Identification of a Dehydrogenase Required for Lactose Metabolism in *Caulobacter crescentus*^{▽†‡}

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Caulobacter crescentus, which thrives in freshwater environments with low nutrient levels, serves as a model system for studying bacterial cell cycle regulation and organelle development. We examined its ability to utilize lactose (i) to gain insight into the metabolic capacities of oligotrophic bacteria and (ii) to obtain an additional genetic tool for studying this model organism, aiming to eliminate the basal enzymatic activity that hydrolyzes the chromogenic substrate 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal). Using a previously isolated transposon mutant, we identified a gene, *lacA*, that is required for growth on lactose as the sole carbon source and for turning colonies blue in the presence of X-gal. LacA, which contains a glucose-methanol-choline (GMC) oxidoreductase domain, has homology to the flavin subunit of *Pectobacterium cypripedii*'s gluconate dehydrogenase. Sequence comparisons indicated that two genes near *lacA*, *lacB* and *lacC*, encode the other subunits of the membrane-bound dehydrogenase. In addition to lactose, all three *lac* genes are involved in the catabolism of three other β -galactosides (lactulose, lactitol, and methyl- β -D-galactoside) and two glucosides (salicin and trehalose). Dehydrogenase assays confirmed that the *lac* gene products oxidize lactose, salicin, and trehalose. This enzymatic activity is inducible, and increased *lac* expression in the presence of lactose and salicin likely contributes to the induction. Expression of *lacA* also depends on the presence of the *lac* genes, implying that the dehydrogenase participates in induction. The involvement of a dehydrogenase suggests that degradation of lactose and other sugars in *C. crescentus* may resemble a proposed pathway in *Agrobacterium tumefaciens*.

Caulobacter species inhabit diverse aquatic and soil environments, their ubiquitous distribution reflecting an ability to prosper despite low nutrient levels (47, 48). Unraveling the physiological adaptations that enable these aerobic chemoheterotrophs to thrive under nutrient-poor conditions will facilitate interpretation of microbial activities in oligotrophic environments, such as water bodies (which make up the majority of the earth's surface). Recent studies of *Caulobacter* have led to novel findings regarding the uptake and catabolism of various carbon sources (6, 14, 38, 44, 52). In addition, one particular member of the group, *Caulobacter crescentus*, has emerged as a prominent model for the study of bacterial cell cycle progression, asymmetric cell division, and organelle development (7, 8, 34, 49). Although factors that regulate the *C. crescentus* cell cycle appear to respond to changes in metabolic states (3, 22, 35), details of the connection remain nebulous. Investigation of *C. crescentus*'s metabolic capabilities can improve understanding of bacterial cell cycle control as well as the biogeochemical contributions of oligotrophs.

We initiated an examination of *C. crescentus*'s ability to utilize lactose for two reasons. (i) *C. crescentus* cells predominantly dwell in freshwater ponds, lakes, and rivers (47, 48). While carbon sources from the degradation of plant materials

might be relatively abundant in such environments (25), lactose should be a rare nutrient source. Understanding how *C. crescentus* metabolizes lactose can reveal how it survives under conditions where the availability of any nutrient is capricious and ephemeral. (ii) *C. crescentus* has native β -galactosidase activity that turns its colonies blue on solid media containing the chromogenic substrate 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal) (32, 42). By identifying and deleting genes required for lactose metabolism, we can abolish the basal activity of X-gal hydrolysis, thus creating a genetic background that allows for blue/white screening of colonies. This additional genetic tool would facilitate the study of this model organism.

By mapping a previously isolated transposon mutant, we have identified a gene, *lacA*, that is essential for lactose utilization in *C. crescentus*. Deletion of *lacA* reduces the basal level of inducible β -galactosidase activity, allowing color-based distinction of wild-type and mutant strains on plates containing X-gal. This β -galactosidase activity also contributes to the hydrolysis of *o*-nitrophenyl- β -D-galactoside (ONPG), another chromogenic analog of lactose, and is sensitive to sodium dodecyl sulfate (SDS) and chloroform. Unexpectedly, *lacA* encodes a protein with an oxidoreductase domain and homology to the flavin subunit of a gluconate dehydrogenase. Sequence evaluation led to the identification of adjacent genes (*lacB* and *lacC*) as ones that encode the other two subunits of the dehydrogenase. Analysis using Phenotype MicroArrays revealed that *lacA* is also involved in the catabolism of three other β -galactosides (lactulose, lactitol, and methyl- β -D-galactoside) and two glucosides (salicin and trehalose). Enzymatic assays suggest that LacA indeed acts as a dehydrogenase, capable of oxidizing lactose, salicin, and trehalose. This dehydrogenase

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‡ Dedicated to the memory of Mei-Long H. Chen.

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant genetic markers, features, and/or description	Construction, source, or reference ^a
<i>E. coli</i> strains		
DH10B	F ⁻ <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) ϕ 80 <i>lacZ</i> Δ M15 Δ <i>lacX74</i> <i>endA1</i> <i>recA1</i> <i>deoR</i> Δ (<i>ara leu</i>)7697 <i>araD139</i> <i>galU</i> <i>galK</i> <i>nupG</i> <i>rpsL</i>	Invitrogen
MT616	MT607/pRK600; helper strain for mobilizing RK2/RP4-derived plasmids	20
<i>C. crescentus</i> strains		
CB15	Wild type	47
NA1000	<i>syn-1000</i> ; previously called CB15N, a synchronizable derivative of CB15	18
SC1140	CB15 <i>lac-101::Tn5 str-152</i>	16, 17
JOE2321	NA1000 Δ <i>lacA</i> (Δ CC1634)	Constructed from NA1000 using pJC343
JOE2323	CB15 Δ <i>lacA</i> (Δ CC1634)	Constructed from CB15 using pJC343
JOE2325	NA1000 Δ <i>lacA::</i> Ω	Constructed from NA1000 using pJC345
JOE2913	CB15 Δ <i>lacA::</i> Ω	CB15 x Φ (JOE2325), select for Sp ^r
JOE3017	NA1000 Δ <i>lacC</i> (Δ CC1635)	Constructed from NA1000 using pJC415
JOE3019	CB15 Δ <i>lacC</i> (Δ CC1635)	Constructed from CB15 using pJC415
JOE3021	NA1000 Δ <i>lacB</i> (Δ CC1632)	Constructed from NA1000 using pJC414
JOE3023	CB15 Δ <i>lacB</i> (Δ CC1632)	Constructed from CB15 using pJC414
JOE3094	NA1000 <i>lacB::</i> pJC418	Mated pJC418 into NA1000
JOE3095	NA1000 <i>lacC::</i> pJC419	Mated pJC419 into NA1000
Plasmids		
pNPTS138	For two-part selection in gene replacement, Km ^r , <i>sacB</i> (confers sensitivity to sucrose)	M. R. K. Alley, unpublished data
pCM62	IncP broad-host-range vector, Tc ^r	40
pJBZ281, 282	Insert <i>E. coli lacZ</i> into <i>C. crescentus</i> genome for expression analysis, Km ^r	M. R. K. Alley, unpublished
pJC324	pCM62 with transcriptional terminator upstream of multiple cloning site	11
pJC326	pJC324- <i>lacZ</i> _{Ec}	11
pJC327	pJC324-P _{perp} - <i>lacZ</i> _{Ec}	11
pJC343	pNPTS138- Δ <i>lacA</i> (Δ CC1634)	See Materials and Methods
pJC345	pNPTS138- Δ <i>lacA::</i> Ω	See Materials and Methods
pJC389	pCM62- <i>lacA</i> (CC1634)	See Materials and Methods
pJC404	pJC324-P _{lacA} - <i>lacZ</i> _{Ec}	See Materials and Methods
pJC414	pNPTS138- Δ <i>lacB</i> (Δ CC1632)	See Materials and Methods
pJC415	pNPTS138- Δ <i>lacC</i> (Δ CC1635)	See Materials and Methods
pJC416	pCM62- <i>lacB</i> (CC1632)	See Materials and Methods
pJC417	pCM62- <i>lacC</i> (CC1635)	See Materials and Methods
pJC418	pJBZ281- <i>lacB</i>	See Materials and Methods
pJC419	pJBZ282- <i>lacC</i>	See Materials and Methods

