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# The Global Carbon Metabolism Regulator Crc Is a Component of a Signal Transduction Pathway Required for Biofilm Development by *Pseudomonas aeruginosa*

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**The transition from a planktonic (free-swimming) existence to growth attached to a surface in a biofilm occurs in response to environmental factors, including the availability of nutrients. We show that the catabolite repression control (Crc) protein, which plays a role in the regulation of carbon metabolism, is necessary for biofilm formation in *Pseudomonas aeruginosa*. Using phase-contrast microscopy, we found that a *crc* mutant only makes a dispersed monolayer of cells on a plastic surface but does not develop the dense monolayer punctuated by microcolonies typical of the wild-type strain. This is a phenotype identical to that observed in mutants defective in type IV pilus biogenesis. Consistent with this observation, *crc* mutants are defective in type IV pilus-mediated twitching motility. We show that this defect in type IV pilus function is due (at least in part) to a decrease in *pilA* (pilin) transcription. We propose that nutritional cues are integrated by Crc as part of a signal transduction pathway that regulates biofilm development.**

Biofilms are communities of microorganisms attached to a surface, and their formation occurs in response to a variety of environmental cues (6, 24, 25, 30, 36). In *Pseudomonas aeruginosa*, these environmental cues include an abundant supply of nutrients, the availability of oxygen, and an osmotically balanced growth medium (24, 26). Biofilm bacteria undergo a developmental program in response to environmental signals that leads to the expression of new phenotypes that distinguish these attached cells from their planktonically growing counterparts. For example, biofilm bacteria have been shown to be up to 1,000-fold more resistant to antibiotics than are planktonically grown cells (13) and gene and protein expression patterns are altered in planktonic versus biofilm-grown cells (6, 7, 8, 26).

A common theme in microbial development involves an input of environmental cues that results in an output of an altered physiological state or behavior. There are several examples of regulatory proteins and signalling pathways which play a central role in the transduction of environmental signals and precipitation of developmental changes that allow a bacterium to adapt to its environment. For example, *Bacillus subtilis* forms spores in response to a lack of nutrients and requires the central regulator Spo0A to integrate and respond to a number of intracellular and extracellular cues (12). In *Myxococcus xanthus*, environmental parameters such as nutrient deprivation, population density, and the presence of a solid surface trigger a series of social behaviors which culminate in the formation of multicellular fruiting bodies. The products of the *asg* genes act early in development to sense the appropriate nutritional signals and initiate the formation of fruiting bodies (33). As mentioned above, environmental signals play an important role in the transition of *P. aeruginosa* from an individual, planktonic existence to a life attached to a surface in a multicellular community. However, the molecular mechanism by which environmental signals trigger the regulatory events required for biofilm formation has not been elucidated.

*P. aeruginosa* uses tricarboxylic acid (TCA) cycle intermediates (which include organic acids such as succinate) as carbon sources preferentially over carbohydrates such as glucose or mannitol and appear do so in a cyclic-AMP-independent manner (29, 34). A locus was identified, designated *crc*, that when mutated results in a strain that can utilize glucose and mannitol even when TCA intermediates are also provided (37). However, the *crc* mutants do not constitutively express the enzymes and transporters required for glucose and mannitol utilization; these enzymes and transporters are only produced when the appropriate inducers are present. Therefore, Crc appears to be required for repression of carbohydrate metabolism in the presence of TCA intermediates (37). Crc has amino acid sequence similarity to a family of apurinic-apyrimidinic endonucleases which have been shown to be involved in DNA repair in *Escherichia coli* and other organisms (2, 9). However, Crc has none of the activities associated with these enzymes (19) and the mechanism by which Crc regulates expression of carbohydrate metabolism is not understood.

Here we present data showing that a *P. aeruginosa* PA14 *crc* mutant is defective in biofilm formation and that the inability to form a biofilm is due to a defect in type IV pilus-mediated twitching motility. Based on the role of Crc in the regulation of carbon metabolism and type IV pilus-mediated twitching motility, we propose that nutritional cues are integrated by Crc as part of a signal transduction pathway that regulates biofilm development.

## MATERIALS AND METHODS

**Bacterial strains, media, and chemicals.** All of the bacterial strains and plasmids used in this study are shown in Table 1. *P. aeruginosa* PA14 was grown on rich (Luria Bertani [LB]) medium or minimal medium (as indicated in each experiment) at 37°C. Unless otherwise stated, the minimal medium used was minimal M63 salts (28) supplemented with glucose (0.2%), $MgSO_4$ (1 mM), and Casamino Acids (CAA; 0.5%). Antibiotics were added at the following concen-

