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Isolation and Characterization of *ilvA*, *ilvBN*, and *ilvD* Mutants of *Caulobacter crescentus*

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***Caulobacter crescentus* strains requiring isoleucine and valine (*ilv*) for growth were shown by transduction and pulsed-field gel electrophoresis to contain mutations at one of two unlinked loci, *ilvB* and *ilvD*. Other *C. crescentus* strains containing mutations at a third locus, *ilvA*, required either isoleucine or methionine for growth. Biochemical assays for threonine deaminase, acetohydroxyacid synthase, and dihydroxyacid dehydratase demonstrated that the *ilvA* locus encodes threonine deaminase, the *ilvB* locus encodes acetohydroxyacid synthase, and the *ilvD* locus encodes dihydroxyacid dehydratase. *C. crescentus* strains resistant to the herbicide sulfometuron methyl, which is known to inhibit the action of certain acetohydroxyacid synthases in a variety of bacteria and plants, were shown to contain mutations at the *ilvB* locus, further suggesting that an acetohydroxyacid synthase gene resides at this locus. Two recombinant plasmids isolated in our laboratory, pPLG389 and pJCT200, were capable of complementing strains containing the *ilvB* and *ilvD* mutations, respectively. The DNA in these plasmids hybridized to the corresponding genes of *Escherichia coli* and *Serratia marcescens*, confirming the presence of *ilvB*-like and *ilvD*-like DNA sequences at the *ilvB* and *ilvD* loci, respectively. However, no hybridization was observed between any of the other enteric *ilv* genes and *C. crescentus* DNA. These results suggest that *C. crescentus* contains an isoleucine-valine biosynthetic pathway which is similar to the corresponding pathway in enteric bacteria but that only the *ilvB* and *ilvD* genes contain sequences which are highly conserved at the DNA level.**

Caulobacter crescentus, a freshwater species in the α division of the purple nonsulfur photosynthetic bacteria (49), has been studied extensively because of its unusual life cycle, in which parental cell division gives rise to two nonidentical daughter cells. One daughter cell possesses a prothecum (or stalk) and is essentially the same as the parental cell. The other daughter, termed a swarmer cell, lacks a stalk and has a flagellum which allows it to swim away from its sibling. Before cell division can occur, this swarmer cell must undergo a period of maturation after which the flagellum is released and a stalk is synthesized in its place. Once the swarmer cell matures into a stalked cell, DNA replication occurs and is followed by cell division to produce both a swarmer cell and a stalked daughter cell. Presumably, the adaptive value of having a flagellated cell as an alternative to the stalked cell is to allow free movement within the environment as an aid to obtaining new surfaces for colonization where nutrients might be more prevalent.

C. crescentus is a unique procaryotic system with which to study genetic programming of cell differentiation. In contrast to other procaryotes, such as *Bacillus* (32) and *Myxococcus* (26) species, which differentiate in response to various environmental cues, *C. crescentus* undergoes cell differentiation as a normal consequence of its cell cycle. Genetic evidence suggests that the asymmetric cell division found in *C. crescentus* is driven by a timing mechanism linked to the DNA synthesis or cell division pathways (34, 36, 42). The genetic program leading to the differentiation of a stalked cell into two different daughters is timed precisely, since the flagellum always is synthesized at the same point in the life cycle (27, 36, 42). In addition to the genetic events leading to differentiation, *C. crescentus* must regulate the genes involved in normal metabolism of the cell to be able to respond to various nutritional conditions. Therefore, the genes involved in regulation of gene expression in this bacterium may be divided conceptually into at least two major classes. One class consists of those genes involved in cell differentiation events, while the other class is composed of genes involved in metabolic processes or maintenance of cell structure. This latter class of genes presumably would be expressed in any of the cell types, while the former likely would be expressed only periodically during the cell differentiation events.

To better understand some of the regulatory signals controlling gene expression in *C. crescentus*, we have chosen to study the isoleucine-valine biosynthetic genes. The genes encoding enzymes which catalyze the formation of isoleucine and valine are well characterized in a number of bacterial species, primarily members of the family *Enterobacteriaceae* (for a review, see reference 46). The flow of carbon skeletons through the *Escherichia coli* K-12 *ilv* pathway (Fig. 1) is regulated by end product inhibition (45), and the control of transcription occurs by a variety of mechanisms. These mechanisms include attenuation of transcription mediated by intracellular levels of aminoacylated branched-chain amino acid tRNAs (22, 31), substrate induction (39, 48), catabolite repression (22), and binding of integration host factor (23, 37). In addition, guanosine-5′-diphosphate-3′-diphosphate (ppGpp) (20) has been shown to affect gene expression of *ilv* genes. Such a diversity of regulatory mechanisms suggests the importance of this pathway in central metabolism. It has been estimated that the input of carbon into the *E. coli* K-12 branched-chain amino acid biosynthetic pathways (including the leucine pathway) may account for as much as 10% of the total carbon available for biosynthesis (as calculated by Calvo [8a] on the basis of data of Dayhoff [11]).

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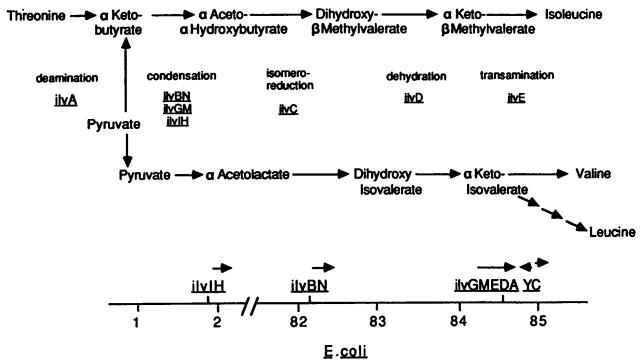

FIG. 1. Isoleucine-valine biosynthetic pathway and locations of the *ilv* genes on the *E. coli* K-12 genetic map. The portion of the pathway common to isoleucine and valine synthesis consists of the last four steps, while the deamination step is specific to isoleucine biosynthesis. Pyruvate condenses either with α-ketobutyrate for synthesis of isoleucine or with a second molecule of pyruvate for synthesis of valine. The numbers below the genetic map indicate the relative positions in minutes of the *ilv* genes, while the arrows above the genes indicate transcriptional units.