^a Φ indicates generalized transduction, as mediated by bacteriophage Φ Cr30. For example, CB15 x Φ (JOE2325) means that a bacteriophage lysate made from JOE2325 was used to infect CB15.

activity is inducible, and elevated expression of *lacA* in the presence of lactose and salicin, but not trehalose, contributes to the increase in activity. Moreover, *lacA* is required for the induction of both gene expression and dehydrogenase activity. We found that the *lacB* and *lacC* deletion strains exhibited characteristics similar to those of the *lacA* deletion mutant, in support of the assertion that products of the three genes act in concert as a dehydrogenase. The involvement of a dehydrogenase in the metabolism of varied sugars provides insight into *C. crescentus*'s ability to scavenge for scarce nutrients. Based on a possible lactose degradation pathway in *Agrobacterium tumefaciens*, we propose that the dehydrogenase catalyzes the formation of 3-keto sugars.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All bacterial strains and plasmids used in this study are listed in Table 1. *C. crescentus* strains were grown at 30°C in peptone-yeast extract (PYE) complex media (27) or M2 minimal media (47) with appropriate supplements, as previously published (12). When testing utili-

zation, individual carbon sources were present at a final concentration of 0.2%. X-gal and isopropyl- β -D-thiogalactopyranoside (IPTG) were used at 20 μ g/ml and 1 mM, respectively. *Escherichia coli* strains were grown at 37°C in LB media with the appropriate antibiotics (12, 43). Procedures for strain construction were described previously (12). Established protocols were used for (i) generalized transduction, as mediated by Φ Cr30 (15); (ii) conjugation, to mobilize plasmids from *E. coli* (20); and (iii) generation of chromosomal deletions by homologous combination (54).

Molecular techniques. Standard techniques were used for DNA manipulation, electroporation, and transformation (1). Primers for this study are included in Table S1 in the supplemental material. DNA sequencing was done by Elim Biopharmaceuticals (Hayward, CA). To determine the location of the *lac-101::Tn5* insertion, we performed arbitrarily primed PCR according to the methods of O'Toole and Kolter (46), except that primers Tn5 151out and Tn5 117out were used as Tn5-specific primers for the first and second rounds of PCR, respectively. Briefly, primers Tn5 151out and ARB1 were used for amplification from chromosomal DNA, and an aliquot of this first reaction mixture was used as the template for the second reaction, which involved Tn5 117out and ARB2. DNA sequences from the PCR were then matched to the *C. crescentus* genome with the Basic Local Alignment Search Tool (BLAST) (26).

pJC343 was constructed as follows: region 5' of CC1634 was amplified with primers CC1634 -497F H and CC1634 14R B and then digested with HindIII

FIG. 1. *lacA*, *lacB*, and *lacC*; their predicted protein products; and plate phenotypes of deletion mutants. (A) *CC1632* (*lacB*), *CC1634* (*lacA*), and *CC1635* (*lacC*) encode three subunits of a dehydrogenase that is involved in lactose metabolism in *C. crescentus*. Arrows indicate the lengths and orientations of open reading frames. *CC1633* encodes a hypothetical protein (45). The bar above *CC1633* and *CC1634* shows the 531-bp fragment (*P_{lacA}*) used in the pJC404 reporter construct for examining expression. (B) Sequence analysis using MEMSAT3 (28) suggested that *LacA*, *LacB*, and *LacC* associate with the cell membrane according to the topologies depicted. All three proteins contain signal peptides (indicated by gray bars) at the amino termini (N). The leader peptides of *LacA* and *LacC* resemble those used for export via the twin-arginine translocation pathway because they each include a motif of two arginines (*) (13). *LacA* and *LacB* are predicted to have additional transmembrane segments (illustrated by white bars). *LacB*, a cytochrome *c* family protein, contains a CXXCH heme-binding motif (represented by a star), while *LacA* contains a GMC oxidoreductase domain (represented by a shaded ellipse) (19, 39). (C) As opposed to wild-type cells, *lac* deletion mutants did not form colonies on M2 minimal media with lactose as the sole carbon source (left panel). On PYE rich media with X-gal, wild-type and $\Delta lacB$ colonies turned blue, whereas $\Delta lacA$ and $\Delta lacC$ colonies stayed white (right panel). $\Delta lacB$ colonies were generally less blue than were the wild-type ones.

