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Genetic Analysis and Characterization of a *Caulobacter crescentus* Mutant Defective in Membrane Biogenesis

DAVID HODGSON,† PENNY SHAW,‡ VERITY LETTS, SUSAN HENRY, AND LUCILLE SHAPIRO*

Departments of Molecular Biology and Genetics, Division of Biological Sciences, Albert Einstein College of Medicine, Bronx, New York 10461

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A mutant of *Caulobacter crescentus* has been isolated which has an auxotrophic requirement for unsaturated fatty acids or biotin for growth on medium containing glucose as the carbon source. This mutant exhibits a pleiotropic phenotype which includes (i) the auxotrophic requirement, (ii) cell death in cultures attempting to grow on glucose in the absence of fatty acids or biotin, and (iii) a major change in the outer membrane protein composition before cell death. This genetic lesion did not appear to affect directly a fatty acid biosynthetic reaction because fatty acid and phospholipid syntheses were found to continue in the absence of supplement. Oleic acid repressed fatty acid biosynthesis and induced fatty acid degradation in the wild-type parent, AE5000. The mutant strain, AE6000, was altered in both of these regulatory functions. The AE6000 mutant also showed specific inhibition of the synthesis of outer membrane and flagellar proteins. Total phospholipid, DNA, RNA, and protein syntheses were unaffected. The multiple phenotypes of the AE6000 mutant were found to cosegregate and to map between *hclA* and *lacA* on the *C. crescentus* chromosome. The defect in this mutant appears to be associated with a regulatory function in membrane biogenesis and provides evidence for a direct coordination of membrane protein synthesis and lipid metabolism in *C. crescentus*.

Caulobacter crescentus synthesizes and assembles surface structures such as pili, a flagellum, a stalk, and phage receptor sites at specific sites on the cell at defined times in the cell cycle. The question of how the cell regulates site-directed assembly of newly synthesized proteins is being approached by determining the role the cell membrane might have in this process. We have defined the composition and biosynthesis of the cell membrane phospholipids and proteins (7, 17, 19, 20). Mutants in membrane lipid and protein synthesis have been isolated (6, 8, 17), and with these we have demonstrated a strict coordination of membrane biogenesis with DNA replication and the expression of temporally regulated genes (8, 19, 28). The synthesis of specific flagellar and membrane proteins, which appear to be dependent on DNA replication (24, 29), has been shown to be indirectly dependent on membrane phospholipid synthesis (8, 28). In addition, the formation of the stalk (20) and the synthesis of two outer membrane proteins synthesized only in the swarmer cell (1) were shown to be independent of DNA replication, but to be dependent on phospholipid synthesis (28).

Initial studies to probe the link between membrane biogenesis and cell cycle functions in *C. crescentus* were done by blocking phospholipid synthesis with glycerol auxotrophs deficient in the biosynthetic glycerol-3-PO_4 dehydrogenase (6, 10) or by treating cultures with the drug cerulenin (6, 22). To further investigate the role of membrane biogenesis, we have isolated mutants that are dependent on unsaturated fatty acids for growth. One of these mutants, AE6000 (*fatA501*), has its auxotrophic requirement also satisfied by biotin. Although the auxotrophic requirement of this mutant was satisfied by two compounds directly related to lipid metabolism, oleate and biotin, we found that fatty acid biosynthesis continued in the absence of supplement and that attempted growth under these conditions led to cell death. These observations suggested that the genetic lesion resides in a regulatory step in membrane biogenesis. We report here the isolation and growth requirements of this mutant and demonstrate genetically that this lesion maps to a single genetic locus.

To define the cellular events that are altered upon starvation of this auxotroph we first examined the effect of exogenous fatty acids on the metabolism of endogenous fatty acids in the wild-type parent strain. Exogenous oleic acid repressed the synthesis of fatty acids and induced the degradation of endogenous unsaturated fatty acids. Examination of mutant AE6000 showed that each of these regulatory functions is altered. Although other major cellular functions such as phospholipid synthesis, DNA replication, RNA synthesis, and total protein synthesis remained unaffected after a shift to unsupplemented medium, this genetic alteration within the fatty acid regulatory system is accompanied by the specific inhibition of the synthesis of two categories of surface proteins: flagellar and membrane proteins.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *C. crescentus* CB15 (AE5000) and derivative strains (Table 1) were grown at 30°C in peptone-yeast extract (PYE) broth or in modified minimal medium (17) and 0.2% glucose as a carbon source (buffered minimal glucose [BMG]). Tergitol Nonidet P-40 (1%) was used to help solubilize the exogenous fatty acids (BMGT). Supplemented liquid media contained 0.5% Tween 40, 0.5% Tween 80, 1% Tergitol Nonidet P-40, and 1 mM oleic acid or biotin (2 ng/ml). To shift cultures from one medium to another, cells were collected on sterile 0.45-μm membrane filters (Millipore Corp., Bedford, Mass.) and washed with twice the volume of BMGT to remove residual fatty acids. The filter was then transferred to a tube contain-

* Corresponding author.
† Present address: Department of Biochemistry, Stanford University School of Medicine, Palo Alto, CA 94301.
‡ Present address: Biogen, Geneva, Switzerland.

ing a small volume of medium and shaken gently, and the washed cells were inoculated into the appropriate medium.

Mutant isolation. AE6000 (*fatA501*) was obtained after *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine mutagenesis of *C. crescentus* AE5000 as previously described (6). A midlog phase culture of AE5000 in PYE broth was added to an equal volume of PYE broth containing *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (400 μg/ml) and shaken at 30°C for 70 min. The cells were then washed twice in PYE and grown to saturation in PYE. The survivors were plated on minimal medium in the presence of Tween and replica plated on minimal medium (BMG) in the presence and absence of Tween. Colonies that grew on the Tween-supplemented plates, but not on BMG alone, were isolated; one of these, AE6000, was chosen for further study.

Conjugation. A rapid method of determining the general map position of mutant genes in *C. crescentus* CB15 was recently devised (3). This method involves RP4-mediated conjugation between the strain containing the mutant allele of the gene to be mapped and a series of strains each containing the transposon Tn*5* inserted a gene of known location (Table 2). The Tn*5*-marked gene is selected because it carries kanamycin resistance, and the frequency of co-transfer of the wild-type allele of the gene being mapped indicates the relative closeness of the two genes.