In this report, we describe the initial characterization of mutations in three *ilv* genes, *ilvA*, *ilvB*, and *ilvD*, and place these loci on the *C. crescentus* genetic and physical maps. The products of these genes are threonine deaminase, acetohydroxyacid synthase (AHAS), and dihydroxyacid dehydratase, respectively. On the basis of the isolation of mutants resistant to the herbicide sulfometuron methyl, a known inhibitor of some AHAS's (9, 28), and Southern hybridization, it appears that *C. crescentus* contains only one AHAS, in contrast to the multiple AHAS isozymes found in enteric bacteria. Hybridization studies using DNA from *Serratia marcescens* and *E. coli* as probes were used to confirm the location of the *ilvB* and *ilvD* genes. However, no hybridization to *ilvA*, *ilvC*, *ilvE*, or *ilvY* was obtained. The interspecies hybridization between the *C. crescentus ilvB* and *ilvD* genes and their counterparts in the enteric bacteria indicates the conservation of these two genes through evolutionary time, since the enteric bacteria are believed to be distantly related to the purple nonsulfur photosynthetic group (49).

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Tables 1 and 2, respectively. All *C. crescentus* strains are derivatives of the wild-type isolate, CB15. Tn*5*-generated mutations in *C. crescentus* were made on the basis of the method of Ely and Croft (15). Other *C. crescentus* mutants were spontaneous isolates obtained by screening survivors of fosfomycin enrichment (25). Plasmids containing *C. crescentus* DNA inserts were constructed from either R300B (3) or the cosmid vector pLAFR1-7 (26b).

Bacterial growth conditions. Peptone-yeast extract (PYE) medium or minimal M2 glucose medium (25) was used to grow CB15 and mutant strains at 33°C. *E. coli* strains were grown at 37°C in either enriched (LB) medium (10) or defined E medium (47). Amino acid or vitamin supplements, purchased from Sigma Chemicals, were used as necessary in the following concentrations unless otherwise noted: isoleucine, 26 mg/liter; leucine, 26 mg/liter; valine, 24 mg/liter; threonine, 36 mg/liter; methionine, 36 mg/liter; and thiamine,

TABLE 1. Bacterial strains

Species and strain	Genotype	Source or reference
C. crescentus		
CB15	Wild type	38
PC7070	*rec-526 zzz*::Tn*5*	33a
SC112	*ilvA101*	Spontaneous in CB15
SC115	*ilvA102*	Spontaneous in CB15
SC125	*ilvBN102*	Spontaneous in CB15
SC136	*ilvBN103*	Spontaneous in CB15
SC147	*ilvD104*	Spontaneous in CB15
SC149	*ilvBN105*	Spontaneous in CB15
SC151	*ilvA103*	Spontaneous in CB15
SC157	*ilvA104*	Spontaneous in CB15
SC160	*ilvBN106*	Spontaneous in CB15
SC163	*ilvA105*	Spontaneous in CB15
SC321	*ilvD107*	Spontaneous in CB15
SC322	*ilvBN108*	Spontaneous in CB15
SC323	*ilvD109*	Spontaneous in CB15
SC325	*ilvD110*	Spontaneous in CB15
SC331	*ilvD111*	Spontaneous in CB15
SC343	*ilvBN112*	Spontaneous in CB15
SC349	*ilvBN113*	Spontaneous in CB15
SC380	*ilvD114*	Spontaneous in CB15
SC382	*cysB102*	Spontaneous in CB15
SC388	*ilvBN115*	Spontaneous in CB15
SC408	*ilvBN116 flaA104*	Spontaneous in CB15
SC418	*ilvBN117*	Spontaneous in CB15
SC453	*ilvBN118*	Spontaneous in CB15
SC456	*ilvBN119*	Spontaneous in CB15
SC464	*ilvBN120*	Spontaneous in CB15
SC466	*ilvBN121*	Spontaneous in CB15
SC482	*ilv-122*	Spontaneous in CB15
SC497	*ilvA106*	Spontaneous in CB15
SC881	*pheA108*::Tn*5*	4
SC1107	*amp-101*::Tn*5 str-142*	4
SC1238	*cysB102 ilvBN128*::Tn*5 str-142*	15
SC1383	*ts104*(pVS1)	4
SC1488	*rif-175*	Spontaneous in CB15
SC1553	*ilvBN127*::Tn*5*	φSC1238 × CB15
SC1905	*pheA108*::Tn*5 ts104*(pVS1)	φSC1238 × CB15
SC1966	*ilvBN128*::Tn*5 str-142*	15
SC2224	*ilvBN129*::Tn*5-132 rif-175*	NC9762 × SC1488
SC2672	*zzz*::Tn*5 ilvD*$^+$	φTn*5* pool × SC331
SC2673	*zzz*::Tn*5 ilvD*$^+$	φTn*5* pool × SC331
SC2674	*zzz*::Tn*5 ilvD*$^+$	φTn*5* pool × SC331
SC2675	*zzz*::Tn*5 ilvD*$^+$	φTn*5* pool × SC331
SC3371	*ilvBN102 rec-526 zzz*::Tn*5*	φPC7070 × SC125
SC3372	*ilvBN103 rec-526 zzz*::Tn*5*	φPC7070 × SC136
SC3373	*ilvBN105 rec-526 zzz*::Tn*5*	φPC7070 × SC149
SC3374	*ilvBN106 rec-526 zzz*::Tn*5*	φPC7070 × SC160
SC3375	*ilvBN108 rec-526 zzz*::Tn*5*	φPC7070 × SC322
SC3376	*ilvBN112 rec-526 zzz*::Tn*5*	φPC7070 × SC343
SC3377	*ilvBN113 rec-526 zzz*::Tn*5*	φPC7070 × SC349
SC3378	*ilvBN115 rec-526 zzz*::Tn*5*	φPC7070 × SC388
SC3379	*ilvBN116 rec-526 zzz*::Tn*5*	φPC7070 × SC408
SC3380	*ilvBN117 rec-526 zzz*::Tn*5*	φPC7070 × SC418
SC3381	*ilvBN118 rec-526 zzz*::Tn*5*	φPC7070 × SC453
SC3382	*ilvBN119 rec-526 zzz*::Tn*5*	φPC7070 × SC456
SC3383	*ilvBN120 rec-526 zzz*::Tn*5*	φPC7070 × SC464
SC3384	*ilvBN121 rec-526 zzz*::Tn*5*	φPC7070 × SC466
SC3385	*ilvBN129*::Tn*5-132 rec-526 zzz*::Tn*5*	φPC7070 × SC2224
SC3427	*ilvD104 rec-526 zzz*::Tn*5*	φPC7070 × SC147
SC3428	*ilvD107 rec-526 zzz*::Tn*5*	φPC7070 × SC321
SC3429	*ilvD109 rec-526 zzz*::Tn*5*	φPC7070 × SC323
SC3430	*ilvD110 rec-526 zzz*::Tn*5*	φPC7070 × SC325
SC3431	*ilvD111 rec-526 zzz*::Tn*5*	φPC7070 × SC331
SC3432	*ilvD114 rec-526 zzz*::Tn*5*	φPC7070 × SC380
SC3433	*ilv-122 rec-526 zzz*::Tn*5*	φPC7070 × SC482