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TABLE 1. Strains and plasmids used in this study

| Strain or plasmid | Relevant genotype | Reference or source |
|---|---|---|
| Strains | | |
| *Pseudomonas aeruginosa* PA14 | | 31 |
| *P. aeruginosa* PA14 | *sad-24*(*crc*)::Tn*5*-B30 Tc$^r$ | This study |
| *P. aeruginosa* PA14 | *sad-31*(*pilB*)::Tn*5*-B30 Tc$^r$ | 24 |
| *P. aeruginosa* PAO1 | | 10 |
| *P. aeruginosa* PAO8023 | *crc*::Cb$^r$ | P. V. Phibbs |
| Plasmids | | |
| pPZ352 | *crc*$^+$ from *P. aeruginosa* PAO1 | 19 |
| pUCP18 | Cloning vector; Ap$^r$ | 32 |
| pSMC31 | *crc*$^+$ from pPZ352 in pUCP18 | This study |
| pMSZ5 | *pilA-lacZ* Ap$^r$ | 16 |
| pPB18 | *pilB-lacZ* Ap$^r$ | 16 |
| pDN19 | Vector; Ap$^r$ | 16 |

trations: tetracycline, 150 μg/ml; ampicillin, 750 μg/ml; carbenicillin, 1 mg/ml. All enzymes for DNA manipulation were purchased from New England Biolabs (Beverly, Mass.). All plasmids were constructed in *E. coli* JM109 by using standard protocols (1) and transferred to *P. aeruginosa* by electroporation (3).

**Enzyme assays.** To generate cell material for enzyme assays, bacterial cultures were grown in 250 ml of minimal medium (18) with 20 mM mannitol as the sole carbon and energy source for at least 9 generations in log phase to fully induce the *hex* regulon (including glucose 6-phosphate dehydrogenase, mannitol dehydrogenase, and NAD-specific glyceraldehyde 3-phosphate dehydrogenase). An aliquot of cells was transferred to minimal medium containing 40 mM succinate and 20 mM mannitol, and growth was continued for 2 to 2.5 generations. Bacteria were chilled, centrifuged, and washed with 1/10 volume of cold minimal medium, and the cell pellets were frozen.

Extracts were prepared from frozen cell pellets by suspending the cells in 2 to 3 volumes of Tris-Cl (50 mM; pH 8)-dithiothreitol (1 mM). The suspended cells were broken by passage through a French press at 16,000 lb/in$^2$ and clarified by centrifugation at 170,000 × *g* for 30 min (50,000 rpm in a Sorvall T-1270 rotor). Mannitol dehydrogenase was assayed on freshly prepared extracts, while the other assays were performed on thawed extracts. Glucose 6-phosphate dehydrogenase and mannitol dehydrogenase were assayed as previously described (14, 34). The NAD- and NADP-dependent glyceraldehyde 3-phosphate dehydrogenase assays were similar to those of Tiwari and Campbell (35). Briefly, 1-ml reaction mixtures containing NAD or NADP at 0.5 mM; Tris-Cl at 100 mM (pH 8), sodium arsenate at 20 mM, L-cysteine at 3.3 mM, and sodium fluoride at 20 mM were incubated at 22°C. Reactions were started with the addition of glyceraldehyde 3-phosphate to 2 mM. For the determination of specific activities, extracts were assayed for protein by the method of Lowry et al. (17) with bovine serum albumin as the standard.

**Twitching motility assays.** Twitching motility was assessed by stabbing cells into a very thin (2-mm) LB agar plate (1.5% agar) with a toothpick and incubating the plate for 24 to 48 h at 37°C. A haze of growth at the agar-plate interface and the characteristic flat, spreading colony morphology indicate strains that are proficient for twitching motility (21, 23).

**Biofilm formation assay. (i) Screen for mutants defective in biofilm formation.** The assay used to screen for biofilm formation-defective mutants is based on the ability of bacteria to initiate biofilm formation on polyvinyl chloride (PVC) plastic. The initiation of biofilm formation was assayed as previously described (25) by determining the ability of cells to adhere to the wells of 96-well microtiter dishes made of PVC (Falcon 3911 Microtest III Flexible Assay Plate; Becton Dickinson Labware, Oxnard, Calif.) using a modification of a previously reported protocol (11). The medium (100 μl/well) was inoculated with either (i) cells patched to LB agar plates using a multiprong device or (ii) a 1:100 dilution from an overnight LB culture. After inoculation, plates were incubated at 37°C for the indicated time and then 25 μl of a 1% crystal violet solution was added to each well (this dye stains the cells but not the PVC). The plates were incubated at room temperature for ~15 min, rinsed four times thoroughly and vigorously with water, blotted on paper towels, and scored for biofilm formation (see Fig. 1).

**(ii) Quantitation of biofilm formation.** Biofilm formation was quantified as previously described (25). Briefly, the crystal violet was solubilized in 95% ethanol and the $A_{570}$ was determined.

**(iii) Microscopy.** Visualization of bacterial cells attached to PVC was performed by phase-contrast microscopy (400× magnification) using a Nikon Diaphot 200 inverted microscope (Nikon Corp., Tokyo, Japan). The images were captured with a black-and-white CCD72 camera integrated with a Power Macintosh 8600/300 computer with video capability. The images were processed with Scion Image software, a modification of NIH Image (National Institutes of Health, Bethesda, Md.) by the Scion Corporation (Frederick, Md.).

**Molecular techniques.** The DNA sequence flanking the transposon mutant insertion site was determined by using arbitrary primed PCR as previously reported (27). All other protocols were performed as previously reported (1, 25).