Spontaneous rifampin- and streptomycin-resistant variants of the AE6000 mutant were selected on PYE containing 50 μg of rifampin per ml and 200 μg of streptomycin per ml. Equal volumes of overnight cultures of one of the Tn*5*-marked donor strains (Table 2) was mixed with the drug-resistant recipient strain (either AE6004 or AE6003). The rifampin-resistant variant was used when the donor strain contained the *ts-104* allele, and the streptomycin variant was used when *ts-140* was involved. This was done to insure that these counterselectable markers did not interfere with the selection of the Tn*5*-marked genes. The mixed strains were collected on a sterile 0.45-μm filter as detailed by Johnson and Ely (12), and the filter incubated on a PYE plate for 12 to 24 h at room temperature. After suspension in 1 ml of PYE medium the cells were plated onto PYE plates containing kanamycin (to select for the Tn*5*-marked gene and against the recipient) and rifampin or streptomycin (to select against the donor strain). The plates were incubated at the restrictive temperature (37°C) (to further select against the donor strains). The transconjugants were then picked onto master plates containing the same additions as the primary selection

TABLE 1. List of strains

Strain	Genotype	Derivation[a] (donor × recipient)
C. crescentus CB15		
AE5000	Wild type	
AE5418	*cysD137*::Tn*5 fatA501 str-500*	SC1382 × AE6000 C
AE5463	*cysD137*::Tn*5 hclA501 pigA501 fat-506 rif-502*	SC1382 × AE6003 C
AE5470	*ts-104* (pVS1)	AEE133 × SC596 C
AE5479	*flaE178*::Tn*5 ts-104* (pVS1)	SC1062 × AE5470 T
AE5513	*cysD137*::Tn*5*	AE5463 × AE5000 T
AE5527	*cysD137*::Tn*5 thrA101 hclA501*	AE5463 × SC126 T
AE5533	*cysD137*::Tn*5 flaF132 ts-104* (pVS1)	SC1708 × AE5470 T
AE5563	*cysD137*::Tn*5 hclA501 ts-104* (pVS1)	AE5527 × AE5470 T
AE5567	*lacA101*::Tn*5 ts-104* (pVS1)	SC1140 × AE5470 T
AE5573	*lacA101*::Tn*5 hclA501 pigA501*	AE5567 × AE6008 C
AE5575	*lacA101*::Tn*5 fatA501 rif-500*	AE5567 × AE6006 C
AE5597	*lacA101*::Tn*5 hclA501 ts-104* (pVS1)	AE5573 × AE5470 T
AE5598	*cysD137*::Tn*5 fatA501*	AE5418 × AE5000 T
AE5623	*lacA101*::Tn*5*	AE5575 × AE5000 T
AE5624	*lacA101*::Tn*5 fatA501*	AE5575 × AE5000 T
AE6000	*fatA501*	AE5000 NTG mutagenesis
AE6002	*pigA501 hclA501 fat-506*	AE5000 NTG mutagenesis
AE6003	*fatA501 str-500*	AE6000 spontaneous
AE6004	*fatA501 rif-500*	AE6000 spontaneous
AE6008	*pigA501 hclA501 fat-506 rif-502*	AE6002 spontaneous
SC126	*thrA101*	Bert Ely (3)
SC596	*ts-104*	Bert Ely (3)
SC1062	*flaE178*::Tn*5 pro103 str-140*	Bert Ely (3)
SC1140	*lacA101*::Tn*5 str-152*	Bert Ely (3)
SC1287	*metB123*::Tn*5 ts-104 str-153* (pVS1)	Bert Ely (4)
SC1288	*hisB137*::Tn*5 ts-104 str-153* (pVS1)	Bert Ely (4)
SC1293	*trpK107*::Tn*5 ts-104 str-153* (pVS1)	Bert Ely (4)
SC1294	*cysB108*::Tn*5 ts-104 str-153* (pVS1)	Bert Ely (4)
SC1300	*pheA108*::Tn*5 ts-104 str-153* (pVS1)	Bert Ely (4)
SC1321	*metF127*::Tn*5 ts-140* (pVS1)	Bert Ely (4)
SC1382	*cysD137*::Tn*5 ts-104 str-153* (pVS1)	Bert Ely (4)
SC1417	*serA113*::Tn*5 ts-104* (pVS1)	Bert Ely (4)
SC1424	*hisD136*::Tn*5 ts-104 aar-131* (pVS3)	Bert Ely (4)
SC1490	*leuA131*::Tn*5 ts-140* (pVS1)	Bert Ely (4)
SC1708	*cysD137*::Tn*5 flaF132 rif-181*	Bert Ely (4)
E. coli		
NC9412	*hsdR hsdM supE44 thr leuB6 lacY tonA21 thi-1* (pVS1)	Bert Ely (4)

[a] C, conjugation; T, transduction (see the text). NTG, *N-methyl-N*-nitro-*N*-nitrosoguanidine.

TABLE 2. Tn5 mapping of *fatA501*[a]

Donor	Selected allele (Kan[r])	Phenotype distribution[b]		% Cotransfer[c]
		fatA501	*fatA*[+]	
SC1300	*pheA*::Tn5	200	0	0
SC1417	*serA*::Tn5	200	0	0
SC1490[d]	*leuA*::Tn5	200	0	0
SC1321[d]	*metF*::Tn5	180	0	0
SC1294	*cysB*::Tn5	149	50	25
AE5479	*flaE*::Tn5	104	96	48
SC1382	*cysD*::Tn5	76	124	62
AE5567	*lacA*::Tn5	102	95	48
SC1293	*trpK*::Tn5	200	0	0
SC1288	*hisB*::Tn5	200	0	0
SC1287	*metB*::Tn5	200	0	0
SC1424	*hisD*::Tn5	199	0	0

[a] The recipient in these crosses was AE6004, and the counterselection was growth in the presence of rifampin at the restrictive temperature, 37°C, unless otherwise noted. Details of how each cross was performed are presented in the text. The gene order is shown in Fig. 2.

[b] Two hundred Kan[r] colonies were selected, and exconjugants were also scored for the presence of the Tn5-induced auxotrophic lesion; any not carrying the lesion were discounted.

[c] Percentage of cotransfer of Tn5-marked gene and the *fatA*[+] allele.

[d] The recipient in the crosses involving SC1490 and SC1321 was AE6003, and the counterselection was growth in the presence of streptomycin at 37°C.

plates and incubated overnight at 37°C. The patches of growth were replicated to appropriate selective media to score mutant alleles. Unmixed donor and recipient cultures were treated in like manner to insure that no spontaneous drug- and temperature-resistant colonies had time to develop before the transconjugants were picked.

Generalized transduction. The ϕCr30-mediated transduction was carried out as described by Ely and Johnson (10). If transduction and subsequent selection of a Tn5-marked gene was to be performed, cells and UV-irradiated phage were mixed and incubated together at 30°C (or 22°C if a temperature-sensitive recipient was used) for 1 h in the absence of kanamycin before spreading onto kanamycin-containing PYE plates. This was to insure time for induction of kanamycin resistance.

Scoring mutant alleles. Failure to grow on BMG plates lacking the following supplements was used to score each respective marker: biotin, (2 ng/ml), Brij 58 (0.4 mg/ml)–oleic acid (0.1 mg/ml), or 0.5% Tween 40–0.5% Tween 80, *fatA*; sodium thiosulfate (0.8 mM), *cysB* and *cysD*; histidine (0.2 mM), *hisB* and *hisD*; leucine (1 mM), *leuA*; methionine (0.2 mM) plus alanine (10 mM), *metB* and *metF*; phenylalanine (1 mM), *pheA*; serine (0.2 mM), *serA*; and tryptophan (1 mM), *trpB*. *lacA* was scored as the inability to grow on 0.3% lactose as a carbon source. Helical cell formation (*hclA*) was scored by direct microscopic examination of cells picked from a master plate into a drop of water on a microscope slide (Hodgson, unpublished data). Nonmotile cells (*flaE* and *flaF*) were scored for production of compact colonies when cells were picked into semisoft PYE agar (13).