Continued

TABLE 1–*Continued*

Species and strain	Genotype	Source or reference
E. coli		
C600	*thr leuB6 lacY1 tonA21 thi hsdR hsdM supE*	1
HB101	*proA2 leu thi lacY1 str endH, hsdR hsdM recA-t3 ara-14 galK2 rpsL20 xyl-5 mtl-2*	7
HB101 (pRK2013)	HB101(pRK2013) Km *tra*$^+$	19
NC1079	C600(pJCT26)	This study
NC1081	C600(pJCT57)	This study
NC1221	S17-1(pJCT200)	This study
NC1464	C600(pJCT58)	This study
NC9720	C600(pPLG389)	41
NC9762	C600(pBEE132)	14
S17-1	*pro recA hsdR hsdM*	43

17 mg/liter. Antibiotics (Sigma) were used at the following concentrations: sulfonamide, 300 to 500 mg/liter; kanamycin, 50 mg/liter in enriched medium and 100 mg/liter in minimal medium; ampicillin, 100 mg/liter in LB medium and 50 mg/liter in E medium; streptomycin, 50 mg/liter; and tetracycline, 5 mg/liter in LB medium and 1 mg/L in PYE medium. The herbicide sulfometuron methyl (SM; Du Pont) was used as a supplement at a concentration of 33 mg/liter. The precursors α-ketoisovalerate and α-keto,β-methyl-*n*-valerate were used at concentrations of 90 and 99 mg/liter, respectively, while the concentration of α-ketobutyrate was 124 mg/liter.

Genetic and physical mapping. Transductional mapping studies with the generalized transducing bacteriophage φCr30 (17) in *C. crescentus* were accomplished by utilizing Tn*5* markers at known locations in the genome (4). The two transductional linkage groups, *ilvB* and *ilvD*, and the *ilvA* locus were assigned to *Dra*I restriction fragments by pulsed-field gel electrophoresis (PFGE) of strains containing Tn*5* inserted in or near the three *ilv* loci. Migration of the fragments containing Tn*5* was altered relative to migration of CB15 DNA restricted with *Dra*I (16).

Since mutagenesis experiments employing Tn*5* (15) had produced no Tn*5* insertions at the *ilvD* locus, the generation of random Tn*5* insertions near the *ilvD* locus was accomplished as follows. Tn*5* was transferred into CB15 by the method of Ely and Croft (15) so that insertion of the transposon in the genome would be essentially random. The resultant kanamycin-resistant (Kan^r) colonies were resuspended and pooled, and an aliquot was infected with φCr30. The lysate, which contained a mixture of random Tn*5* insertions, was used in transduction experiments with *ilvD* strains so that transduction of a Tn*5* marker close to a functional *ilvD* locus might occasionally occur. Kanamycin-resistant colonies were grown on minimal medium supplemented with kanamycin to select for those colonies which also were *ilv*$^+$. φCr30 was then used to infect these resulting Kan^r *ilv*$^+$ colonies, and the lysate was used to determine cotransduction frequencies for each of the *ilvD* mutants individually. The strategy for mapping the *ilvA* locus was similar to that employed for mapping *ilvD*.