**Plasmid construction.** Plasmid pSMC31 (*crc*$^+$ Cb$^r$) carries a wild-type copy of the *crc* locus derived from *P. aeruginosa* PAO1. This plasmid was built by first amplifying *crc*$^+$ from pPZ352 (19) by PCR using primers which generated a *Bam*HI site at the 5′ end and an *Eco*RI site at the 3′ end of the *crc* gene. The PCR product was digested with *Eco*RI and *Bam*HI and then ligated to pUCP18, which had also previously been digested with *Eco*RI and *Bam*HI.

**EM.** LB agar plates without NaCl were prepared and inoculated as described for the twitching motility assays above. Approximately 10 μl of distilled water ($dH_2O$) was dropped on the edge of the colony, the bacteria were allowed to become suspended for ~30 s, and then the copper-coated grid was floated on the cell-containing $dH_2O$ drop for ~30 s, rinsed for 5 s with $dH_2O$, stained for 45 s with phosphotungstate (2%), rinsed for 5 s with $dH_2O$, and then gently blotted and air dried (D. Wozniak, personal communication). For each strain, at least 100 cells were examined. The pili are fragile structures; they are apparently easily lost during the preparation of electron microscopy (EM) grids. Samples were analyzed with a JEOL 1200 EX transmission electron microscope at 80 kV.

**β-Galactosidase assays.** β-Galactosidase assays were performed as previously described without modification (22). The data presented are averages of two cultures assayed in duplicate (a total of four data points).

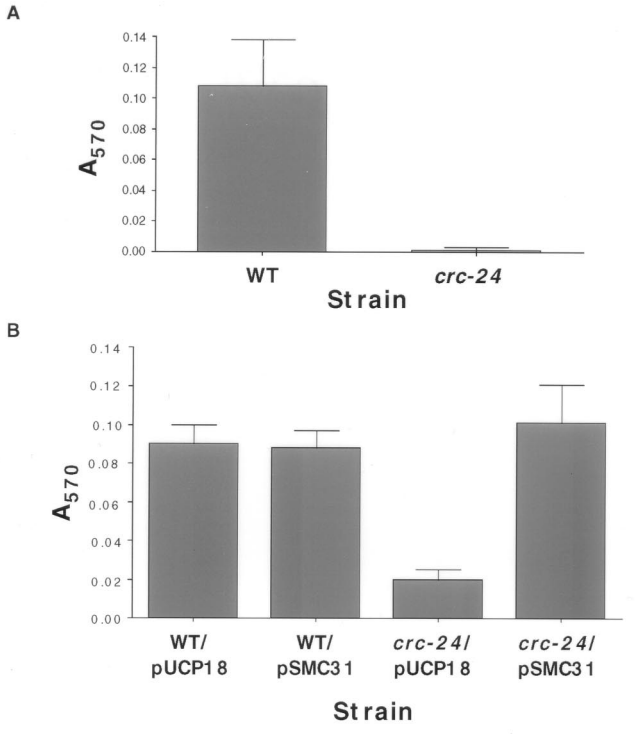
## RESULTS

**Isolation of the *crc* mutant.** We previously performed a screen for mutants of *P. aeruginosa* PA14 defective in biofilm formation and identified mutations in genes required for flagellum-mediated swimming and type IV pilus-mediated twitching motility (24). In this search for biofilm formation mutants, we also obtained a *sad-24* mutant strain with an insertion in the *crc* gene. The biofilm formation phenotype of this mutant is shown in Fig. 1A.

Comparison of the DNA sequence flanking the *sad-24*::Tn*5* insertion to DNA sequences in the GenBank database revealed 100% identity over a 34-amino-acid region to the previously described *crc* locus of *P. aeruginosa* PAO1 (19), and this allele was therefore designated *crc-24*. The location of the insertion is near the 3′ end of the *crc* gene, and the mutated gene is predicted to code for a protein whose C-terminal 38 amino acids are deleted and replaced with 9 amino acids derived from a transposon sequence. We observed other phenotypes in the strain carrying the *crc-24*::Tn*5* mutation, including overproduction of a pigment (presumably pyocyanin [4]) and a decrease in type IV pilus-mediated twitching motility.

It is important to note that the growth rate of the *crc* mutant is indistinguishable from that of the wild type in glucose-containing minimal medium supplemented with CAA (the same medium and growth conditions used in the screen to isolate this mutant; data not shown). Therefore, the defect in the *crc* mutant appears to be specific to biofilm formation and not a general defect in growth. Finally, in addition to its defect in biofilm formation on PVC plastic, this strain is also defective for biofilm formation on polystyrene, polycarbonate, and polypropylene (data not shown).