Measurement of fatty acid synthesis. AE6000 was grown in minimal medium with glucose as the carbon source and supplemented with 0.2 ng of biotin per ml. The culture was then collected on Millipore filters, washed, and suspended in minimal medium plus glucose either in the presence or absence of biotin. Samples were withdrawn from each culture at the times indicated in Fig. 1A and assayed for CFU. Concomitantly, samples were pulse-labeled with either [^{14}C]acetate or $^{32}P_i$. In each case the incorporation of label into total trichloroacetic acid (TCA)-precipitable material and into total lipid was determined.

Measurement of net synthesis of DNA, RNA, phospholipid, and protein. To measure the net synthesis of DNA, RNA, and phospholipid, cultures were grown in BMGT-oleate (1 mM) for five generations in the presence of $^{32}P_i$ (10 μCi/ml) as described previously (8). The generation time under these growth conditions was 240 min; after five generations of growth in the presence of $^{32}P_i$, steady-state labeling was achieved (7). The cells were then collected on sterile filters and washed with BMGT and suspended in BMGT–$^{32}P_i$ (10 μCi/ml) with or without 1 mM oleic acid so that the specific activity of the label was maintained. The cultures were

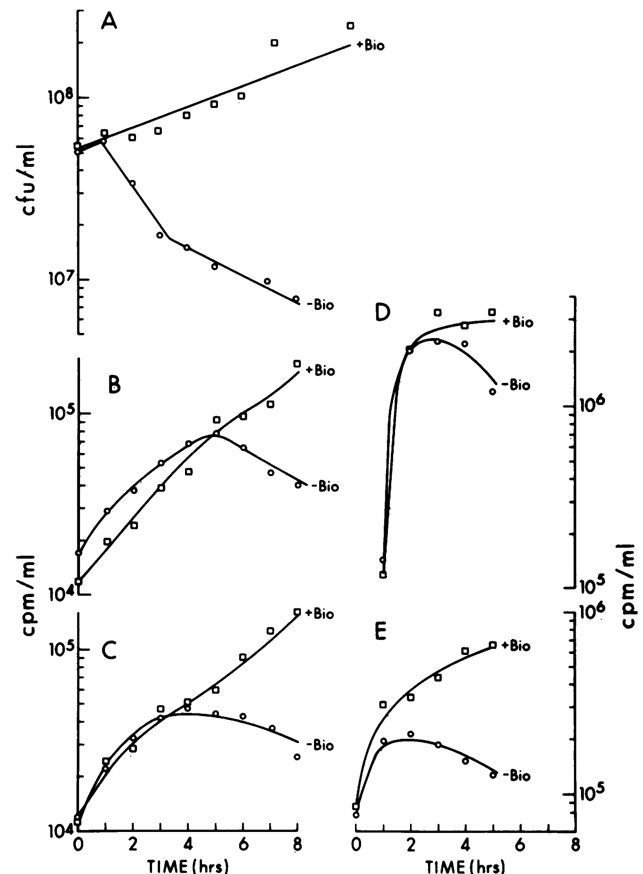

FIG. 1. Viability and fatty acid and phospholipid synthesis in AE6000 grown in the absence or presence of biotin with glucose as the carbon source. Cultures of AE6000 were grown to the midlog phase in BMG plus 2 ng of biotin per ml. At time zero the cells were washed and inoculated into BMG alone (○) or BMG plus biotin (□). CFU per milliliter were determined at the times indicated by plating samples on PYE medium (A). At these same times either [1-^{14}C]acetate (54 mCi/mmol) (B and C) or $^{32}P_i$ (0.5 mCi/mmol) (D and E) were added to cultures growing in the absence or presence of biotin. After incubation of the labeled samples for 15 min at 30°C, a 1.0-ml portion was added to an equal volume of cold 20% (wt/vol) TCA, filtered through glass fiber disks, washed, and counted (B and D), and lipids were then extracted from the remaining 2.0-ml portion (C and E).

shaken at 30°C, and samples were withdrawn at various times. To measure the incorporation of counts into DNA and RNA the samples were precipitated with cold 20% TCA and treated as previously described (6). Phospholipids were extracted with methanol-chloroform as described by Ames (2), and the radioactivity incorporated at various intervals after the removal of oleic acid was measured by liquid scintillation as described previously (6).

The synthesis of bulk proteins was measured in cultures grown in BMGT-oleate and prelabeled for 1 h with 10 μCi of ^{3}H-labeled, reconstituted protein hydrolysate per ml. To remove the oleic acid the cells were collected on filters, washed with BMGT, and then suspended in BMGT and 10 μCi of ^{3}H-labeled, reconstituted protein hydrolysate per ml with or without oleic acid. The cultures were incubated at 30°C with shaking, and 0.1-ml samples were removed at various times and added to 2.0 ml of cold 20% TCA. The precipitates were collected on glass fiber filters, washed with 5% TCA–95% ethanol, and counted. The immunoprecipitation of flagellin proteins was carried out as described previously (28).

Lipid analysis. Lipids were extracted from cells by the procedure of Bligh and Dyer (5) as modified by Ames (2). Fatty acid methyl esters were prepared as previously described (17). Separation of the methyl esters was achieved by argentation chromatography with silver nitrate-impregnated silica gel-G chromatography plates (Supelco Inc., Bellefonte, Pa.), and the developing solvent was petroleum ether-diethyl ether (95:5, vol/vol). Radioactively labeled areas were located by autoradiography with Kodak X-Omat AR film (Eastman Kodak Co., Rochester, N.Y.) and counted as previously described (17).

Materials. Biotin and ^{14}C- and ^{3}H-labeled, reconstituted protein hydrolysate were obtained from Schwarz/Mann (Orangeburg, N.Y.). $^{32}P_i$ and [1-^{14}C]acetate (54.9 mCi/mmol) were from New England Nuclear Corp. (Boston, Mass.). Formalin-fixed *Staphylococcus aureus* cells were from Bethesda Research Laboratories (Rockville, Md.). Antibody to *C. crescentus* CB15 outer membrane proteins and flagellin A was prepared as described previously (28).

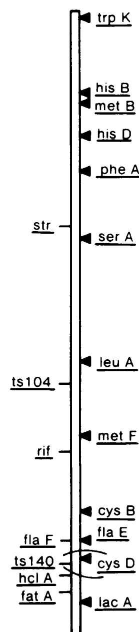

FIG. 2. Partial genetic map of *C. crescentus* CB15 (AE5000) showing gene order of several Tn*5* insertion mutants on the right (3, 4; B. Ely, personal communication). Other markers used in Tables 2 and 3 are shown on the left.

RESULTS

Growth requirements. A mutant of *C. crescentus* CB15 was isolated which was dependent on exogenous fatty acids for growth in minimal medium with glucose as the carbon source. The growth requirement was satisfied by the unsaturated fatty acids, oleic acid, vaccinic acid, linoleic acid, or palmitoleic acid. It was not possible to test the supplementary effects of saturated fatty acids because they were not soluble in BMG minimal medium. Neither glycerol, glycerol-3-PO_4, acetate, nor Tergitol could supplement the growth of AE6000 in minimal glucose medium, but the mutant was able to grow in unsupplemented rich PYE broth. A group of vitamins and cofactors were tested for their ability to satisfy the auxotrophic requirement. We found that concentrations of biotin of 0.2 ng/ml or greater gave maximal growth rates in glucose minimal medium, comparable to supplementation with 1 mM oleic acid. Biotin precursors were unable to support the growth of AE6000.