TABLE 2. Plasmids

Plasmid	Description	Reference
pJCT26	pPLG389 with 2.0-kb *Bam*HI-*Bam*HI deletion	This study
pJCT57	pPLG389 with 2.5-kb *Bam*HI-*Bam*HI deletion	This study
pJCT58	R300B with 4.0-kb *Sma*I-*Sma*I insert from pPLG389	This study
pJCT200	pLAFR1-7 with 23-kb insert containing *C. crescentus ilvD* DNA	This study
pLAFR1-7	Cosmid vector containing multiple cloning sites and RK2 replicon	26b
pPLG389	R300B with 5.2-kb *Sst*I-*Sst*I *C. crescentus ilvBN* DNA insert in *Sst*I site	41
pPU143	pBR322 with 3.7-kb *S. marcescens* DNA insert (*ilv'GMED'*)	24
pRD129	pBR322 with 1.4-kb *Hin*dIII-*Sal*I *E. coli* DNA insert (*ilv'DA'*)	12
pRK2013	ColE1 Km *tra*(RK2)	19

Biochemical assays. *C. crescentus* strains were grown at 33°C in 250 ml of minimal medium, supplemented with isoleucine, valine, or both for *ilv* auxotrophs, to a density of approximately 10^8 cells per ml, collected by centrifugation, washed, and resuspended in 2 ml of 0.5 M KH_2PO_4 buffer (pH 7.2). Extracts were made by sonication followed by centrifugation at 12,060 × *g* for 10 min at 4°C. Protein determinations for all extracts tested were made by the Bradford method (8). Since the original conditions for the *ilv* enzyme assays were determined with enteric bacteria, it is possible that these assay conditions are not optimal for the analogous enzyme activity in *C. crescentus*. However, the conditions described below were sufficient to allow the detection of each particular enzyme activity in the wild-type strain, CB15, while each set of mutants had one enzyme activity which was not detectable under the same conditions.

Threonine deaminase activity was assayed to measure the catalytic conversion of threonine to α-ketobutyrate (30). The reaction cocktail consisted of 100 mM KH_2PO_4 (pH 8.0), 100 mM NH_4Cl, and 80 mM threonine in a total volume of 1 ml, and the reaction was initiated by the addition of 0.3 ml of total extract. After 15 or 30 min of incubation, the reaction was terminated by the addition of 3 ml of 0.025% 2,4-dinitrophenylhydrazine (in 0.5 M HCl), and the reaction mixture was allowed to stand at room temperature for 15 min. Color development occurred when 1 ml of 40% KOH was added and was measured as a change in optical density at 600 nm.

Dihydroxyacid dehydratase activity was assayed by a similar method which also measured the production of a keto acid, α-ketoisovalerate (21). The reaction cocktail consisted of 20 mM KPO_4 (pH 7.5), 100 mM dihydroxyisovalerate, and 10 mM $MgCl_2$ in a total volume of 1 ml. The reaction was started by the addition of 0.2 ml of total extract, the mixture was incubated at 33°C for 10 to 20 min, and the reaction was terminated by the addition of 0.1 ml of 50% trichloroacetic acid and 3 ml of 2,4-dinitrophenylhydrazine. After 15 min at room temperature, 1 ml of 40% KOH was added and color development was measured as a change in optical density at 540 nm.

Acetohydroxyacid synthase activity was assayed (5, 44) with a 0.5-ml reaction cocktail with the following final concentrations of constituents: 40 mM sodium pyruvate, 10 mM $MgCl_2$, 0.1 mM thiamine pyrophosphate (cocarboxylase), 20 μg of flavin adenine dinucleotide per ml, and 0.1 M KH_2PO_4 buffer (pH 7.2). After 0.5 ml of total extract was added, the mixture was incubated for 10 to 30 min at 33°C. The reaction was terminated by the addition of 0.1 ml of 50% H_2SO_4, and the mixture was incubated at 60°C for 45 min.

Color development occurred with the addition of 1.0 ml of a 0.5% creatine hydrate solution and 1.0 ml of 5% α-naphthol (5 g in 100 ml of 4 N NaOH). After a brief vortexing, the reaction tubes were allowed to site for 1 h at room temperature, and then spun for 5 min to remove any precipitate, and the optical density was measured at 535 nm.

Complementation analysis. Bacterial matings were performed by the cross-streak method as described by Ely (13). M2 glucose medium supplemented with sulfonamide or tetracycline was used for selection of strains containing antibiotic resistance plasmids. Upon purification, the resultant colonies were subjected to segregation analysis to verify that complementation had occurred. After overnight growth in nonselective (PYE) medium, 0.1 ml of a 10^{-6} dilution was plated onto PYE agar, M2 glucose agar (single selection), and M2 glucose agar supplemented with sulfonamide or tetracycline (double selection) to verify that loss of the plasmid coincided with restoration of the mutant phenotype.

Recombinant DNA techniques. Agarose and acrylamide gel electrophoresis, restriction mapping, nick translation, and Southern hybridizations were performed essentially as described by Maniatis et al. (33) and Davis et al. (10) with the following exceptions. The hybridization and wash temperature for *C. crescentus* DNA with *E. coli* or *S. marcescens* DNA was 44 to 46°C. Washes were performed with 1× SSPE–0.1% sodium dodecyl sulfate (33) at the hybridization temperature and were repeated four times for 15 min each time.

RESULTS

Isolation and characterization of *ilv* mutants. The analysis of more than 300 *C. crescentus* auxotrophic strains led to the identification of 30 strains deficient in isoleucine and valine biosynthesis as a result of spontaneous mutations or Tn*5* insertions. Of these mutants, 24 were able to grow on minimal medium supplemented with isoleucine and valine but not on minimal medium supplemented with either amino acid alone. The remaining six mutants were capable of growth on minimal medium supplemented with either isoleucine or methionine, a response which has not been reported previously for *ilv* mutants in other bacteria. Precursor feeding studies were undertaken to examine the response of each of the mutants to certain pathway intermediates. All the mutants grew on minimal medium supplemented with α-ketoisovalerate and α-keto,β-methyl-*n*-valerate, the common substrates for the final transamination step. Thus, all strains possess a functional transaminase. When the strains were tested on minimal medium supplemented with α-ketobutyrate, only the mutants which responded to either isoleucine or methionine grew. Other intermediates were not commercially available and were not tested. Since α-ketobutyrate is the product of the *ilvA*-encoded threonine deaminase, these strains appear to be *ilvA* mutants. Enzyme assays confirmed that all six of these strains lack threonine deaminase activity (see below). The substitution of methionine for isoleucine in these strains has not been investigated extensively, but it is possible that methionine can be metabolized intracellularly to produce cystathionine in the cysteine biosynthetic pathway. The cleavage of cystathionine by cystathioninase would yield cysteine and α-ketobutyrate, circumventing the need for threonine deaminase.