**Complementation of the *crc* mutation.** In order to confirm that the phenotypes of the *crc-24* mutant strain were indeed caused by the mutation in the *crc* gene, we performed complementation analysis. The *crc* gene of *P. aeruginosa* PAO1 had been cloned previously (19), and using the cloned gene, we built a plasmid construct which carries only *crc*$^+$ and no other complete open reading frame (pSMC31; see Methods and Materials for details of the plasmid construction). Figure 1B shows the results of the complementation assay performed on these strains. The plot in Fig. 1B shows that based on quantitation of biofilm formation using the crystal violet assay, plasmid pSMC31 (*crc*$^+$) fully rescued the biofilm formation defect of the *crc-24* mutant strain to levels equivalent to those of the wild type (carrying either plasmid). As expected, the mutant



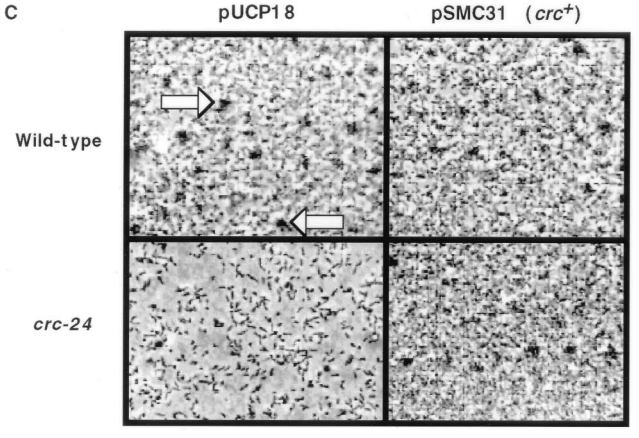




FIG. 1. Biofilm formation phenotypes. (A) Biofilm formation phenotypes of the wild type (WT) and a *crc-24*::Tn*5* mutant. Cells attached to PVC plastic after 8 h of growth were stained with crystal violet, and then the cell-associated crystal violet was solubilized in ethanol. The $A_{570}$ of the ethanol-solubilized crystal violet was measured (see Materials and Methods for a detailed explanation of this protocol). (B) Complementation of the biofilm formation defect of the *crc-24* mutant by providing a wild-type copy of *crc* in *trans*. Biofilm formation was quantitated as described above. The extent of biofilm formation was determined for the wild-type strain or the *crc-24* mutant carrying either a *crc*-containing plasmid (pSMC31 [$crc^+$]) or the vector control (pUCP18). Cells were grown in glucose-containing minimal medium plus CAA at 37°C for 8 h. The data presented are averages of four replicate experiments. (C) The direct observation of biofilm formation on PVC by phase-contrast microscopy (400-fold magnification). The dark spots (indicated by arrows) are microcolonies of bacteria. The light gray regions are the surface of the PVC plastic. Cells were grown in glucose-containing minimal medium plus CAA at 37°C for 8 h.

carrying the vector control remained defective in biofilm formation.

The result above was confirmed and extended by directly visualizing biofilm formation by these strains using phase-contrast microscopy (Fig. 1C). After incubation for 8 h, the wild type (carrying either plasmid) had formed a dense monolayer of cells punctuated with microcolonies on the surface of the PVC plastic. Examination of the *crc-24* strain carrying pUCP18 (vector-only control) revealed a dispersed monolayer of cells markedly different from that of the wild-type strain. However, providing the *crc-24* mutant with a wild-type copy of *crc* on pSMC31($crc^+$) resulted in a biofilm formation phenotype indistinguishable from that of the wild-type strain.

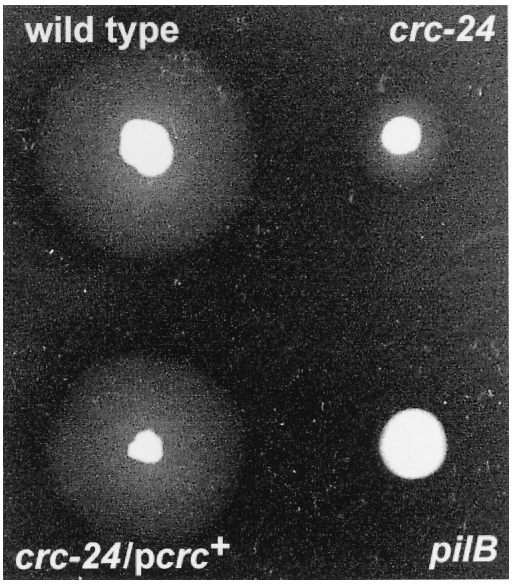



FIG. 2. The *crc-24* mutant is defective in twitching motility. Shown are twitching motility assays of the wild type and the *crc-24* mutant strain carrying plasmid-borne $crc^+$ (pSMC31) or the vector control (pUCP18). The light haze surrounding the colonies is the zone of twitching, which is occupied by cells that have migrated away from the point of inoculation using type IV pilus-mediated twitching motility. In a strain completely lacking type IV pili (the *pilB* mutant), no twitching zone is observed. The assays were performed as described in Materials and Methods.