Cell viability in the absence of supplement was tested by growing AE6000 to the midlog phase in glucose minimal medium plus biotin (2 ng/ml), washing the culture, and then inoculating into BMG alone or in the presence of biotin (2 ng/ml) (Fig. 1A). In the absence of a supplement, AE6000 cultures lost viability. Cultures of AE6000 also lost viability upon a shift to unsupplemented medium if they had first been grown in glucose minimal medium supplemented with oleic acid and Tergitol. Tergitol alone could not supplement growth. Biotin in the presence or absence of Tergitol efficiently supplemented growth.

Mapping of the mutation. Tn*5*-marked genes were used to determine the general location of the *fatA* gene (Table 2). The results demonstrate that *fatA* is close to *cysD*, *flaE*, and *lacA*. The map position of each of the Tn*5* reference mutations is shown in Fig. 2. To map *fatA* more precisely, three-factor crosses were performed. Cotransduction frequencies between *fatA* and *cysD* and *fatA* and *lacA* were too low to allow meaningful analysis (4 to 2%, respectively). Therefore, *cysD flaF*, *cysD hclA*, and *lacA hclA* donors were constructed by transduction, and the conjugations were performed (Table 3). Inspection of the cotransfer frequency in cross A indicates that *cysD* is closer to *fatA* than it is to *flaF*. Therefore, the two possible positions for *fatA* are *flaF fatA cysD* or *flaF cysD flaA*. If the arrangement *flaF fatA cysD* is correct, we would expect that the frequency of *fatA501* versus *fatA*$^+$ would be the same in both the *flaF132* and the *flaF*$^+$ populations, i.e., independent segregation. If the alternative position is correct, i.e., *flaF cysD fatA*, this would not be the case as *fatA501 flaF132* exconjugants would require a quadruple crossover event for this formation, an event rare in *C. crescentus* (4). Because *fatA*$^+$ and *fatA501* were found to segregate independently, the position *flaA fatA cysD* is preferred on the basis of cross A.

The results of cross B indicate that *cysD* is closer to *hclA* than it is to *fatA* (Table 3). In this case we would expect independent segregation if the position *fatA cysD hclA* were correct and dependent segregation if the alternative, *cysD hclA fatA*, were correct. Examination of the segregation data shows clear dependent segregation, and the deficient class of exconjugants, *fatA*$^+$ *hclA*$^+$, is precisely the one we would predict would result from a quadruple crossover. Hence, the

TABLE 3. Three-factor crosses involving the *fatA501* allele[a]

Cross	Strains		Selected allele	Unselected allele	Cross	Genotype distribution		% C transfer frequency	
						fatA501	*fatA*$^+$	Tn*5* and *fatA*$^+$	Tn*5* and *flaF132* of *hclA501*
A	Donor[b]	^AE5533	*cysD137*::Tn*5*	*flaF132*	*flaF cysD*^{c}	41	59 *flaF132*	62	53
	Recipient[d]	AE6004		*fatA501*	*fatA*	32	58 *flaF*$^+$		
B	Donor	AE5563	*cysD137*::Tn*5*	*hclA501*	*cysD*^{c} *hclA*	33	89 *hcA501*	48	64
	Recipient	AE6004		*fatA501*	*fatA*	66	3 *hclA*$^+$		
C	Donor	AE5597	*lacA101*::Tn*5*	*hclA501*	*hclA lacA*^{c}	0	149 *hclA501*	54	37
	Recipient	AE6004		*fatA501*	*fatA*	187	70 *hclA*$^+$		

[a] The gene order was *flaF cysD hclA fatA lacA*.
[b] In each case the donor strains contained plasmid pVS1 to mobilize the chromosome. *ts-104* was used as a counterselectable marker.
[c] Indicates a Tn*5* insertion in the gene.
[d] An *rif*^{r} marker in the recipient was used to counterselect the donor strain.

position *cysD hclA fatA* has been confirmed by two independent crosses; *fatA* must be to the right of *hclA*.

Turning now to cross C, *lacA* is closer to *fatA* than it is to *hclA* (Table 3). Examination of the genotype distribution data demonstrates that dependent segregation has occurred. The position of *fatA* that could lead to dependent segregation (*hclA fatA lacA*) predicts that *fatA501 hclA501* exconjugants should be rare, which indeed they are. Therefore *fatA* must be between *hclA* and *lacA*, as shown in the final gene order (Table 3; Fig. 2).

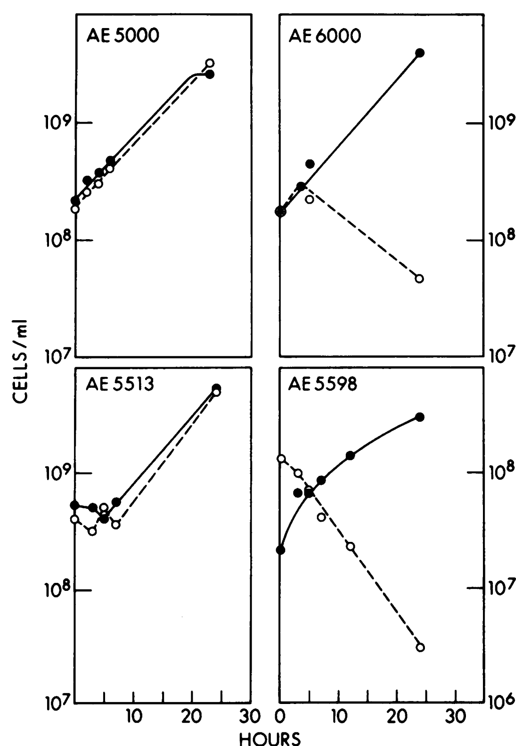

FIG. 3. Cell viability studies of the transductants *cysD137*::Tn*5* *fatA*$^+$ (AE5513) and *cysD137*::Tn*5* *fatA501* (AE5598) as compared with the parent strain AE5000 and mutant strain AE6000. Cells were grown in BMGT plus 1 mM oleic acid and transferred to BMGT plus (●) or minus (○) oleic acid with suitable supplements for *cysD* strains. CFU per milliliter were determined on PYE medium.