Genetic mapping and physical mapping by PFGE. Transduction experiments with the 24 mutants which require both isoleucine and valine indicated that the mutations in these strains are clustered at two loci, which were designated *ilvB* and *ilvD*. Initial mapping studies employed conjugation experiments to determine the approximate map location of these two groups of *ilv* mutations. The seven *ilvD* mutations were linked to *pheA*, while the 17 *ilvB* mutations were linked to *amp* (Fig. 2). The location of *ilvB* was confirmed by transductional linkage to *amp* (20 to 40%), but no transductional linkage was obtained between *pheA* and the *ilvD* cluster. Presumably, *pheA* and *ilvD* are not linked closely enough to allow cotransduction to occur. Therefore, an alternative method for mapping these strains was needed. Bacteriophage φCr30 was grown on a pool of colonies containing random Tn*5* insertions and was used to infect the *ilvD* strain SC331. Four *ilvD*$^+$ Km^r strains were obtained, and each of the Tn*5* insertions was shown to be linked to the *ilvD* mutations. By PFGE, the Tn*5* insertions linked to the *ilvD* locus were demonstrated to be in a 240-kb *Dra*I fragment of the *C. crescentus* genome (Fig. 3B, lane 3). Since the 240-kb *Dra*I fragment also contains the *pheA* gene (16), these results confirm the genetic analysis, which indicated that the *ilvD* locus was linked to *pheA*.

Other PFGE experiments demonstrated that the three Tn*5*-derived *ilvB* mutations in SC1553, SC1966, and SC2224 were located on a 310-kb *Dra*I fragment of *C. crescentus* genomic DNA (Fig. 3A). This result is in agreement with the genetic map location, since the *amp* gene also is located on this fragment (16). Two of the transposon insertions, those contained in SC1966 (*ilvB-127*::Tn*5*) and SC2224 (*ilvB-129*::Tn*5-132*), carried *Dra*I sites, since digestion with *Dra*I resulted in the cleavage of the 310-kb chromosomal *Dra*I fragment (Fig. 3B, lanes 2 and 3). (Tn*5-132* contains a *Dra*I site in the portion derived from Tn*10*. The Tn*5* element in SC1966 contains a spontaneous mutation which generated a *Dra*I site [14a].) In these cases, two new fragments appeared: a fragment of approximately 270 kb and one of approximately 46 kb which can be seen in gels run with a shorter pulse time. Thus, the *ilvB* locus is situated 46 kb from one end of the 310-kb *Dra*I fragment.

The location of the *ilvA* gene also was determined by using linked Tn*5* insertions. In this case, the Tn*5* insertions were located on a 305-kb *Dra*I fragment (Fig. 3B, lane 2) which was known to contain *trpB* (16). The *trpB* locus was found to be tightly linked to the *hunH* locus (97% by transduction), which in turn was found to be distantly linked to *ilvA* (3% by transduction). Subsequently, all six *ilvA* strains were found to be linked by transduction to *hunH*. Additional crosses demonstrated that the *ilvA* locus was 20% linked to *flbU*, 36% linked to *gltA*, and 4% linked to *hisG*. Since *flbU* is 77% linked to *gltA* and 1% linked to *hisG* (26a), the order of these genes must be *hunH-flbU-gltA-ilvA-hisG*.

Enzyme assays. Each of the 30 *ilv* strains was tested for the presence or absence of certain *ilv* pathway enzymatic functions. All of the *ilvB* strains lacked AHAS activity, indicating that mutations at the *ilvB* locus result in loss of AHAS activity. In contrast, all of the *ilvD* strains were found to be deficient for dihydroxyacid dehydratase, the product of the *ilvD* gene, and all of the mutations in *ilvA* resulted in loss of threonine deaminase activity. Taken together, these results indicate that an AHAS gene resides at the *ilvB* locus, a dihydroxyacid dehydratase gene resides at the *ilvD* locus, and the mutants which require either isoleucine or methionine for growth lack the *ilvA*-encoded threonine deaminase. The AHAS locus was designated *ilvBN*, since the organization of the *C. crescentus* AHAS locus is similar to those of the enteric bacteria, in which the AHAS large subunit (*ilvB*) and the small subunit (*ilvN*) genes are organized into an operon (44a).