In addition to assessing biofilm formation, we also investigated whether pSMC31 ($crc^+$) could complement the defect in twitching motility of the *crc-24* mutant (Fig. 2). The hazy zone of growth around the wild-type colonies is the "twitching zone"; that is, the area containing cells which have used type IV pilus-mediated twitching motility to move away from the point of inoculation. The wild-type strain (carrying either plasmid) and the *crc-24* mutant carrying pSMC31 ($crc^+$) both formed an obvious twitching zone with an average size of 9.5 mm (measured as the distance from the colony edge to the edge of the twitching zone). In contrast, the *crc-24* mutant carrying pUCP18 (vector control) produces a twitching zone about sixfold smaller (1.5 mm) than that produced by the wild-type strain. Furthermore, the *crc-24* mutant carrying pSMC31 ($crc^+$) no longer overproduced pyocyanin (data not shown). Based on these results, we conclude that the *crc-24* mutation caused the observed phenotypes.

**The biofilm formation phenotype of a *crc* mutant is identical to that of strains carrying mutations in genes required for type IV pilus biogenesis.** The *crc-24*::Tn*5* mutant of *P. aeruginosa* PA14 is defective for twitching motility. This observation suggested that *crc* is required for the synthesis or function of type IV pili. As assessed by phase-contrast microscopy, the *crc-24* mutant showed a pattern of dispersed cells on the PVC plastic surface after incubation for up to 8 h (Fig. 1C), a phenotype identical to that observed previously for mutants lacking type

IV pili (24). Therefore, the *crc* mutant has a biofilm formation defect indistinguishable from that of a strain which lacks type IV pili.

**The *crc-24*::Tn*5* mutant produces type IV pili.** Three lines of evidence demonstrated that although the *crc-24* mutant is defective in twitching motility, this strain still has at least some functional type IV pili. The first line of evidence is that the *crc-24* mutant retains residual twitching motility, as shown in Fig. 2. Second, the *crc-24* mutant remains sensitive to infection by bacteriophage SN-T (15), which utilizes type IV pili as a receptor (26). The abilities of phage SN-T to form plaques on the wild-type strain and the *crc-24* mutant are indistinguishable (data not shown). It is interesting that although the *crc-24* mutant has decreased twitching motility, it is still sensitive to phage SN-T infection. This observation may reflect the sensitivity of the phage infection assays, that is, the possibility that only a single phage or a few phage are sufficient for infection and lysis. The wild-type strain may be bound by many more phage particles than are required for cell lysis. No plaques were formed on other type IV pilus mutants (such as *pilB* and *pilC* mutants), even using a phage lysate at a titer of $\sim 3 \times 10^7$ PFU/ml. Finally, we directly visualized type IV pili by using EM. Type IV pili are polar structures found at the same end of the cell as the flagellum. As shown in Fig. 3, in the wild-type strain the type IV pili are readily visible as multiple, polarly localized rods which are somewhat thinner than the single polar flagellum. These pili are also visible in the *crc* mutant, consistent with the results of the phage susceptibility tests and twitching motility assays. However, in the *pilB* strain, which is not susceptible to lysis by phage SN-T and retains no detectable twitching motility, no pili were visible.

**A *crc* null mutant of *P. aeruginosa* PAO1 is also defective for biofilm formation.** As mentioned above, mutations in *crc* were originally isolated in *P. aeruginosa* PAO1 based on their defects in the regulation of carbon metabolism. However, these mutations were not tested for defects in biofilm formation. The constructed *crc*::Cb$^r$ mutation in *P. aeruginosa* PAO1, like the *crc*::Tn*5* mutation in *P. aeruginosa* PA14, results in a strain defective in biofilm formation (data not shown). We have also shown that a strain carrying the *crc*::Cb$^r$ allele is also defective for twitching motility and remains sensitive to lysis by type IV pilus-specific bacteriophage SN-T. Prince and coworkers had also indicated that a *crc* mutant of *P. aeruginosa* PAO1 is defective in twitching motility (4). These data further confirm a role for *crc* in biofilm formation and demonstrate that the defect in biofilm formation is not specific to *P. aeruginosa* PA14.

**Effects of *crc-24* on carbon metabolism in *P. aeruginosa* PA14.** The *crc* locus was originally identified in *P. aeruginosa* PAO1 during a screen for genes that participate in catabolite repression control of enzymes involved in mannitol utilization (37). In order to determine if a *P. aeruginosa* PA14 *crc* mutant is also defective in the regulation of carbon metabolism (in addition to the other observed phenotypes), we assessed the *crc-24* mutant isolated in the biofilm mutant screen for its effects on catabolite repression in *P. aeruginosa* PA14. These data are summarized in Table 2. In wild-type *P. aeruginosa* PAO1, the levels of enzyme activities required for glucose and mannitol utilization are repressed in the presence of succinate (29, 34). Similarly, levels of representative enzymes involved in mannitol and glucose catabolism in *P. aeruginosa* PA14 (mannitol dehydrogenase, glucose 6-phosphate dehydrogenase, and NAD-specific glyceraldehyde 3-phosphate dehydrogenase) are up to eightfold higher when cells are grown in the absence versus the presence of succinate. The level of these enzymes in *P. aeruginosa* PA14 carrying the *crc-24* allele is no longer as strongly repressed (or not repressed at all) when cells are grown in the presence of succinate. This is the phenotype that originally defined the *crc* locus (37). The NADP-specific glyceraldehyde 3-phosphate dehydrogenase (as opposed to the NAD-specific enzyme) is not regulated by Crc, and the enzyme level shows a less-than-twofold change when cells are grown in the presence versus the absence of succinate.