Maintenance of the pleiotropic phenotype in transductants. The mutant AE6000 exhibited at least three distinct phenotypes. These were its specific auxotrophic requirements, cell death in the absence of supplement, and altered membrane protein metabolism (28). Strains were constructed which transferred the *fatA501* allele into different genetic backgrounds. Generalized transduction was used to create *cysD137*::Tn*5* *fatA*$^+$ (AE5513) and *cysD137*::Tn*5* *fatA501* (AE5598 strains with AE5000 (wild type) as the recipient. The frequency of cotransduction of *cysD* and *fatA* was 4%, which implies that very little of AE6000 DNA between *fatA* and *lacA* was now present in these studies. The analogous experiment was used to create the *lacA101*::Tn*5* containing pair, AE5623 (*fatA*$^+$) and AE5624 (*fatA501*). The frequency of cotransduction of *lacA* and *fatA* was 2%. All *fatA* strains could be supplemented with either oleic acid or biotin. Each of these strains was tested for its ability to survive removal of oleic acid in minimal medium with glucose as the carbon source. As would be expected, strain AE5513, like wild-type AE5000, was unaffected by the removal of oleic acid, but strain AE5598 died at about the same rate as AE6000 (Fig. 3). Seven other *cysD137*::Tn*5* *fatA501* transductants were also tested and gave identical results (data not shown). As shown in Fig. 4, when strain AE5624 was deprived of oleic acid viability was lost, but not as quickly as that seen with AE6000 or AE5598 (Fig. 3). The analogous *fatA*$^+$ (AE5623)

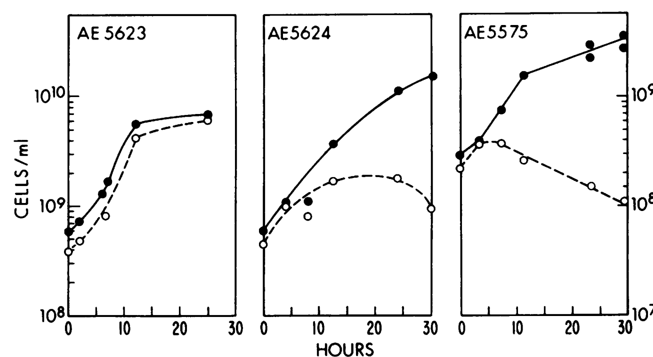

FIG. 4. Cell viability studies of the transductant pair *lacA101*::Tn*5* *fatA*$^+$ (AE5623) and *lacA101*::Tn*5* *fatA501* (AE5624) as compared with their parental strain, AE5575. Growth conditions were as described in the legend to Fig. 3. Cultures were grown in BMGT plus (●) or minus (○) or 1 mM oleic acid.

TABLE 4. Distribution of ^{14}C-labeled fatty acids of AE5000 and AE6000 (*fatA501*) grown in the presence and absence of biotin[a]

Fatty acids	% of total fatty acid			
	AE5000		AE6000	
	+ Biotin	− Biotin	+ Biotin	− Biotin
Unsaturated	68.3	71.3	68.3	58.2
X[b]	6.0	3.5	4.2	6.8
Saturated	25.6	25.2	27.5	34.9

[a] Cultures of *C. crescentus* wild-type Ae5000 or mutant AE6000 were grown to the midlog phase in BMG plus 0.2 ng/ml biotin. Cells were washed and suspended in BMG in either the presence or absence of biotin and in the presence of 2 μCi of [1-^{14}C]acetate (54.9 mCi/mmol) per ml. Samples were taken at 3 h, the lipids were extracted and saponified, and the fatty acid methyl esters were separated by argentation chromatography as described in the text. Similar results were obtained when Tergitol Nonidet P-40 (1%, wt/vol) was present in the growth medium.

[b] X, Unidentified fatty acid. This fatty acid appears as a shoulder preceding the 18:0 peak of fatty acids when analyzed by gas-liquid chromatography.

strain remained viable (Fig. 4). Six other *lacA101*::Tn*5* *fatA501* strains gave results identical to those with AE5624 (data not shown). This raises the possibility then that there is some "enhancer" mutation for the *fatA501* allele that was lost upon transduction of *fatA501*, the implication being that it falls in the *cysD fatA* region. To test this possibility the same experiment was performed on AE5575, the parental strain of AE5623 and AE5624. The results (Fig. 4) show the same delay in death, demonstrating that the transduction did not generate this effect. Cells carrying *fatA501* and the *lacA101*::Tn*5* allele had an increased doubling time (6.0 ± 0.82 h, eight determinations) when compared with that of cells carrying *fatA501* and the *cysD137*::Tn*5* allele (4.0 ± 0.40 h, eight determinations) in adequately supplemented minimal medium. Therefore, the presence of the *lacA101*::Tn*5* allele itself appears to slow down the rate of growth.

These experiments demonstrate, however, that the auxotrophic requirements and cell viability phenotype of mutant AE6000 are due to a genetic lesion within or overlapping the *fatA* gene. We describe below that outer membrane proteins are also altered in these strains upon starvation for supplement.

Fatty acid synthesis in wild-type and the mutant strain AE6000 grown in the presence and absence of supplement. Fatty acid synthesis was measured in cultures of AE6000 grown in the presence or absence of biotin by pulse-labeling with [^{14}C]acetate (Fig. 1B and C). In parallel experiments cultures were pulse-labeled with $^{32}P_i$ to measure phospholipid synthesis (Fig. 1D and E). Although the cell viability began to decrease soon after the shift to unsupplemented medium (Fig. 1A), the incorporation of the ^{14}C isotope into both TCA-precipitable material and into total lipid continued for many hours. The same results were observed when the experiments were repeated with oleic acid instead of biotin as supplement (data not shown). Thus, the AE6000 auxotroph has the surprising phenotype of being able to continue fatty acid synthesis in the absence of a supplement, even though the auxotrophic requirements of this mutant are the lipid metabolites, biotin, or fatty acids. Because a defect in the synthesis of one or more fatty acids might not be apparent when only total lipid is studied, we determined the fatty acids synthesized by mutant AE6000 grown in the presence and absence of a supplement (Table 4). Fatty acid synthesized in wild-type AE5000 and mutant AE6000 was determined by measuring the incorporation of [^{14}C]acetate into fatty acids separated by thin-layer chromatography. The relative proportion of saturated and unsaturated fatty acids synthesized by the mutant and parent strains incubated with and without biotin supplement was not remarkably different (Table 4).

These results suggest that AE6000 is not a simple mutant in fatty acid biosynthesis. We therefore determined the regulatory effects of exogenous fatty acid on fatty acid synthesis and turnover in wild-type and mutant cultures.

Effect of oleate on fatty acid synthesis and turnover in wild-type AE5000 and mutant AE6000. Cultures of AE5000 and AE6000 labeled continuously with [^{14}C]acetate for several generations in the presence of oleate incorporated reduced amounts of [^{14}C]acetate into lipid compared with cultures incubated in the presence of biotin. Cells were grown in the presence of either oleate or biotin since the mutant cells must be supplemented with one of these two supplements in continuous-labeling experiments. Incubation of either mu-

TABLE 5. Lipid turnover in *C. crescentus* wild-type AE5000 and mutant AE6000 grown in the absence and presence of oleic acid or biotin[a]

Strain	Pregrowth medium	Test medium	[^{14}C]acetate remaining in fatty acids (cpm × 10^4/ml of culture)		
			0 h	4 h	6 h
AE5000	Oleate	+ Oleate	4.5	2.6 (58)[b]	2.0[c] (44)
	Oleate	− Oleate	4.1	2.8 (68)	1.3 (31)
	Biotin	+ Biotin	23.4		23.0 (98)
	Biotin	− Biotin	31.4		24.1 (77)
AE6000	Oleate	+ Oleate	14.2	12.9 (91)	10.8 (76)
	Oleate	− Oleate	12.1	10.6 (88)	11.4 (94)
	Biotin	+ Biotin	29.8		25.6 (86)
	Biotin	+ Biotin	21.9		24.1 (110)

[a] The cells were pregrown overnight in BMGT-oleic acid or in BMGT-biotin. The cultures were then divided, half of each was washed free of supplement, and [1-^{14}C]acetate (2 μCi/ml) was added to both. The zero time point in each case represents [1-^{14}C]acetate incorporated into fatty acids after 2 h of incubation in BMGT in the presence of oleate (1 mM) or biotin (2 ng/ml). At 0 h the cultures were washed free of label and incubated for a further 4 or 6 h as indicated. The amount of [1-^{14}C]acetate remaining isolated in fatty acids was determined at each time period.