and BamHI; region 3' of *CC1634* was amplified with primers CC1634 1722F B and CC1634 2253R E and then digested with BamHI and EcoRI; and the two regions were inserted into pNPTS138 digested with HindIII and EcoRI by triple ligation. pJC345 was constructed by inserting the Ω cassette (4) into the BamHI site of pJC343, between regions 5' and 3' of *CC1634* (*lacA*). pJC389 was made by amplifying *CC1634* (*lacA*) with primers CC1634 -34F X and CC1634 +14R S, digesting the fragment with XbaI and SacI, and inserting it into pCM62 digested with the same enzymes. pJC404 was made by amplifying the promoter region 5' of *CC1634* (*lacA*) with primers CC1634 -480F B and CC1634 49R P, digesting the fragment with BglII and PstI, and inserting it into pJC326 cut with the same enzymes; the fragment contains the first 17 codons of *CC1634* (translationally fused to *lacZ* on the plasmid) as well as 480 bp upstream of the start codon (Fig. 1A). pJC414 was constructed by amplifying the region 5' of *CC1632* with primers CC1631 591F SpeI and CC1632 5R HindIII and digesting with SpeI and HindIII; amplifying the region 3' of *CC1632* with primers CC1632 315F HindIII and CC1632 + 524R SphI and digesting with HindIII and SphI; and then inserting both fragments into pNPTS138 digested with SpeI and SphI. pJC415 was constructed by amplifying the region upstream of *CC1635* with primers CC1634 1231F SphI and CC1635 17R HindIII and digesting with SphI and HindIII; amplifying the downstream region with primers CC1635 531F HindIII

and CC1636 399R SpeI and digesting with HindIII and SpeI; and then inserting both fragments into pNPTS138 digested with SpeI and SphI. pJC416 and pJC417 were made by amplifying *CC1632* with primers CC1632 -42F SphI and CC1632 +18R XbaI or amplifying *CC1635* with primers CC1635 -18F SphI and CC1635 +15R XbaI, cutting the fragments with SphI and XbaI, and inserting each into the corresponding sites of pCM62. pJC418 was constructed by amplifying a 557-bp fragment that contains the entire *CC1632* gene and a portion of *CC1631* with primers CC1631 904F BglII and CC1632 +15R HindIII, cutting the fragment with BglII and HindIII, and inserting the fragment into pJBZ281 cut with BamHI and HindIII. pJC419 was constructed by amplifying a 581-bp fragment that contains the entire *CC1635* gene with primers CC1635 -18F BglII and CC1635 +16R PstI, cutting the fragment with BglII and PstI, and inserting the fragment into pJBZ282 cut with BamHI and PstI. pJC418 and pJC419 were constructed such that chromosomal integration of the plasmid places *E. coli lacZ* (with its own ATG start codon) 14 bp after *CC1632* or 10 bp after *CC1635*.

Phenotype MicroArrays. Phenotype MicroArrays from Biolog were used according to the manufacturer's instructions and as previously described (5, 52), with modifications. Cells were grown overnight in M2 media plus 0.02% glucose and then washed and diluted in M2 media without carbon source, so that optical density at 570 nm (OD_{570}) was 0.02. Each well of the PM1 and PM2A plates was inoculated with 145 μ l of this cell suspension, and OD_{570} values of individual wells were measured with a microtiter plate reader after incubation at 30°C for 48 and 72 h. We performed four independent growth comparisons between wild-type and mutant strains. Consistent differences in OD_{570} values were recorded as variations in carbon source utilization.

β -Galactosidase assay and ONPG hydrolysis. β -Galactosidase activity, as encoded by the *E. coli lacZ* gene, was measured by the method of Miller (43); cells were grown in the appropriate media to mid-logarithmic phase and harvested for analysis. Each sugar being tested was present in the growth media at a final concentration of 0.2%, except for salicin, which was present at 0.02%. At least three independent triplicates were averaged for each measurement. To determine the endogenous enzymatic activity that hydrolyzes *o*-nitrophenyl- β -D-galactoside (ONPG) in *C. crescentus*, we modified Miller's β -galactosidase assay to exclude cell lysis with SDS and chloroform. Cells were grown in M2 plus 0.1% xylose, with or without 0.05% lactose, to late log phase or early stationary phase. They were then resuspended and diluted in M2 media without a carbon source, so that the OD_{600} of the dilution was approximately 0.08. ONPG was incubated directly with the cell suspension at a final concentration of 0.1 mg/ml at 30°C. If testing inhibition, sodium azide (1 mM), chloramphenicol (1 μ g/ml), SDS (0.01%), or chloroform (5%) was added to the reaction mixture. When it turned visibly yellow, the cell suspension was centrifuged, and the supernatant's OD_{420} and OD_{550} were measured. Enzymatic activity was calculated according to Miller (43), except the reaction duration was in hours, not minutes. Wild-type NA1000 cells that were induced with lactose usually exhibited 45 to 50 units of activity. At least three independent measurements were averaged for each data point.

Dehydrogenase assay. Dehydrogenase activity was measured in permeabilized cells spectrophotometrically, similar to methods previously described for membrane-bound gluconate dehydrogenases (41, 59). Cells were grown in M2 minimal media plus 0.2% xylose, with or without an inducer (0.1% lactose or 0.02% salicin), until mid- to late log phase. They were washed and resuspended in M2 to an OD_{600} of 0.15. For each 1.5-ml reaction mixture, we mixed 1 ml of cell resuspension, 150 μ l 0.1 M sodium phosphate buffer (pH 6), 10 μ l 10 mM 2,6-dichloroindophenol (DCIP), 10 μ l 3 mM phenazine methosulfate (PMS), 150 μ l 67 mM sugar (electron donor), 150 μ l 1% Triton X-100, and 30 μ l water. Reference reaction mixtures contained all components except cells or sugar (replaced with M2 medium or water instead). After incubation at 30°C, the reaction mixture was centrifuged, and the OD_{600} of the supernatant measured. Enzymatic activity was calculated as the initial rate of DCIP reduction, which resulted in the reaction mixture turning from blue to colorless, using the following formula: $1,000 \times (OD_{600} \text{ of reference} - OD_{600} \text{ of reaction mixture}) / [\text{elapsed time} \times (OD_{600} \text{ of cell suspension}/1.5)]$, where elapsed time was measured in minutes. Reference reaction mixtures gave similar readings, regardless of whether cells or sugars were omitted. At least three independent measurements were averaged. We found that enzymatic activity was reduced in the absence of Triton X-100, in agreement with previous results indicating that certain detergents can stimulate membrane-associated enzymes (33, 41).