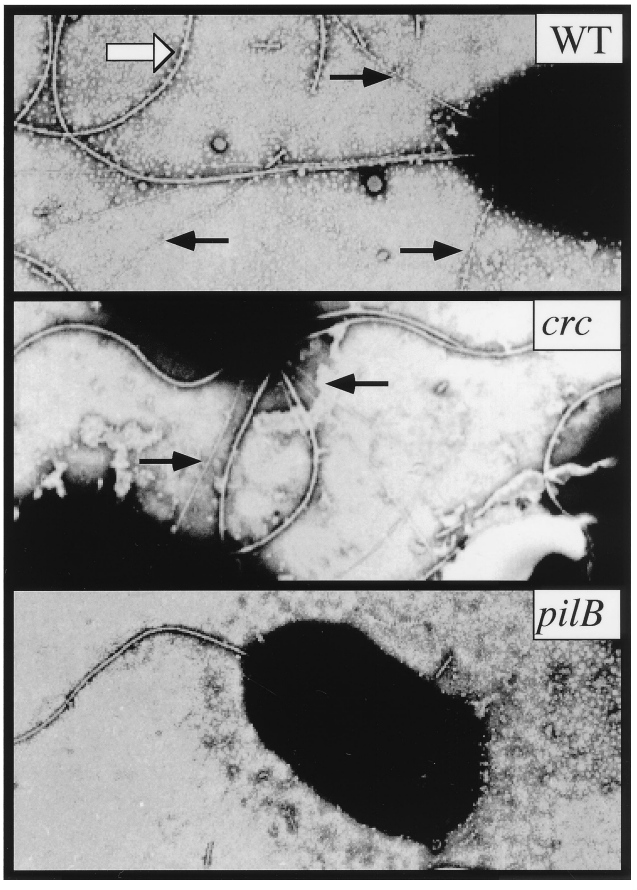



FIG. 3. Transmission EM. Shown are electron micrographs of cells stained with phosphotungstate and viewed at a magnification of ×15,000. Black arrows indicate the type IV pili, and the white arrow points to the relatively thicker flagellum. Pili can be seen in both the wild type (WT) and the *crc-24* mutant, but no pili were observed in the *pilB* mutant. See Materials and Methods for details. Not all of the wild-type cells had observable pili. However, pili could be observed on a subset of wild-type and *crc-24* mutant cells (~5 to 10%) but were never observed on *pilB* mutant cells.

Strains which express amidase are sensitive to the amide analog fluoroacetamide (FAA), which is converted to the toxic metabolite fluoroacetate by this enzyme (5). Expression of amidase is normally repressed when cells are grown on succinate, and this repression is dependent on Crc. Strains without functional Crc express amidase even in the presence of succinate and die when FAA is included in the medium. Thus, FAA sensitivity acts as an indirect assay of Crc function. Figure 4 shows the growth of the wild type, the *crc-24* mutant, and the *crc-24* mutant carrying pSMC31 (*crc*$^+$) on minimal medium containing succinate with (A) or without (B) FAA. All of the strains grew equally well on minimal medium containing succinate without FAA (Fig. 4B). In contrast, the *crc-24* mutant showed no growth on the FAA-containing medium (Fig. 4A). The wild type and the *crc-24* mutant carrying the wild-type copy of *crc* on a plasmid grew well on this medium. Therefore,

TABLE 2. Effects of *crc-24* mutation on levels of carbohydrate catabolism enzymes in *P. aeruginosa* PA14

| Genotype | Addition[a] | Avg activity ± SD[b] | | | |
|---|---|---|---|---|---|
| | | Gluc-6-P DH | Mannitol DH | NAD-specific Gly-3-P DH | NADP-specific Gly-3-P DH[c] |
| *crc*$^+$ | None | 256 ± 23[d] | 56 ± 11 | 1,014 ± 102 | 130 ± 67 |
| | Succinate | 32 ± 5 | 15 ± 1.4 | 125 ± 5 | 76 ± 4 |
| *crc-24* | None | 335 ± 70 | 100 ± 13 | 1,203 ± 103 | 168 ± 8 |
| | Succinate | 191 ± 48 | 73 ± 11 | 755 ± 67 | 130 ± 20 |

[a] Bacteria were grown on minimal medium with 30 mM mannitol with or without the addition of 40 mM succinate. See Materials and Methods for details.
[b] Abbreviations: Gluc-6-P DH, glucose 6-phosphate dehydrogenase; Gly-3-P DH, glyceraldehyde 3-phosphate dehydrogenase.
[c] The level of NADP-specific glyceraldehyde 3-phosphate dehydrogenase activity is Crc independent and served as a control.
[d] The enzyme assays monitored the reduction of NAD(P) to NAD(P)H, and specific activity is expressed as nanomoles of NAD(P)H produced per minute per milligram of protein. The data presented are for duplicate (NAD- and NADP-dependent glyceraldehyde 3-phosphate dehydrogenase) or triplicate (glucose 6-phosphate dehydrogenase and mannitol dehydrogenase) assays.

although the strain carrying the *crc-24* mutation was isolated in a screen for mutants unable to form a biofilm, this strain is also defective in the Crc-mediated, succinate-dependent catabolite repression of enzymes required for carbohydrate catabolism.