[b] Numbers within parentheses indicate the percentage of [^{14}C]acetate measured at 0 h remaining at the time indicated.

[c] This sample was assayed 8 h after the pulse.

tant or wild-type cells in the presence of oleate resulted in incorporation of 1.5×10^4 to 3.3×10^4 cpm of ^{14}C per ml from acetate into lipid, whereas the biotin-supplemented cultures incorporated 6.8×10^4 to 9.9×10^4 cpm/ml, suggesting that exogenous oleate repressed fatty acid synthesis. To analyze the effect of exogenous oleate on the synthesis of lipid, the cells were incubated with [^{14}C]acetate for a shorter period of time in the presence and absence of the growth supplements. In these experiments (Table 5) the cells were first grown overnight in either oleate- or biotin-supplemented medium and then split into cultures with and without the respective supplements and labeled for 2 h with [^{14}C]acetate. The wild-type cells pregrown in biotin (in the absence of oleate) incorporated approximately four- to fivefold more label than did AE5000 cells incubated in the presence of oleate. In the mutant AE6000, the cells exposed to oleate in the pregrowth period also incorporated less ^{14}C from acetate into lipid during a 2-h pulse than did cultures grown in the presence of biotin. However, the difference was less striking in the mutant and represented a reduction of

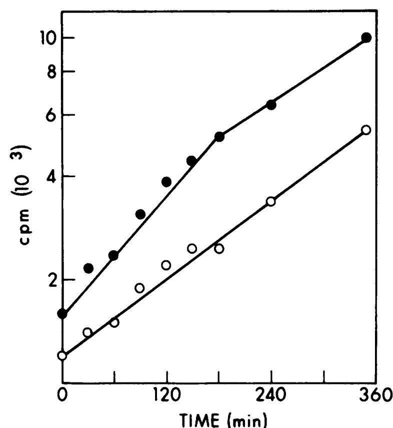

FIG. 6. Effect of fatty acid deprivation on net protein synthesis in AE6000. Cultures were grown in BMGT-oleate and labeled for 1 h with ^3H-labeled reconstituted protein hydrolysate. The cells were washed and suspended in the same medium with ^3H-labeled reconstituted protein hydrolysate at the same final specific activity, but with (●) or without (○) oleic acid. The incorporation of counts into TCA-precipitable material was determined at indicated times.

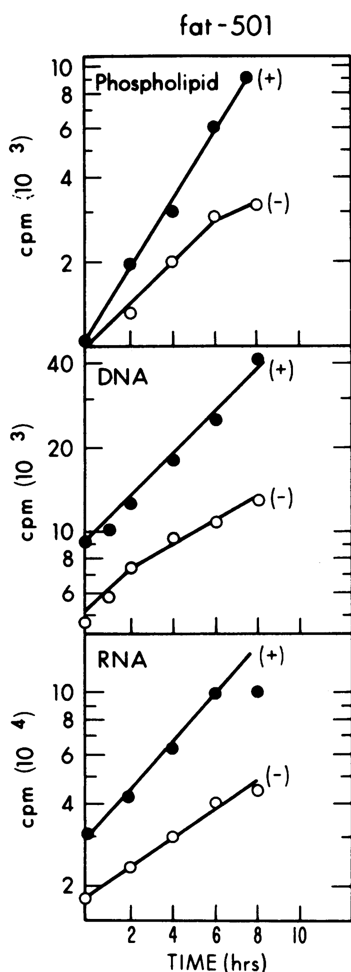

FIG. 5. Effect of fatty acid deprivation on net synthesis of phospholipid, DNA and RNA in AE6000 *fatA501*. Cultures were grown in oleic acid-supplemented minimal medium in the presence of $^{32}P_i$ for five generations. The cells were washed and suspended in medium containing $^{32}P_i$ in the presence (●) or absence (○) of oleic acid. Net syntheses of phospholipid, DNA, and RNA were determined at the times indicated as described in the text.

approximately twofold compared with the four- to fivefold reduction observed in the parent strain (Table 5). Whereas the composition of the medium in which the cells were pregrown (oleate versus biotin supplement) affected the rate of fatty acid synthesis, the absence of a supplement during the actual 2-h labeling period had no effect.

These experiments demonstrate that mutant and wild-type cells pregrown in biotin incorporated comparable amounts of label into lipid, whereas mutant cells grown in the presence of exogenous oleate incorporated about three times as much label as did the oleate-grown parent strain, suggesting that exogenous oleate functions to repress fatty acid synthesis in *C. crescentus* and that this repression is partially nullified in the AE6000 mutant.

The turnover of the label in these cells was also examined, and again a difference was observed in wild-type AE5000 cells pregrown in oleate as compared with those pregrown in biotin-supplemented medium (Table 5). At 6 h after the removal of [1-^{14}C]acetate, greater than 50% of the label was turned over in oleate-grown wild-type cells, whereas in the biotin-grown cells more than 80% of the label was retained. In the mutant AE6000, however, this difference was not observed. Both oleate- and biotin-grown cells retained approximately 80% or more of the label in lipid in the 6-h period after the removal of [^{14}C]acetate. These results indicate that in wild-type *C. crescentus* exogenous oleate functions to induce fatty acid turnover and that this regulatory mechanism is altered in the AE6000 mutant.

Net phospholipid, DNA, and RNA syntheses in mutant AE6000 grown in the presence or absence of a supplement. Net synthesis of phospholipid was measured in cultures uniformly labeled with $^{32}P_i$ to the steady state. After five generations of growth in the presence of the label the cells were shifted to medium with or without oleic acid containing ^{32}P at the same specific activity, so that the radioactivity obtained from phospholipid extracted at various times reflects the relative amount of total phospholipid in each culture (Fig. 5). In cultures of AE6000 net phospholipid increased for 6 h after the removal of fatty acid from the medium, although at a slightly slower rate than that of

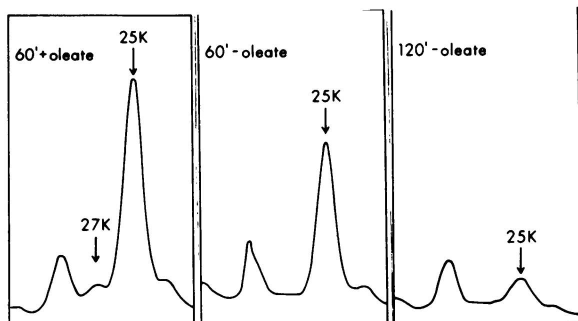

FIG. 7. Densitometer tracings of an autoradiogram of immunoprecipitates prepared with anti-flagellin antibody and analyzed by polyacrylamide-sodium dodecyl sulfate gel electrophoresis. Samples were taken from cultures of AE6000 grown in BMGT in the presence or absence of 1 mM oleic acid at 60 and 120 min and then pulse-labeled for 15 min with ^{14}C-amino acids. Flagellin synthesis was determined by immunoprecipitation of cell extracts as described in the text. ^{14}C-labeled proteins (Bethesda Research Laboratories) for molecular weight standards were ovalbumin (M_r, 43,000), α-chymotrypsinogen (M_r, 25,700), and β-lactoglobulin (M_r 25,000).

supplemented cultures. The subsequent decrease in the rate of net accumulation of phospholipid is likely due to the decrease in viable cells (Fig. 1A). These results confirm the pulse-label experiments described in Fig. 1D and E, which demonstrate that AE6000 is able, in the absence of supplement, to continue de novo phospholipid synthesis.