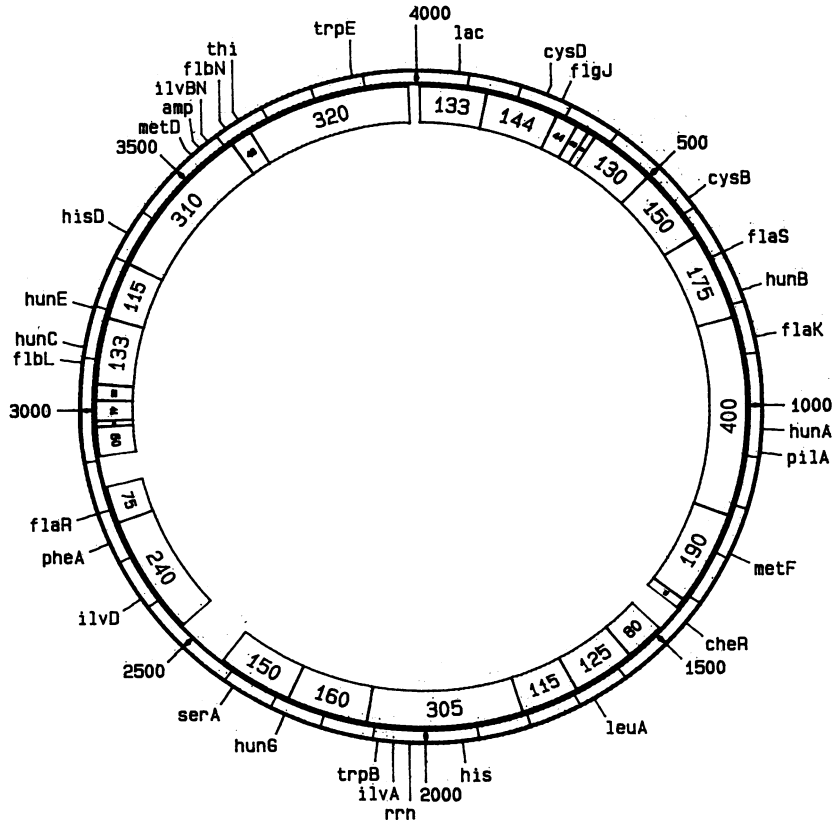

FIG. 2. Transductional linkage map and physical map of *C. crescentus*. The *C. crescentus* genome is approximately 4,000 kb long, as noted by numbers placed periodically along the outside of the circle that indicate the relative physical distance in kilobases. The gene order indicated on the outside of the map was generated by transductional linkage. The numbered blocks on the inside of the circle represent *Dra*I fragments (sizes in kilobases) composing a physical map derived from PFGE. The three known *ilv* loci reside on the 310-kb (*ilvBN*), 240-kb (*ilvD*), and 305-kb (*ilvA*) *Dra*I fragments. Blocks in the physical map having no numbers represent regions where no fragments have been placed on the map.

Mutants resistant to SM. The herbicide SM is believed to cause allosteric inhibition of the action of acetohydroxyacid synthases from a variety of biological systems (9, 18, 29). Some naturally SM-resistant AHAS's, such as *E. coli* AHAS I and *Salmonella typhimurium* AHAS I (both encoded by the respective *ilvBN* genes), have been characterized (29). When the *C. crescentus* wild-type strain, CB15, was tested, it was shown to be sensitive to SM, indicating that *C. crescentus* has no naturally occurring AHAS which is resistant to the action of SM. Three SM-resistant *C. crescentus* mutants were isolated by spreading 0.1 ml of an overnight culture of CB15 on minimal medium plates containing SM. Transduction experiments showed that the mutations in these strains were closely linked to the *ilvBN* locus, confirming that this locus contains the AHAS gene. Enzyme assays for AHAS activity in extracts in the presence and in the absence of SM confirmed that AHAS activity was inhibited by SM. In contrast, when an extract of one of the SM-resistant mutants, SC2668, was assayed in the presence of SM, AHAS activity was approximately equal to that of CB15.

Complementation and recombination analysis of *ilvBN* and *ilvD* mutants. Genomic libraries constructed from *C. crescentus* CB15 were screened, and recombinant plasmids containing DNA from the *ilvBN* (pPLG389) and *ilvD* (pJCT200) loci were identified (41). Recombination-deficient strains containing the *ilvD* mutations were constructed for complementation analysis (Table 1), and studies with the *ilvD rec* mutants revealed that pJCT200 can complement all but one of the *ilvD* mutants, SC3433. Therefore, pJCT200 must contain the entire *ilvD* gene. Experiments with strain SC482 (isogenic to SC3433 but rec^+) yielded prototrophs by recombination between pJCT200 and the bacterial chromosome. Thus, pJCT200 may contain a portion of a second *ilv* gene as the upstream part of an operon containing *ilvD*. The mutation in SC482 may lie in this upstream gene and impart a polar effect on *ilvD* expression. Alternatively, the lack of complementation of SC3433 by pJCT200 may be a result of the production in this strain of a mutant product which interacts with the *ilvD* product produced by pJCT200 to yield an inactive enzyme. Thus, it is not possible to determine whether pJCT200 contains another *ilv* gene on the basis of these complementation experiments, and resolution of the nature of the defect in SC482 awaits further study.

Recombination-deficient strains containing the *ilvBN* mutations also were constructed. Complementation experiments with *ilvBN rec* mutants indicated that pPLG389 must contain the entire AHAS gene, since it could complement all of the *ilvBN* mutations. Two deletion subclones of pPLG389, pJCT26 and pJCT57 (Fig. 4), were constructed by using a partial digest to delete a *Bam*HI fragment, but neither could complement the *ilvBN* mutants. Since both subclones contain a common *Bam*HI restriction site, it is likely that the AHAS gene spans this restriction site. A third subclone, pJCT58, contains a 4-kb *Sma*I-*Sma*I insert which includes