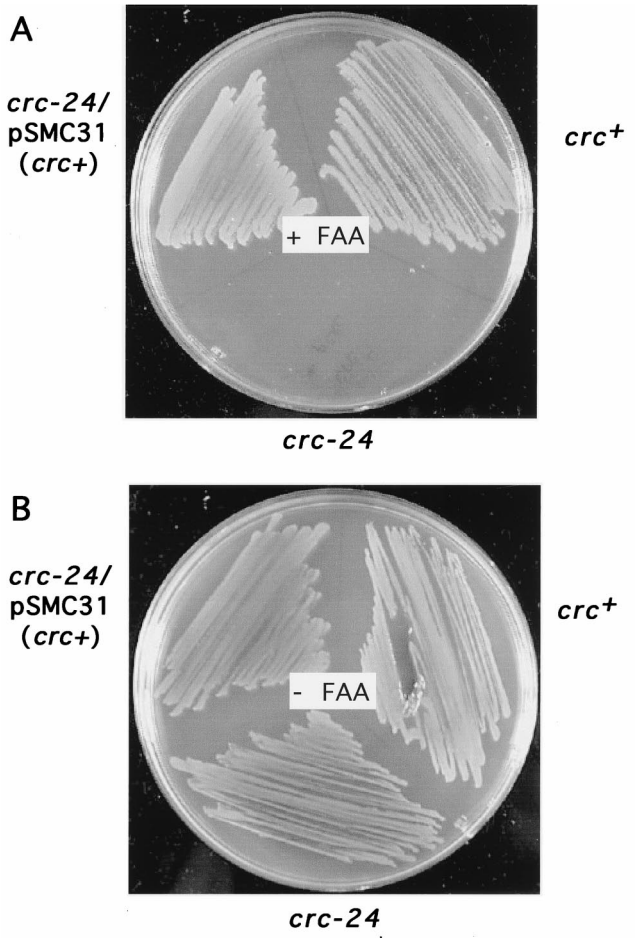



FIG. 4. *crc* mutants are sensitive to growth on succinate-containing minimal medium supplemented with FAA. (A) Shown is a minimal-medium agar plate supplemented with succinate (40 mM) and FAA (2.5 mg/ml). The wild-type strain (*crc*$^+$) and the *crc-24* mutant carrying pSMC31 (*crc*$^+$) grew well on this medium. In contrast, the *crc-24* mutant was completely defective in growth on FAA-containing medium. The plate was incubated for 48 h at 37°C. (B) All strains grew equally well on a minimal-medium agar plate supplemented with succinate alone (no FAA). The plate was incubated for 24 h at 37°C.

**Mutations in *crc* decrease the expression of *pilA*, the pilin structural gene.** Phibbs and coworkers reported that *P. aeruginosa* PAO1 carrying mutations in *crc* had increased levels of enzymes and transporters involved in carbohydrate metabolism even under conditions in which expression of these functions should have been repressed (i.e., in the presence of TCA intermediates such as succinate) (20, 37). However, the mechanism by which Crc could alter gene expression has not yet been determined.

We have obtained evidence that Crc can function to regulate gene expression at the level of transcription. Specifically, strains with mutations in *crc* have markedly lower levels of expression of *pilA*, which codes for the major pilin subunit, and *pilB*, which codes for an accessory factor required for pilus assembly. These genes are positioned adjacent to each other on the chromosome but are transcribed divergently (21, 23). We introduced plasmid-borne *pilA-lacZ* (pMSZ25) and *pilB-lacZ* (pPB18) transcriptional fusions (along with a vector control, pDN19) into wild-type *P. aeruginosa* PAO1 and its *crc*::Cb$^r$ derivative. The results of β-galactosidase assays performed on these strains are summarized in Fig. 5. Expression of the *pilA-lacZ* transcriptional fusion activity was 132-fold higher in the wild type than in the *crc* mutant strain (*crc*$^+$, 3,965 miller units [MU]; *crc* mutant, 30 MU). Similarly, the expression of the *pilB-lacZ* transcriptional fusion in the wild type was 641 MU, compared to 98 MU from this same fusion in the *crc*::Cb$^r$ mutant (a decrease of 6.5-fold). As expected,