Net syntheses of DNA and RNA in AE6000 appeared virtually unaffected by the removal of fatty acid from the medium (Fig. 5).

Syntheses of total cellular proteins and specific surface proteins in unsupplemented cultures of AE6000. Total protein synthesis was measured in AE6000 grown in the presence and absence of oleic acid (Fig. 6). The net synthesis of cellular protein, as measured by the incorporation of ^{3}H-amino acids, was relatively insensitive to fatty acid deprivation. We found, however, that in AE6000 the syntheses of specific surface proteins, such as the components of the flagellum and the cell membrane, was dependent on supplementation with either oleic acid or biotin.

Flagellar synthesis. Cultures of AE6000 showed a sharp reduction in the proportion of motile cells between 60 and 120 min after the removal of either oleate or biotin. Immunoprecipitation of flagellar proteins from cultures of AE6000 pulse-labeled with ^{14}C-amino acids at various times after the removal of oleic acid showed that flagellin biosynthesis decreased sharply between 60 and 120 min of starvation (Fig. 7). Immunoprecipitation with anti-hook antibody showed the simultaneous loss of hook protein (data not shown). In confirmation of the continued net synthesis of total protein observed in starved cultures, however, the amount of ^{14}C-amino acids incorporated into bulk proteins during each pulse demonstrated ongoing protein synthesis after the removal of oleic acid. These results suggest that early after the removal of supplement there is a specific decrease in flagellar protein synthesis.

Outer membrane protein composition and synthesis. Examination of the incorporation of labeled amino acids into outer membrane proteins in unsupplemented cultures of AE6000 cells showed that with the exception of the 75-kilodalton protein, the high-molecular-weight proteins were significantly reduced by 60 min after oleic acid (Fig. 8A) or biotin (Fig. 8B) deprivation. The incorporation of label into the outer membrane proteins of wild-type AE5000 grown in the absence or in the presence of oleic acid was unchanged (Fig. 8A). Unsupplemented cultures of AE6000, therefore, fail to incorporate some proteins into the outer membrane at about the same time that flagellin synthesis is shut off. To determine whether this mutant phenotype is maintained when the *fatA501* allele is in other genetic backgrounds we analyzed the outer membrane protein composition of the transductants AE5598 and AE5624 as well as their control strains, AE5513 and AE5623. When AE5598 or AE5624 was deprived of oleic acid several outer membrane proteins were lost, parallel to what was observed with AE6000 (data not shown). The control strains AE5513 and AE5623 showed the same pattern of outer membrane protein synthesis as the parent strain AE5000 grown in the presence or absence of oleic acid.

To determine whether the observed decrease in specific labeled outer membrane proteins was due to a block in processing and assembly or whether the defect lay in the synthesis of those proteins, we examined cytoplasmic proteins precipitated with anti-outer membrane protein antibody (Fig. 9). Precursor membrane proteins were not detected in immunoprecipitates of cytoplasmic extracts of AE6000 grown in the presence or in the absence of biotin (Fig. 9, lanes b and c). Total cytoplasmic proteins from extracts of mutant cells grown in unsupplemented medium showed protein profiles identical to those of the supplemented cultures. Therefore, in the absence of supplement the synthesis of cytoplasmic proteins was not altered in the amount of label incorporated (Fig. 6) or in the distribution of label into specific proteins. It thus appears that the change in membrane protein composition in unsupplemented cultures of the AE6000 mutant is due either to a specific inhibition of the synthesis of certain membrane proteins or to their unusually rapid degradation.

DISCUSSION

The *fatA501* mutation present in strain AE6000 results in a pleiotropic phenotype which includes, in addition to its auxotrophic requirement for oleic acid or biotin, cell death

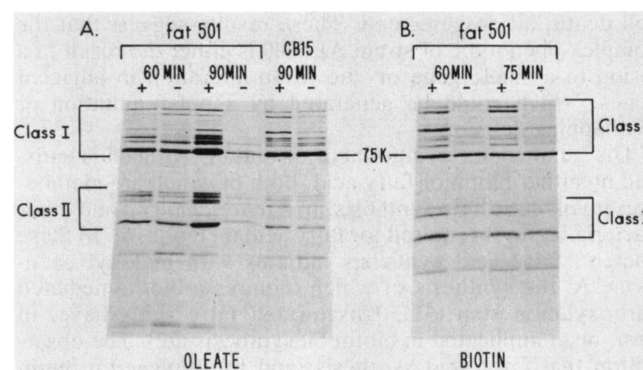

FIG. 8. Outer membrane protein synthesis during oleic acid or biotin starvation of the mutant AE6000. (A) AE6000 *fatA501* and *C. crescentus* CB15 wild-type AE5000 were grown in BMGT in the presence or in the absence of oleic acid (1 mM) for 60 and 90 min and then pulse-labeled for 15 min with ^{14}C-amino acids. (B) AE6000 *fatA501* was grown in the presence and in the absence of biotin (0.1 ng/ml) and at 60 and 75 min was pulse-labeled was 15 min with ^{14}C amino acids. The outer membrane fractionation and polyacrylamide gel electrophoresis were as described in the text. The region of the gel depicting the class I (high-molecular-weight) and class II (low-molecular-weight) outer membrane proteins is indicated.

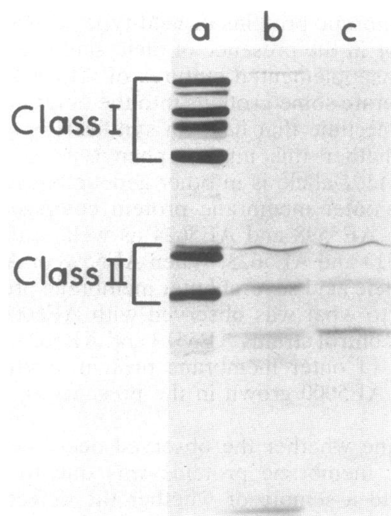

FIG. 9. Autoradiograms of polyacrylamide-sodium dodecyl sulfate gels of immunoprecipitates prepared with anti-outer membrane protein antibody. Cultures of AE6000 were incubated for 90 min in the presence or absence of biotin and then pulse-labeld for 15 min with ^{14}C-labeled, reconstituted protein hydrolysate as described in the text. The samples were then separated into an outer membrane fraction and a cytoplasmic fraction. (A) Immunoprecipitates of the outer membrane fraction; (B) cytoplasmic fraction of AE6000 grown in the presence of biotin; (C) immunoprecipitate of the cytoplasmic fraction of AE6000 incubated in the absence of supplement. The region of the gel depicting the class I (high-molecular-weight) and class II (low-molecular-weight) outer membrane proteins is indicated.

during growth on glucose in the absence of supplement and a marked alteration in flagellar and outer membrane protein syntheses upon starvation for fatty acid or biotin. The auxotrophic requirement of *fatA501* maps between *hclA* and *lacA* on the chromosome. Studies with transductants that placed *fatA501* in different genetic backgrounds demonstrated that the multiple phenotypes, the auxotrophic requirement, alterations in outer membrane protein synthesis, and cell death, all cosegregated. These results suggest that the complex phenotype of strain AE6000 is either the result of a lesion in a single gene or due to an alteration in adjacent genes, which could be generated by a polar mutation or overlapping deletion.

