phosphatidylcholine (5). One of the potential roles for PLC in *P. aeruginosa* infections of CF patients' lungs is to provide nutrients for growth. From phosphatidylcholine, PLC will liberate PC, which could then serve as a source of carbon and energy. The breakdown products choline and glycine betaine could also act as osmoprotectants for *P. aeruginosa*, shielding the bacteria against the inhibitory effects of the hyperosmolar environment of the lungs of CF patients (9, 10, 22). Since PLC can act to exploit a source of carbon and energy for *P. aeruginosa*, it seemed possible that its expression is subject to CRC.

In this study, it was demonstrated that osmoprotectant-dependent expression of *plcH* is subject to CRC which acts at the transcriptional level. It also was shown that CRC of phosphate starvation-dependent expression of *plcH* or *plcN* does not occur. The CRC-deficient mutants PAO8023 and PAO8024 exhibit a carbon source-dependent repression of *plcH* osmoprotectant-dependent induction similar to that of the wild-type strain PAO1. This indicates that CRC of osmoprotectant-dependent *plcH* expression is mediated by a mechanism distinct from the previously described CRC system in *P. aeruginosa* (6, 28) that affects the expression of other catabolic pathways, such as those involved in the utilization of glucose or mannitol. One regulatory protein, the *E. coli* Crp homolog Vfr, plays no role in CRC of *plcH* expression.

Several different experimental avenues were pursued to determine if CRC regulated osmoprotectant-dependent *plcH* expression. First, total accumulated PLC activity in supernatants from choline- or glycine betaine-induced cultures of bacteria supplemented with lactate or succinate was assayed. In order to more directly examine *plcH* expression early in log phase,

when PLC levels have not accumulated, a *plH::lacZYA* operon fusion was introduced into *P. aeruginosa*. Levels of osmoprotectant-dependent induction of *plcH* in cultures of bacteria supplemented with either lactate or succinate were then compared (Fig. 2). The effect of succinate addition on osmoprotectant-induced *plcH* expression was also examined. Choline uptake has been shown to be subject to CRC; this experiment addressed the possibility that the uptake of an osmoprotectant is repressed by succinate, preventing these compounds from entering the cell and inducing *plcH*. If only the expression of the choline or glycine betaine transport machinery was affected, then the β -galactosidase activity would have gradually fallen as the uptake apparatus for these compounds was diluted by the growth of the culture. Instead, results from these experiments indicate that osmoprotectant-dependent *plcH* expression is directly affected by CRC. RNase protection analyses confirmed that *plcH* transcription is immediately shut down by the addition of succinate to osmoprotectant-induced *P. aeruginosa*. Cultures supplemented with the nonrepressive substrate lactate still produced *plcH* mRNA at levels near those seen in cultures prior to exposure to lactate or succinate.

plcH is also induced under phosphate starvation conditions. The possibility that CRC affects *plcH* induction under these conditions was also examined. Phosphate starvation-dependent *plcH* expression was not affected by CRC. This is not surprising considering that CRC would interfere with the role PLC plays as a phosphate-scavenging enzyme. These results are consistent with the preferential use of the phosphate starvation-dependent promoter of *plcH* over the osmoprotectant-dependent promoter (44–46).

Expression of *plcH* was also examined in CRC-deficient mutants. It was predicted that CRC of *plcH* by succinate would be decoupled in these bacteria, reflecting the observed phenotypes of other catabolic pathways. Another possibility was that *plcH* expression would be reduced as has been reported previously (3). Surprisingly, this mutant exhibited the same phenotype as the wild-type strain PAO1 with respect to both osmoprotectant-dependent expression of *plcH* and CRC of *plcH*. This indicates that catabolite repression of *plcH* is not subject to the same mechanism of control as that which regulates the enzymes of carbohydrate catabolism. Interestingly, the histidine-degradative pathway involving histidase and urokinase also appears to be subject to catabolite repression by a mechanism distinct from that which regulates carbohydrate metabolism (29). Thus, it appears that another distinct system exists in *P. aeruginosa*.

One possible candidate for mediation of this regulation was the Crp homolog Vfr. Crp mediates cyclic AMP-dependent CRC of carbon source metabolism in *E. coli*. Vfr is able to restore a wild-type phenotype to Crp-deficient strains of *E. coli*; however, the Crp gene could not complement a *P. aeruginosa* *vfr* mutant. Also, total PLC levels were reduced in a *vfr* mutant (48). However, the *vfr* mutant exhibited no differences from strain PAO1 with respect to osmoprotectant-dependent *plcH* expression or CRC of *plcH* under these conditions. This was consistent with a recent report indicating that CRC of neither the carbohydrate-catabolic enzymes nor the histidine utilization pathway in a *vfr*-deficient mutant was affected (38).

Direct regulation of the formation of virulence factors by CRC in *P. aeruginosa* has not been reported previously. PlcH becomes the first *P. aeruginosa*-produced virulence factor whose expression is directly regulated in this manner. It is possible that in certain cases of *P. aeruginosa* infection of the lungs of CF patients, succinate or another TCA cycle intermediate is used to reduce the formation of PLC. This could have three effects. First, *P. aeruginosa* would be denied a means of

acquiring a source of osmoprotectant with which to protect itself against the hyperosmolar conditions of the CF-affected lung. Second, the bacteria might be denied an important carbon, nitrogen, and energy source (phosphatidylcholine). Finally, a reduced level of PLC in that environment could lead to a lower level of inflammation due to the action of the phosphatidylcholine cleavage product diacylglycerol.

The regulation of the virulence factor PLC by osmoprotectants remains largely uncharacterized. The factors involved in its control are still largely unidentified. CRC is one of perhaps several regulatory mechanisms that affect PLC formation. It is possible that several other virulence factors besides *PlcH* are subject to CRC. It would be of interest to identify the factor(s) controlling this novel form of catabolite repression in *P. aeruginosa* and to characterize its interaction with *plcH*. It is also of interest to determine if CRC affects the expression of other virulence factors, especially those that may play a role in energy acquisition, such as elastase or protease. Further work in this laboratory will be directed toward this end.

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