RESULTS

Identification and initial characterization of *lacA*, *-B*, and *-C*. To investigate lactose metabolism in *C. crescentus*, we took advantage of transposon mutagenesis screens previously

FIG. 2. Comparison of wild-type and $\Delta lacA$ strains on agar media containing X-gal. Wild-type and mutant strains carrying the empty vector, mutants carrying a plasmid with *lacA*, and mutants carrying a plasmid with *E. coli lacZ* (*lacZ_{Ec}*) were grown on M2 minimal media plus 0.2% lactose, PYE media plus tetracycline, or PYE alone, as indicated below each panel. The far right panel [PYE (zoom)] signposts the strain genotype in each quadrant of all panels and shows the close-up of colonies grown on PYE media with X-gal. Mutant strains with the empty vector or *lacZ_{Ec}* failed to grow on M2 plus lactose. The plasmid vector (pCM62) and its derivatives (pJC327, pJC389) all confer resistance to tetracycline. Loss of the plasmid carrying *lacA* or *E. coli lacZ* from $\Delta lacA$ strains can be detected visually as growth of white or partially white colonies on PYE media.

conducted by Ely and Croft, which had yielded a mutant unable to use lactose as a carbon source (16, 17). First, to confirm linkage to the mutant phenotype, we transduced the insertion mutation (*lac-101::Tn5*) into CB15 and its derivative NA1000, two commonly used and sequenced wild-type strains, selecting for kanamycin resistance conferred by the Tn5 transposon. The inability to use lactose was assessed subsequently by streaking the transductants onto M2 minimal media containing lactose as the sole carbon source; the defect in lactose utilization always cotransduced with the resistance marker (data not shown). In addition, all Km^r transductants formed white colonies on PYE rich media containing X-gal, as opposed to their wild-type parents, which formed blue colonies (data not shown). Because X-gal serves as a chromogenic substrate for monitoring the processing of lactose, these results indicated that the Tn5 insertion was indeed in a locus required for lactose catabolism.

Using arbitrarily primed PCR, we determined that the *lac-101::Tn5* insertion disrupted *CC1634*, originally annotated to encode an oxidoreductase in the CB15 genome (45) (Fig. 1A). In the more recently sequenced NA1000 genome, the same gene is annotated as a dehydrogluconate dehydrogenase (M. E. Marks, C. Teiling, L. Du, V. Kapatral, T. L. Walunas, and S. Crosson, unpublished data). Our independent analysis using BLAST (26) indicated that the *CC1634* gene product has homology to the flavoprotein subunit of the biochemically characterized gluconate dehydrogenase from *Pectobacterium* (formerly *Erwinia*) *cyprripedii* (59). Like its homolog, the gene product is predicted to be membrane bound and to contain a conserved protein domain that belongs to the widespread glucose-methanol-choline (GMC) oxidoreductase superfamily (10, 39, 60) (Fig. 1B). Sequence evaluation using MEMSAT3 (28) suggested that at least one transmembrane segment exists in the *CC1634* protein. We also found that the N terminus of *CC1634* may contain a signal sequence suitable for the twin-arginine translocation (TAT) pathway, which allows export of folded proteins; in particular, substrates of the TAT pathway often consist of redox proteins and those that require incorporation of a cofactor, such as flavin adenine dinucleotide (FAD), for proper folding (13). To ascertain that the gene is involved in lactose utilization, we constructed a null deletion of *CC1634*. This in-frame deletion produced the same phenotypes as the *lac-101::Tn5* mutation; $\Delta CC1634$ mutants were

unable to use lactose as a carbon source and formed white colonies on rich media containing X-gal (Fig. 1C). Thus, *CC1634* was named *lacA* for its involvement in lactose metabolism.

Complementation analysis verified that the *lacA* deletion was responsible for both the failure to grow on M2 lactose and the white color of colonies on plates with X-gal (Fig. 2). The same results were obtained in both the CB15 and NA1000 backgrounds. A plasmid carrying a copy of the *lacA* gene allowed the $\Delta lacA$ mutants to grow on M2 lactose and form blue colonies. Wild-type strains carrying the vector alone exhibited the same phenotypes, whereas $\Delta lacA$ mutants carrying the vector did not grow on M2 lactose and formed white colonies on rich media with X-gal. Finally, introduction of a plasmid carrying the *lacZ* gene from *E. coli* allowed the $\Delta lacA$ mutants to produce the distinctive blue color from X-gal but did not complement the defect in lactose utilization. This last result is not surprising, considering that *E. coli lacZ* encodes a β -galactosidase, while *C. crescentus lacA* appears to encode a distinct enzymatic activity. Because the presence of *E. coli* β -galactosidase in the *C. crescentus* cytoplasm should enable the breakdown of lactose into glucose and galactose, both of which can be used by *C. crescentus* (25, 31, 47), the failure of *E. coli lacZ* to support the growth of $\Delta lacA$ mutants on M2 lactose suggests that *lacA* is necessary for converting lactose into molecules that can be imported into the cytoplasm. This deduction dovetails with the finding that the *lacA* gene product shares the predominantly periplasmic membrane topology of *P. cyprripedii*'s gluconate dehydrogenase.

The gluconate dehydrogenase of *P. cyprripedii*, as well as related enzymes purified from other bacterial species, consists of three protein subunits: (i) a flavoprotein that contains the primary dehydrogenase activity, (ii) cytochrome *c*, and (iii) a small subunit with unknown function (41, 59). In *P. cyprripedii*, the three subunits are encoded by adjacent genes in the same operon. In *C. crescentus*, *lacA* (*CC1634*) encodes the flavoprotein subunit, while nearby genes appear to encode the other two subunits (45) (Fig. 1A and B). The gene immediately downstream of *lacA*, *CC1635*, whose putative start codon overlaps with the stop codon of *lacA*, encodes a protein that has homology to the smallest subunit of the *P. cyprripedii* gluconate dehydrogenase (26). Computational analysis indicated that the *CC1635* protein also contains a leader peptide that may serve

FIG. 3. ONPG hydrolysis activity in wild-type and Δ *lacA* strains. (A) Wild-type (*lacA*⁺) cells induced with lactose showed the highest level of ONPG hydrolysis, compared with uninduced wild-type cells and induced or uninduced Δ *lacA* cells. (B) ONPG hydrolysis by induced *lacA*⁺ cells and uninduced Δ *lacA* cells was assessed in the absence or presence of different inhibitors, as follows: sodium azide (azide), chloramphenicol (Cm), or SDS and chloroform (SDS+CHCl₃). Levels of ONPG hydrolysis were measured as described in Materials and Methods and normalized according to the average activity in induced *lacA*⁺ cells, without metabolic inhibitor. Error bars indicate standard deviations.

as a twin-arginine translocation signal (13), allowing export into the periplasm. On the other side, two genes upstream of *lacA*, *CC1632* encodes a cytochrome *c* family protein (39), which is predicted to be membrane bound as well (28). The intervening *CC1633* gene encodes a hypothetical protein with a conserved domain of unknown function (39).