The auxotrophic requirement of mutant AE6000 is satisfied by either biotin or fatty acid, both of which are metabolites involved in lipid synthesis. In *Escherichia coli* and other bacteria biotin is required for fatty acid metabolism. In these bacteria fatty acid synthesis initiates with malonyl coenzyme A, the synthesis of which requires a biotin-mediated carboxylation step (31). Unsaturated fatty acids have, in turn, been implicated in biotin biosynthesis (22). The observation that fatty acid synthesis (and by implication biotin synthesis as well) continues under conditions of starvation is inconsistent with the interpretation that the lesion in AE6000 lies in either biosynthetic pathway. The finding that none of the intermediates in biotin biosynthesis can substitute for biotin implies (assuming that they are transported into the cell) that it is some regulatory property of biotin that is involved. We have recently found that the auxotrophic requirement is affected by the carbon source. For example, preliminary results suggest that the auxotrophic requirement is alleviated by growth on xylose as opposed to glucose. In *E. coli* certain aspects of fatty acid metabolism have been shown to be regulated in coordination with enzymes involved in carbon metabolism (21)

The studies reported here demonstrate that in wild-type *C. crescentus* the pattern of fatty acid metabolism is modified in response to exogenous unsaturated fatty acid. The metabolic changes in response to supplied fatty acid include (i) repression of total fatty acid synthesis and (ii) an increase in the rate of fatty acid degradation. The mutant strain AE6000 is altered in both of these responses to exogenous fatty acid. Exogenous unsaturated fatty acid has been reported to have similar, although not identical, effects upon fatty acid metabolism in *E. coli* (14, 26, 30, 32). The addition of oleate to the growth medium of *E. coli* leads to a decrease in total fatty acid biosynthesis as well as a marked decrease in the proportion of unsaturated fatty acid being synthesized (30). In *C. crescentus* a decrease in total fatty acid biosynthesis was observed, but the proportions of the various saturated and unsaturated fatty acids synthesized were not markedly altered.

The enzymes for fatty acid degradation in *E. coli* are induced by exogenous fatty acid (14, 26, 32). Although the fatty acid degradation pathway in *C. crescentus* has not yet been characterized, it is clear that fatty acid degradation occurs. The organism is able to use fatty acid supplements as carbon sources, and mutants blocked in fatty acid utilization have been isolated (M. O'Connell, unpublished results). The data reported here demonstrate that active turnover of fatty acids occurs in *C. crescentus*. The rate of turnover of fatty acids was increased in cells grown in the presence of oleic acid, but not biotin, suggesting that the degradative pathway is induced, as it is in *E. coli*, by exogenous unsaturated fatty acid.

We have shown here that each of the two responses to exogenous fatty acid observed in the wild type (i.e., repression of fatty acid synthesis and increased fatty acid turnover) is less profound in the mutant AE6000. These results indicate that the mutant is defective in the regulatory responses to exogenous fatty acids and that the several responses are coordinately controlled. An analogous result has recently been reported in mutant *fadR* of *E. coli* (21). This mutant was first identified as being constitutive for the fatty acid degradation enzymes (25). Subsequently, it was shown that the *fadR* mutant also had a decreased level of unsaturated fatty acid biosynthesis (21). Thus, the *fadR* mutation simultaneously affects regulation of two separate responses to unsaturated fatty acid biosynthesis in *E. coli*. The *fadR* cell expresses the metabolic pattern induced by unsaturated fatty acid even when fatty acid is not present. The phenotype of mutant AE6000 may be viewed as being the converse of the *fadR* phenotype. In other words, mutant AE6000 fails to induce degradation and reduce synthesis of fatty acid when exposed to exogenous fatty acid.

The mutation of *fadR* in *E. coli* also affects the expression of glycolytic shunt enzymes (18). Thus, its phenotype is very complex and no doubt reflects the coordinate regulation of several related metabolic pathways. The *C. crescentus* mutant AE6000 also has a pleiotrophic phenotype that may reflect the complex coordination of lipid metabolism, membrane biogenesis, and the cell cycle which has been previously observed in this organism (28). The mutation in strain AE6000, in addition to its effect on regulation of fatty acid metabolism, also affects the expression of several cell surface proteins, including flagellar and outer membrane proteins. In this report we have demonstrated that when mutant AE6000 is deprived of fatty acid or biotin, it fails to synthe-

size several membrane proteins. In cells of *C. crescentus* blocked in total phospholipid synthesis it has previously been demonstrated that several membrane proteins are not synthesized (28). One subset of membrane proteins is not made because its synthesis is dependent upon DNA replication, which, in turn, in dependent upon membrane lipid synthesis. However, synthesis of another set of membrane proteins was demonstrated to be directly dependent upon ongoing membrane lipid synthesis (28). Thus there is evidence in *C. crescentus* of direct coordination of the synthesis of membrane proteins and membrane lipid. The failure to synthesize a set of outer membrane proteins in mutant AE6000 when it is deprived of oleate or biotin no doubt reflects some aspect of this coordination. We tentatively view mutant AE6000 as a regulatory mutant, and its effect upon membrane protein expression may be interpreted in that light. In wild-type cells these membrane proteins are apparently made constitutively, whereas in mutant AE6000 their synthesis has now become dependent upon the exogenous addition of the lipid metabolites biotin or oleic acid. This might imply that mutant AE6000 has uncovered a regulatory role of these metabolites in the control of specific membrane protein expression in *C. crescentus*. The failure to express these membrane proteins may be sufficient to explain the death of the mutant cells in the absence of supplement. Indeed no substantial inhibition of any other metabolic process (other than regulation of fatty acid metabolism) has been observed in the mutant cells during starvation. Total lipid, protein, RNA, and DNA syntheses continue relatively unaffected until the cells die. Thus, inhibition of synthesis of the membrane proteins cannot be a secondary result of a block in DNA or total phospholipid synthesis. Rather, we feel that the inhibition of membrane protein synthesis in strain AE6000 probably results from the regulatory effects of the mutation in the *fatA* gene. This hypothesis can now be tested by isolating mutants with mutations that prevent cell death during oleate or biotin deprivation of AE6000. These suppressor mutants, if our hypothesis is correct, should reflect uncoupling of membrane protein synthesis and regulation by oleate or biotin in the *fatA* mutant. Such mutants might be expected to have alterations in the mechanism of coordination of membrane protein and membrane lipid synthesis.

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