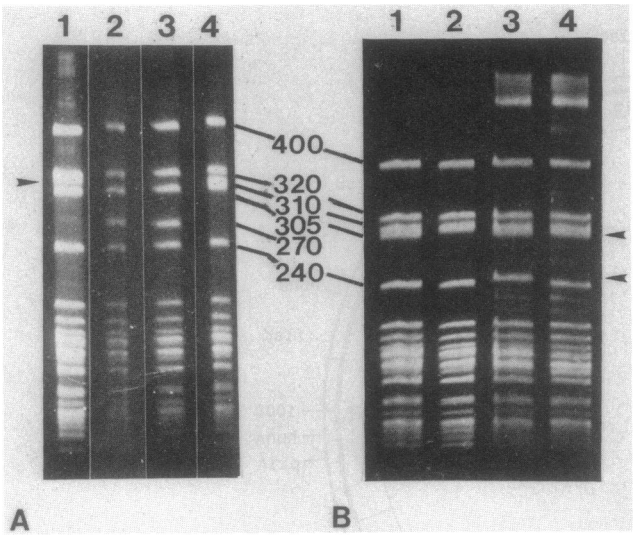

FIG. 3. PFGE of various Tn*5* insertions at or near the three *ilv* loci. All lanes contain genomic DNA digested with *Dra*I and separated by PFGE. The sizes of the largest *Dra*I fragments are denoted by the numbers (in kilobases) between panels A and B. (A) Tn*5* insertions at the *ilvBN* locus. Lanes 1 through 3 contain the strains which have Tn*5* inserted at the *ilvBN* locus (SC1553, SC1966, and SC2224, respectively). The faint bands in lane 1 may be due to partial digestion products. Lane 4 contains CB15 genomic DNA. The arrow on the left indicates the 310-kb fragment containing the *ilvBN* locus, which has altered migration because of the Tn*5* insertions. A 270-kb fragment appearing in lanes 2 and 3 results from a *Dra*I site within the transposons contained in SC1966 and SC2224. (B) Tn*5* insertions at the *ilvD* and *ilvA* loci. Lanes 1 and 4 contain CB15 DNA. The arrowheads on the right indicate *Dra*I fragments with altered migration relative to the CB15 genomic fragments. Lane 2 contains DNA isolated from the *ilvA* strain SC3000. In this strain the *ilvA* locus is linked to a flanking Tn*5* insertion (see text). The 305-kb fragment migrates higher than the corresponding fragment from CB15. Lane 3 contains SC2674 DNA. In this strain the *ilvD* locus is linked to a flanking Tn*5* insertion (see text). The 240-kb fragment in this lane migrates higher than the corresponding fragment from CB15 (lane 4).

the common *Bam*HI site and complements all of the *ilvBN* mutants. Thus, all of the *ilvBN* mutations must lie within this 4-kb region. When pJCT26 and pJCT57 were transferred to *ilvBN rec*$^+$ strains, prototrophic colonies were obtained with some of the strains, suggesting that recombination had occurred. As a result of these experiments, it was possible to distinguish two subgroups of *ilvBN* mutations on the basis of their ability to be corrected by either pJCT26 or pJCT57 (Fig. 4). These results indicate that pJCT26 and pJCT57 contain portions of the AHAS gene and confirm that the *Bam*HI site is within the coding region.

Southern analysis of the *ilvBN* and *ilvD* mutations. Since clones containing the *ilvBN* and *ilvD* regions were available, genomic DNA was isolated from the corresponding *ilvBN* and *ilvD* mutants and was used for Southern analyses. Initially, each of the isoleucine- and valine-requiring mutants which arose from spontaneous mutations were subjected to reversion analysis, and these studies revealed that 11 of 21 mutants (SC125, SC147, SC149, SC321, SC322, SC323, SC380, SC388, SC408, SC464, and SC466) reverted at frequencies higher than 10^{-9} while the remaining 10 (SC136, SC160, SC325, SC331, SC343, SC349, SC418, SC453, SC456, and SC482) had no detectable reversion. These results suggest that some *ilv* mutations may arise from deletions or DNA rearrangements. As expected, DNA from SC1553, SC1966, and SC2224 contained Tn*5* insertions in the 5.2-kb *Sst*I-*Sst*I DNA fragment which corresponds to the *ilvBN* region contained in pPLG389. All but one of the remaining *ilvBN* and *ilvD* mutations showed no significant changes in the migration of the appropriate DNA fragment, indicating that these mutations arose from single-base changes or minor alterations in their DNA. The one exceptional strain, SC125, was found to contain an insertion of approximately 1.3 kb. This insertion was investigated by DNA sequence analysis as well as by Southern hybridization studies and appears to be a transposable element, designated IS*511* (35).

Interspecific hybridization of the *ilv* genes. Cloned *ilv* DNA from *E. coli* and *S. marcescens* hybridized to both genomic and cloned *C. crescentus* DNA and led to the identification of *ilvD*-like and AHAS-like sequences. To locate the *C. crescentus ilvD* gene, an 817-bp *Hin*dIII-*Xho*I internal fragment from the *ilvD* gene of *E. coli* was used to probe various restriction digests of the *ilvD* clone, pJCT200, and was shown to hybridize to a 1.4-kb *Sal*I-*Sal*I DNA fragment (Fig. 5A). This heterologous hybridization of the *E. coli ilvD* sequences to pJCT200 affirms the conclusion from genetic analysis and indicates that part of the *ilvD* gene is located between two *Sal*I sites which are 1.4 kb apart.

Similar DNA hybridization experiments using cloned *S. marcescens ilv* DNA (pPU143 [24]) confirmed that the *ilvBN* locus includes a *C. crescentus* AHAS gene, since pPU143 hybridized to a 0.6-kb *Bam*HI-*Sal*I fragment from pPLG389 (Fig. 5B). Conversely, when labeled pPLG389 was used to probe pPU143 digested with *Pst*I, two fragments, one of 0.6 kb and the other of 1.2 kb, gave hybridization signals (Fig. 5C). Both fragments contain DNA sequences from the *ilvG* gene, which forms the large subunit of AHAS II in *S. marcescens*. In addition, the 0.6-kb fragment contains DNA sequences from the *ilvM* gene, which forms the small subunit of the AHAS II. Since only one AHAS-like locus was detected, these results suggest that only one set of AHAS genes is present in *C. crescentus*. No hybridization was detected when probes for *ilvA*, *ilvC*, *ilvE*, and *ilvY* from either *E. coli* or *S. marcescens* were used in Southern experiments with genomic or cloned DNA from *C. crescentus*. Presumably, the lack of hybridization reflects divergence of DNA sequence in these genes between the enteric bacteria and *C. crescentus*.