Based on sequence homology and their proximity to *lacA*, we postulated that *CC1632* and *CC1635* also contribute to lactose metabolism in *C. crescentus*. Indeed, in-frame deletions of *CC1632* (named *lacB*) and *CC1635* (*lacC*) prevented growth on minimal media containing lactose as the sole carbon source (Fig. 1C). The Δ *lacC* mutant, like the Δ *lacA* mutant, also formed white colonies in the presence of X-gal, while the Δ *lacB* mutant formed blue colonies, albeit generally less blue than those formed by the wild type (Fig. 1C). One possible explanation is that other cytochrome *c* family proteins (such as CC1210, which shares 35% identity with LacB [26]) can serve as part of the dehydrogenase, but with lower efficiency, such that lactose is processed insufficiently to support growth, whereas X-gal can be processed over longer time periods to turn the colonies blue. No similar substitutes appear to exist for LacA and LacC. *lacC* does not have close paralogs in the *C. crescentus* genome, and while there are at least four other genes that encode GMC oxidoreductases (*CC0945*, *CC1278*, *CC1638*, and *CC2642*), all of them encode proteins that share low homology with LacA (<24% similarity) (26, 45). Moreover, none of the other oxidoreductase genes has the same gene arrangements as those of *lacA*, *lacB*, and *lacC*.

We confirmed that LacB and LacC participate in lactose metabolism in *C. crescentus* by performing a complementation analysis similar to that described above for LacA (see Fig. S1 in the supplemental material). A plasmid carrying *lacB* or *lacC* supported growth of the Δ *lacB* or Δ *lacC* mutant, respectively, on lactose. The presence of either *lacC* or *E. coli lacZ* allowed the Δ *lacC* mutant to turn blue on X-gal. Because the Δ *lacB* mutant already appeared blue on X-gal, the presence of *lacB* or *E. coli lacZ* did not have an obvious effect on colony color for this strain. However, as for the Δ *lacA* mutant, *lacZ* did not restore lactose utilization in either the Δ *lacB* or Δ *lacC* mutant. In conjunction with information about homology and gene

location, these phenotypic results suggest that, along with LacA, LacB and LacC form subunits of a dehydrogenase involved in lactose degradation. Since the three proteins appear to be parts of the same enzyme, our subsequent investigation focused primarily on one of the components, LacA.

While performing the complementation analysis, we also noticed that plasmid loss could be detected visually in the Δ *lacA* and Δ *lacC* mutants (Fig. 2; also see Fig. S1 in the supplemental material). Specifically, in the presence of tetracycline, which selected for maintenance of the complementing plasmid, Δ *lacA* strains carrying either *lacA* or *lacZ* on the plasmid formed only colonies that were completely blue on PYE rich media (Fig. 2). On the other hand, without tetracycline in the media, plasmid loss led to formation of white or partially white (sectorized) colonies (Fig. 2). Wild-type strains carrying the vector or the complementing plasmids produced only blue colonies, regardless of whether tetracycline was present in the media (Fig. 2 and data not shown). We obtained analogous results with Δ *lacC* strains (see Fig. S1 in the supplemental material). The sectorized colony phenotype seen in the Δ *lacA* and Δ *lacC* mutants due to plasmid loss may prove particularly useful in future genetic studies.

Inducible ONPG hydrolysis depends on *lacA*. As in many other bacteria, the lactose utilization pathway appears to be inducible in *C. crescentus* (32). For example, we observed colonies that were bluer on X-gal plates containing lactose than on those lacking lactose (data not shown). To assess the role of *lacA* in lactose utilization and induction, we modified Miller's β -galactosidase assay (43) to measure hydrolysis of ONPG, a colorimetric analog of lactose; cells were grown in M2 minimal media plus xylose with or without lactose, resuspended in M2 without carbon sources, and incubated directly with ONPG. ONPG hydrolysis activity in wild-type cells grown without lactose was about 50% of that in cells induced with lactose (Fig. 3A). On the other hand, regardless of whether they were induced with lactose, Δ *lacA* strains exhibited enzymatic activities at about 30% of that in induced *lacA*⁺ cells. These results suggest that the enzymes required for lactose catabolism are inducible in *C. crescentus*. Furthermore, *lacA* is required for the induction.

FIG. 4. *lac* genes required for utilization of specific carbon sources (A) Phenotype MicroArray analysis indicated that the *lacA* deletion inhibited growth on six carbon sources, the chemical structures of which are depicted. Salicin is a β-glucoside, and trehalose is an α-glucoside, while lactose, lactulose, lactitol, and methyl-β-D-galactoside are all β-galactosides. (B) Deletion of any *lac* gene prevented colony formation on M2 agar with salicin in the NA1000 background (top panel) but reduced growth only in the CB15 background (middle panel). The bottom panel indicates the genotype in each sector of the plate. For the other five sugars, deletion of any *lac* gene prevented growth in both strain backgrounds.

Originally, we were puzzled by *C. crescentus*'s ability to hydrolyze X-gal on solid media but not ONPG in Miller's β-galactosidase assay; wild-type cells exhibited minimal activity (<1 Miller unit) in the assay (data not shown). We suspected that the presence of SDS and chloroform in the assay inhibited enzymatic activity in *C. crescentus*. That was why we modified the β-galactosidase assay by excluding cell lysis, to allow measurement of the ONPG hydrolysis described above. To confirm this suspicion, we tested the effects of different metabolic inhibitors with our modified assay, by adding them to washed cells that were being incubated with ONPG. Indeed, the addition of SDS and chloroform (together or individually) diminished ONPG hydrolysis to the same level as that in the *ΔlacA* mutants (Fig. 3B and data not shown). In contrast, the addition of sodium azide or chloramphenicol, which inhibits electron transport or protein synthesis, respectively, did not reduce the enzymatic activity significantly (Fig. 3B). Thus, in *C. crescentus*, SDS and chloroform appear to be detrimental to endogenous enzymes involved in lactose catabolism. Use of *E. coli lacZ* as a reporter in traditional β-galactosidase assays has been feasible because there is an insignificant level of intrinsic activity, even in wild-type strains, under these conditions.