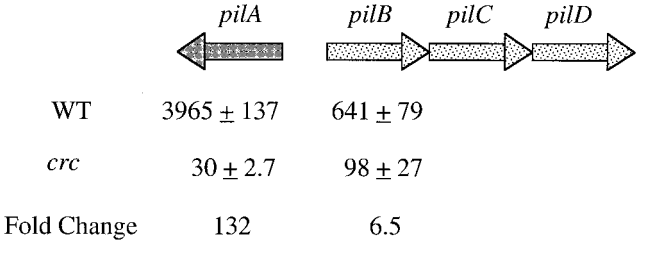



FIG. 5. Effect of a *crc* mutation on expression of genes required for type IV pilus biogenesis. At the top is the organization of the chromosomal region of *pilA* and *pilB* in *P. aeruginosa*. Shown below is the expression of the *pilA-lacZ* and *pilB-lacZ* fusions in either the wild-type (WT) or the *crc*::Cb$^r$ mutant background (expressed in MU). Averages and standard errors are shown, and the fold change in expression between the wild-type and *crc* mutant strains is shown in the last line. Cells were grown to late exponential phase in M63 minimal medium containing glucose (0.2%) and CAA (0.5%) and supplemented with tetracycline to maintain selection of plasmids carrying the transcriptional fusions. These growth conditions are similar to those used to assay biofilm formation. The representative data presented are averages of two cultures assayed in duplicate (a total of four data points).

the vector control showed only minimal activity in both genetic backgrounds.

## DISCUSSION

Environmental signals play an important role in regulating the formation of biofilms; however, very little is known about the molecular mechanisms necessary to transduce the environmental signals that trigger biofilm development. We report here the isolation of a strain defective in biofilm formation which has a mutation in *crc*, a locus which may participate in the linking of environmental signals with biofilm development. It has been shown previously that the *crc* gene is involved in the regulation of carbon metabolism in *P. aeruginosa* (20, 37). We show here that Crc has a role in the formation of biofilms on abiotic surfaces and that *P. aeruginosa* PA14 *crc* mutants not only are defective in type IV pilus-mediated twitching motility (Fig. 1 and 2) but also have all of the carbon source utilization phenotypes described for the *crc* mutants of *P. aeruginosa* PAO1 (Table 2 and Fig. 4).

We propose that the *crc* mutant is defective in biofilm formation because it lacks fully functional type IV pili. Two lines of evidence support this conclusion. First, we have shown previously that type IV pili and type IV pilus-mediated twitching motility are necessary for the development of biofilms in *P. aeruginosa* PA14 (24). In fact, the observation that the *crc-24* mutant has a defect in twitching motility but still retains at least some type IV pili on its surface (based on phage sensitivity and EM studies) further supports our previous hypothesis that twitching motility (and not just the presence of the pili on the cell surface) plays an important role in biofilm development (24). The direct observation of the biofilm formed by a *crc* mutant on PVC plastic using phase-contrast microscopy also supports the contention that it is the defect in type IV pilus assembly and/or function that leads to the biofilm formation defect. The phase-contrast micrograph in Fig. 1C shows that the phenotype of the *crc-24* mutant is indistinguishable from that reported for a *pilB* mutant; strains carrying mutations in *pilB* have been shown previously to make no detectable type IV pili (23) and to be defective in biofilm formation (24). Therefore, the biofilm formation phenotype of the *crc-24* mutant can be accounted for by its defect in type IV pilus assembly and/or function.

The results of twitching motility assays suggested that *crc* mutants have a defect in the synthesis, assembly, and/or function of type IV pili. Based on previous studies of the role of Crc in the regulation of carbon metabolism (20, 37), it was possible that Crc exerts its effect via gene expression at some level. As shown in Fig. 5, the expression of genes whose products are essential for type IV pilus biogenesis (*pilA* and *pilB*) is markedly reduced in *crc* mutant backgrounds compared to that in the wild-type strain. This is the first report of Crc regulation of a non-carbohydrate metabolism target and the first demonstration that Crc can affect regulation at the transcriptional level. It is possible, therefore, that decreased levels of the pilin subunit, accessory factors required for assembly of the pilin subunit into functional pili, and/or expression of gene products required for extension-retraction of pili cause the defect in twitching motility and biofilm formation observed in *P. aeruginosa* PA14 and PAO1 *crc* mutants.

It is clear from work in many laboratories that biofilm formation occurs in response to various environmental cues (6, 24, 25, 30, 36). However, how these environmental signals are sensed and transduced by the biofilm-forming bacteria and the molecular mechanism(s) utilized to initiate the development of a biofilm in response to these cues are not understood.

We propose that Crc may play a role in the transduction of nutritional signals that trigger biofilm development by *P. aeruginosa*. Crc has been shown to be involved in the regulation of carbon utilization by preventing the utilization of sugars such as glucose when *P. aeruginosa* PAO1 cells are also provided with TCA cycle intermediates (20, 37). We have shown that a *crc* mutant of *P. aeruginosa* PA14, like its *P. aeruginosa* PAO1 counterpart, is defective in the succinate-dependent catabolite repression of enzymes required for carbohydrate catabolism. We have also shown here that Crc is necessary for biofilm formation, possibly by controlling the transcription of genes required for type IV pilus biogenesis. Therefore, Crc may be part of a signal transduction pathway that can sense and respond to nutritional signals and thereby play a role in the bacterium's transition from planktonic to biofilm growth in response to these nutritional signals. Additional work is needed to determine the precise mechanism by which Crc regulates its various targets and to identify other members of this signalling pathway. Finally, the fact that *crc* cross-hybridizing sequences are also found in a variety of fluorescent pseudomonads (19) suggests that Crc plays a similar role in biofilm formation in these related organisms.

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