Phenotype MicroArray analyses reveal *lac* mutants' defect in utilization of several carbon sources. Since the *lacA* gene product belongs to the family of GMC oxidoreductases, members of which catalyze diverse reactions and individually can often act on a number of different substrates (10, 60, 61), we asked if it is involved in the metabolism of other carbon sources. Phenotype MicroArrays (Biolog) were used to compare the growth of wild-type and *ΔlacA* strains on each of 190 different carbon sources. The *lacA* deletion prevented utilization of four β-galactopyranosides, lactose, lactulose, lactitol, and methyl-β-D-galactoside (Fig. 4A). Somewhat surprisingly,

ΔlacA mutants were also unable to use salicin, a β-glucopyranoside, and trehalose, an α-glucopyranoside (Fig. 4). However, the mutation did not affect the utilization of other glucosides, such as cellobiose or sucrose. We confirmed these findings by streaking the *lac* deletion strains on M2 minimal media containing select sugars as the sole carbon source (Fig. 4B and data not shown). When incubated at 30°C, wild-type strains formed visible colonies (1 mm in diameter) on glucose or xylose within 2 to 3 days; on lactose, lactulose, lactitol, or salicin within 5 to 7 days; and on trehalose or methyl-β-D-galactoside within 7 to 10 days. (*C. crescentus* was unable to use methyl-α-D-galactoside as a sole carbon source.) The *ΔlacA* strains did not grow if the carbon source present was one of those identified in our Phenotype MicroArray experiments. One notable exception was that the *lacA* gene was necessary in NA1000 but not in CB15 for growth on salicin. While the CB15 *ΔlacA* mutant grew on salicin, its colonies were smaller than those of the CB15 wild-type strain. Introduction of a plasmid carrying *lacA* was able to complement these defects in sugar utilization. Similar results were also obtained with *ΔlacB* and *ΔlacC* strains (Fig. 4B and data not shown). Therefore, the *lac* genes appear to contribute to the catabolism of distinct carbon sources, possibly by serving as a nexus in branching degradation pathways.

***lac* genes required for inducible dehydrogenase activity.** Based on sequence homology and the Phenotype MicroArray results, we hypothesized that the three *lac* gene products act in concert as a dehydrogenase for different sugars. To validate this idea, we assessed enzymatic activity by using protocols previously established for membrane-bound gluconate dehydrogenases (41, 59). Wild-type and *lac* deletion strains were grown in M2 minimal medium plus xylose, with or without lactose as the inducer. Dehydrogenase activities in harvested

FIG. 5. Dehydrogenase activity, as measured by reduction of DCIP, in wild-type (*lacA*⁺) and Δ *lacA* mutant strains under different conditions. (A) Dehydrogenase activities of wild-type and mutant cells, grown in the absence or presence of the lactose inducer (uninduced or induced), were assessed with xylose, lactose, or salicin as the electron donor. Induced *lacA*⁺ cells incubated with lactose or salicin exhibited the highest levels of activity. (B) A plasmid carrying the *lacA* gene complements the deficiency in dehydrogenase activity in the Δ *lacA* mutant. Wild-type and mutant cells with the complementing *lacA*⁺ plasmid (pJC389) or the empty vector (pCM62) were grown in the absence or presence of lactose (uninduced or induced) and then analyzed with lactose as the electron donor. Dehydrogenase activities were measured as described in Materials and Methods, with 1 unit of activity defined as the change in OD₆₀₀ per minute per unit of cell density. Reactions without cells showed no activity. Error bars indicate standard deviations.

cells were then measured spectrophotometrically as the rate of reduction of DCIP, mediated via PMS, in the presence of equimolar concentrations of individual carbon sources (lactose, salicin, trehalose, xylose, or gluconate). If the carbon source served as an appropriate electron donor, electrons flowed from the flavin-dependent dehydrogenase to PMS, a carrier, to the terminal acceptor DCIP, which turned from blue to colorless when it was reduced.

When incubated with lactose or salicin, wild-type cells exhibited the largest increases in dehydrogenase activities, 6- to 8-fold higher in induced versus uninduced cells (Fig. 5A). Incubation with trehalose led to an intermediate level of increase in activity, approximately 3-fold, when cells were induced, but the induced activity associated with trehalose was relatively low compared to that associated with lactose or salicin (8 versus 29 or 31 units of activity, respectively) (data not shown). Finally, when incubated with gluconate or xylose, wild-type cells, whether induced or uninduced, showed low levels of dehydrogenase activity in this particular assay, similar to those of uninduced wild-type cells incubated with any carbon source (Fig. 5A and data not shown). The inability of gluconate to stimulate DCIP reduction is consistent with a previous observation that *C. crescentus* cannot utilize gluconate for growth (25). Parallel patterns of activity levels were obtained when cells were induced with salicin instead of lactose (data not shown).

In contrast to wild-type cells, Δ *lacA* mutants exhibited only basal levels of dehydrogenase activity, regardless of induction or the carbon source being tested (Fig. 5A and data not shown). These basal levels were similar to those seen when no sugar was present in the assay, whether wild-type or mutant strains were used (data not shown). Furthermore, deletion of *lacC* abolished dehydrogenase activity to the same levels as those seen in the Δ *lacA* mutants (see Fig. S2 in the supplemental material). While deletion of *lacB* also reduced enzyme activity, the levels were slightly higher than those observed in Δ *lacA* and Δ *lacC* strains and similar to that observed in uninduced wild-type cells (see Fig. S2 in the supplemental material). This slightly higher residual activity in Δ *lacB* cells may again be attributed to possible substitution by other cyto-

chrome *c* family proteins (see above). Nevertheless, results from these enzymatic assays suggest that *lacA*, *-B*, and *-C* together encode an inducible dehydrogenase that oxidizes specific carbon sources, such as lactose and salicin, or their derivatives.

We conducted complementation analysis to confirm that *lacA* encodes specific dehydrogenase activity. Introduction of a plasmid carrying *lacA* under the control of a constitutive promoter increased the levels of dehydrogenase activity in the Δ *lacA* mutant, under both uninduced and induced conditions, above corresponding levels in the wild-type strain (Fig. 5B). The plasmid vector alone had no such effect on enzymatic activity in either the wild-type or mutant strain. Thus, *lacA* appears to be responsible for the specific dehydrogenase activity detected by our assay. Intriguingly, introduction of the complementing plasmid, which constitutively expressed *lacA*, into the wild-type strain did not alter activity levels. One possible explanation for the difference in the complementing plasmid's effects on wild-type and mutant strains is that *cis* elements at the endogenous *lacA* locus may contribute to the regulation of dehydrogenase activity; this regulation would remain intact in the wild-type strain but be lost in the Δ *lacA* mutant (see Discussion).

Regulation of *lac* expression. Our results indicated that the *lac* genes are required for the inducible catabolism of different sugars. To obtain better understanding of how the metabolic pathway is regulated, we examined *lacA* expression under different conditions. A reporter construct containing *E. coli lacZ* translationally fused to the first 17 codons of *lacA*, as well as the 480-bp region upstream (Fig. 1A), was placed on a plasmid and introduced into *C. crescentus* to assess variation in expression levels by the use of Miller's β -galactosidase assay. First, wild-type cells carrying the plasmid were grown in different media, with or without lactose for induction (Fig. 6A). Expression levels were lowest when cells were grown in PYE complex media, and the presence of lactose did not significantly increase expression. Expression levels in M2 minimal media plus glucose (M2G) without the inducer were similar to those in PYE, but the addition of lactose increased expression ~2-fold.

FIG. 6. Variations in *lacA* expression in different growth media, as determined by β -galactosidase assays using a P_{lacA} -*lacZ*_{Ec} reporter fusion. (A) Wild-type cells carrying the reporter construct (pJC404) were grown in PYE broth, M2 minimal media plus 0.2% glucose (M2G), or M2 plus 0.2% xylose (M2X), in the absence or presence of 0.2% lactose as the inducer; cells grown to mid-log phase were harvested to measure levels of β -galactosidase activity. (B) Wild-type (*lacA*⁺) and Δ *lacA* cells were grown in M2G with no other sugar, 0.2% xylose, 0.2% lactose, or 0.02% salicin and harvested for β -galactosidase assays. Error bars indicate standard deviations.

Finally, while expression levels in M2 plus xylose (M2X) without lactose were only slightly higher than, if not similar to, uninduced levels in PYE and M2G, the presence of lactose in M2X produced the highest level of expression, five times the basal level in PYE. These results suggest that *lacA* expression is dictated by a complex hierarchy of nutrient preference; the availability of glucose and other carbon sources in M2G and PYE may limit the degree of induction by lactose.

Having determined that induction is detectable in M2 minimal media but not in PYE rich media, we next assessed the ability of different compounds to serve as inducers (Fig. 6B). In M2G, salicin led to an almost 4-fold increase in expression, compared to lactose's 2-fold increase. On the other hand, trehalose and IPTG (commonly used as an inducer for the *lac* operon in *E. coli*) did not produce any detectable changes in expression (data not shown), whereas addition of xylose to M2G actually reduced the level of expression slightly. Deletion of *lacA* prevented induction by lactose or salicin but did not alter the reduced level of expression in the presence of xylose (Fig. 6B). Deletion of *lacB* or *lacC* also prevented induction of this P_{lacA} -*lacZ* reporter construct by lactose (data not shown). Thus, the *lac* genes are required for lactose and salicin to serve as inducers for promoting *lacA* expression. In addition, our results indicate that the levels of other nutrients, such as glucose and xylose, can also affect *lacA* expression, possibly via catabolic repression, as previously suggested (32, 42, 51). Expression of *lacB* and *lacC* is likely under similar types of control. When we inserted *E. coli lacZ* (with its own start codon) into the *C. crescentus* chromosome immediately after *lacB* or *lacC*, we also observed induction of *lacZ* expression in the presence of lactose, to the same extent as that seen for the P_{lacA} -*lacZ* reporter (see Fig. S3 in the supplemental material). Like metabolic genes in other systems, expression of the *lac* genes appears to be under complex regulation to ensure optimal fitness with changing resource availability.

DISCUSSION

We have determined that, in *C. crescentus*, *lacA*, *lacB*, and *lacC* are required for the utilization of lactose and three other β -galactosides, as well as salicin and trehalose, two glucosides.

They appear to act as subunits of a dehydrogenase that oxidizes these sugars or their derivatives. When cells are grown in the presence of the appropriate inducer, such as lactose, the dehydrogenase activity encoded by the *lac* genes is elevated, concomitant with a rise in β -galactosidase activity, as detected by ONPG hydrolysis. The increase in dehydrogenase activity is attributable, at least in part, to an increase in transcription in the presence of the inducer. In the *lac* deletion mutants, neither gene expression nor enzymatic activity can be induced. Thus, the Lac dehydrogenase appears necessary for processing substrates into physiologically relevant inducers inside the cell. Taken together, these results indicate that *lacA*, *-B*, and *-C* encode a critical component of the metabolic pathways that dissimilate lactose and other sugars, and its dehydrogenase activity contributes to the substrate-dependent regulation of these pathways.

Two lines of evidence support the assertion that *lacA*, *-B*, and *-C* encode subunits of a dehydrogenase. First, analysis of gene sequence and arrangement indicated that LacA, *-B*, and *-C* are homologous to components of the biochemically characterized gluconate dehydrogenase from *P. cyripedii* (59). Second, enzymatic assay results suggest that they act as a dehydrogenase that specifically oxidizes lactose, salicin, and, to a lesser extent, trehalose. The addition of these sugars stimulated the ability of permeabilized, induced wild-type cells to reduce DCIP, whereas no such activity was detected in the *lac* deletion mutants or when other sugars were used. While *C. crescentus* contains xylose dehydrogenase activity (47, 52), our assay appears to detect only the enzymatic activity encoded by the *lac* genes.

Results from complementation analysis further bolster LacA's role as a subunit of the dehydrogenase, as the introduction of a plasmid that constitutively expressed *lacA* into the mutant cells raised enzymatic activity above that of wild-type cells. Confoundingly, the introduction of the same plasmid into wild-type cells did not produce a congruent increase in activity. One possible explanation is that *lacA* encodes only one of three subunits of the dehydrogenase and expression of all three subunits has to be increased in the correct ratio for enhanced activity. In wild-type cells, all levels of the regulation are intact,

preventing excess expression. On the other hand, in the $\Delta lacA$ mutant, some requirements of the regulation, such as *cis* elements within the *lacA* locus that affect expression of the downstream *CC1635* gene, may be lost, allowing an increase in expression of the other subunits and consequent enhancement in enzymatic activity.

Although LacA, -B, and -C apparently work together as a dehydrogenase, the exact function of the enzyme in the utilization of lactose and other sugars remains speculative. In bacteria, lactose metabolism typically involves one of two well-defined pathways (9, 29, 36, 58): (i) lactose may be imported by a specific permease into the cytoplasm and split by β -galactosidase into glucose and galactose, which are converted to glucose-6-phosphate for glycolysis; or (ii) lactose may be taken up by a phosphoenolpyruvate (PEP)-dependent phosphotransferase system (PTS), forming lactose phosphate, which is subsequently hydrolyzed by phospho- β -galactosidase into glucose and D-galactose 6-phosphate. Neither pathway appears to involve a dehydrogenase. However, a dehydrogenase/oxidoreductase participates in a proposed pathway for lactose degradation in *A. tumefaciens* (9, 23, 56): (i) hexopyranoside:cytochrome *c* oxidoreductase catalyzes the oxidation of lactose to generate 3'-ketolactose; (ii) 3-ketolactose hydrolase then breaks down 3'-ketolactose into glucose and 3-keto- β -D-galactose; and (iii) a hypothetical, as-yet-unidentified enzyme converts 3-keto- β -D-galactose to β -D-galactose. While hexopyranoside:cytochrome *c* oxidoreductase has been purified and characterized, 3-ketolactose hydrolase activity has been only partially defined, and the corresponding genes for these enzymes have not been determined.

Despite the absence of assigned genes for sequence comparison, various pieces of evidence suggest that the Lac dehydrogenase of *C. crescentus* serves the same function as the hexopyranoside:cytochrome *c* oxidoreductase of *A. tumefaciens* (55). First, as for the *C. crescentus* dehydrogenase, one of the enzymatic assays for the *A. tumefaciens* oxidoreductase employs DCIP as an electron acceptor. Second, hexopyranoside:cytochrome *c* oxidoreductase uses FAD as a cofactor. Third, it can oxidize a number of different sugars, including those identified in this study. Fourth, three consecutive genes in *A. tumefaciens* encode products that are homologous to *C. crescentus* LacA, -B, and -C: Atu4377 (561 amino acids [aa]) is 56% identical to LacA, Atu4378 (186 aa) is 27% identical to LacC, and Atu4379 (cytochrome *c2*; 131 aa) is 35% identical to LacB (21, 26, 57). Fifth, the combined predicted molecular weight of Atu4377 (63,350) and Atu4378 (20,085) is similar to that of purified hexopyranoside:cytochrome *c* oxidoreductase ($85,000 \pm 7,700$ without cytochrome *c*) (55). Therefore, homologs of LacA, -B, and -C in *A. tumefaciens* likely form subunits of the hexopyranoside:cytochrome *c* oxidoreductase. We propose that *C. crescentus* uses the same pathway as that prescribed for *A. tumefaciens* to degrade lactose. Other sugars are similarly converted to their corresponding 3-keto derivatives and subsequently hydrolyzed, as suggested for a candidate sucrose degradation pathway in *A. tumefaciens* (9, 50).

While the model above is tantalizing, questions remain. For example, how is 3'-ketolactose transported into the cytoplasm, and do the other enzymes in the proposed pathway and their corresponding genes actually exist in *C. crescentus*? Identification of additional genes required for utilization of lactose and

other sugars would help clarify the pathway. One approach is to exploit the genetic difference between the CB15 and NA1000 strains. Deletion of the *lac* genes prevented NA1000 from growing on salicin as the sole carbon source, whereas it only weakened growth of CB15. The genomes of both strains have been completely sequenced, and a limited number of genetic variations exist between the two (45; Marks et al., unpublished). Future analysis of these genetic variations should reveal which is responsible for the phenotypic difference between CB15 and NA1000 Δlac strains when grown on salicin. This and other prospective means of identifying more genes involved, such as additional transposon mutagenesis screens, will help clarify the biochemical pathways for catabolism of salicin, lactose, and other sugars.

The ecological niche that *Caulobacter* occupies may explain why the Lac dehydrogenase is involved in the catabolism of different sugars as well as why the induction of its expression and enzymatic activity is relatively modest. First, *Caulobacter* may have developed enzymes with versatile substrate specificities because it lives in low-nutrient-concentration environments, and the ability of a single gene to play multiple roles would be an economical way to digest the diverse and rare nutrients that it encounters. Intriguingly, a number of sugar oxidases and dehydrogenases, from plant-associated fungal species in particular, have been characterized biochemically, and their ability to oxidize cellobiose as well as lactose draws tantalizing parallels with LacA's involvement in the utilization of salicin and lactose (2, 24, 30, 37, 61). Like LacA, fungal cellobiose dehydrogenases contain a flavoprotein domain of the GMC oxidoreductase superfamily; in addition, they contain a cytochrome domain due to an ancestral gene fusion event (60, 61). *Caulobacter* and fungal species may have acquired enzymes with similar functions and substrate versatility because they both thrive in ecological niches where plant-derived carbon sources, such as salicin and cellobiose, are readily available. The same logic applies to *A. tumefaciens*, a plant pathogen.

Second, although induction of *lac* expression and dehydrogenase activity is clearly observable in *C. crescentus*, the level of induction appears modest in comparison to other metabolic systems, both in other bacteria and in *C. crescentus*, including that used for xylose catabolism (the *xyl* system) (25, 42, 53). Poindexter (48) has noted that, for the majority of sugar substrates, the quantitative response of the *Caulobacter* cell to the sudden availability of a new substrate is not impressive and has suggested that, in dilute environments where a nutrient is likely to be available for only a short time at irregular intervals, dramatic shifts to dedicated pathways may be maladaptive; low-level maintenance of different catabolic systems at all times would be more advantageous. This type of physiological response may be particularly applicable to nutrients such as lactose that are rarer and considered secondary by *Caulobacter*. This environmental adaptation would also help explain the catabolic repression of *lacA* transcription; *Caulobacter* would not invest in enhancement of pathways for other carbon sources if favored nutrients are present, yet there is no complete shutdown of expression. Tempered responses to the presence of particular nutrients may have evolved as a consequence of *Caulobacter*'s natural environment. Unraveling the complex regulation of such responses would help elucidate

how an oligotroph controls its metabolic flux to achieve optimal fitness. One avenue of investigation would be identification of the transcriptional factors that control *lac* expression. Inspection of genomic sequences surrounding *lacA*, *-B*, and *-C* readily yielded potential regulators; *CC1627* encodes a regulator of the LacI family, while *CC1640* encodes a regulator of the BlaI family. Characterization of these and other potential regulators of *lac* expression will further our understanding of how metabolic capacity is controlled in *C. crescentus*.

Finally, while regulation of *lac* expression and the metabolic role of the Lac dehydrogenase await further investigation, identification of the gene has immediate applicability for genetic studies of *C. crescentus*. By removing the background level of X-gal hydrolysis, the Δ *lacA* mutation facilitates use of visual assays, such as blue/white screening on solid media (43). For example, mutants with enhanced expression of a *lacZ* reporter construct can be detected as colonies that turn bluer on X-gal plates than do those of the parental strain. We have also used the visual observation of plasmid loss to confirm that specific genes are essential for viability in *C. crescentus*. Figure 2 illustrates such a visual test of growth requirement. In the presence of tetracycline, the plasmid-borne *tetA* gene, which confers resistance to the antibiotic, is required for growth. Hence, all colonies are completely blue because, to be viable, cells need to maintain the plasmid that carries both *tetA* and *lacZ*. However, in the absence of selection, cells can lose the plasmid. This simple and inexpensive assay has diverse applications, the extent of which relies solely on the user's imagination.

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