DISCUSSION

Mutations resulting in *ilv* auxotrophy isolated in our laboratory occurred in the *ilvA*, *ilvBN*, and *ilvD* genes. It is somewhat surprising that none of 30 spontaneous or randomly generated Tn*5* *ilv* mutations is in the *ilvC* gene. Explanations for the absence of *ilvC* mutants include the possibilities that more than one isomeroreductase may be present, that the accumulation of the product of the AHAS-catalyzed step may be toxic to the cell, or that the isomeroreduction step in *ilv* synthesis may be carried out by a component of another metabolic pathway which is essential for growth. Alternatively, one mutation, SC482, which maps to the region near *ilvD* and causes a deficiency in the corresponding dihydroxyacid dehydratase activity was not complemented by pJCT200 and could contain a mutation in the *ilvC* gene. An assay for the isomeroreductase activity in mutant SC482 has not been performed, since the substrates for such an assay are not commercially available. Since

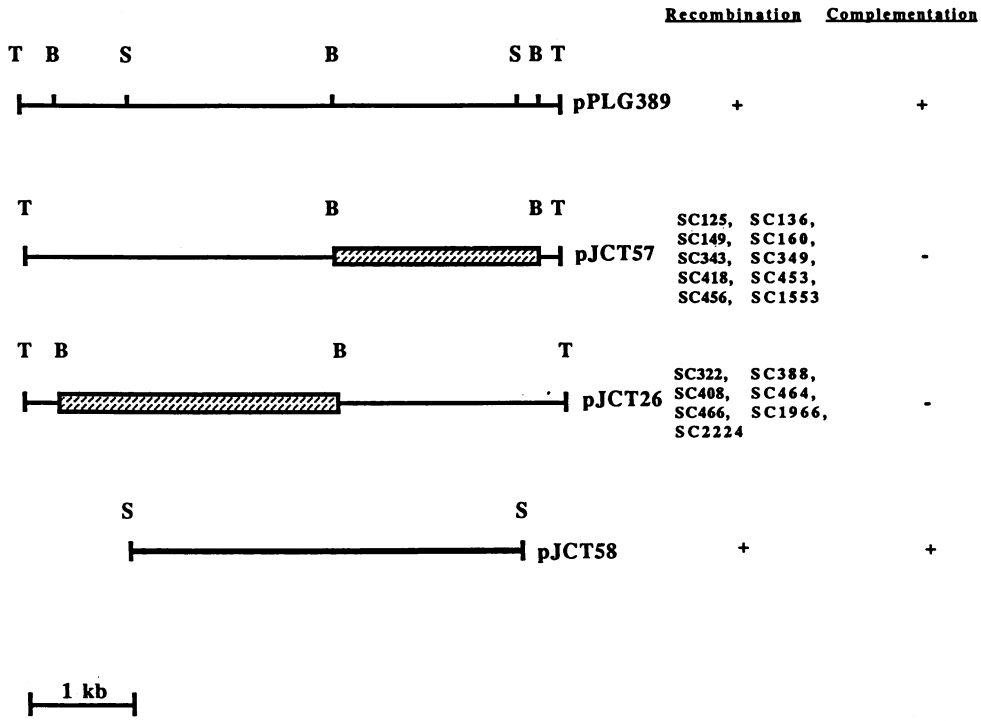

FIG. 4. Complementation and recombination analysis of the *ilvBN* mutants. A partial restriction map for the *ilvBN* locus is given at the top. Abbreviations for restriction enzymes: B, *Bam*HI; S, *Sma*I; T, *Sst*I. DNA was originally cloned from the *ilvBN* locus to produce pPLG389 (a 5.2-kb *Sst*I-*Sst*I fragment), from which other subclones, pJCT26, pJCT57, and pJCT58, were constructed. Hatched areas represent DNA regions contained in pPLG389 which are deleted in pJCT26 and pJCT57. + indicates that all of the *ilvBN* strains were corrected by recombination in a *rec*$^+$ background; − indicates that they were complemented in a *rec* genetic background. Strain numbers for subgroups of *ilvBN* mutants which can be corrected to prototrophy by pJCT57 or pJCT26 in a *rec*$^+$ background are listed beside each recombinant plasmid. pJCT58, which contains 4 kb of contiguous DNA subcloned from pPLG389, is sufficient to restore prototrophy to all *ilvBN* mutants in both *rec* and *rec*$^+$ genetic backgrounds.

pJCT200 contains DNA sequences that can correct the *ilv* mutation in SC482 by homologous recombination, a mutation in this putative *ilvC* gene would be located near the *ilvD* locus and could affect expression of the *ilvD* product through a polar effect on the *ilvD* gene. However, SC482 could also have a mutation in the *ilvD* gene which causes the production of a polypeptide which interferes with the *ilvD* product from pJCT200 by forming an aberrant quaternary structure that would prevent the dehydratase from functioning.

All the *C. crescentus ilv* strains were able to grow on minimal medium supplemented with α-ketoisovalerate and α-keto,β-methyl-*n*-valerate demonstrating the presence of transaminase activity. Thus, presumably we have no *ilv* strains with mutations in *ilvE*. The presence of an alanine-valine-specific transaminase C (the product of the *avtA* gene) in *E. coli* confers an isoleucine auxotrophy on *ilvE* strains of this species, since valine synthesis may still occur (6). However, all the *C. crescentus* strains requiring isoleucine (or methionine) alone have been demonstrated to be deficient in threonine deaminase activity. Since *E. coli* is known to have at least four transaminases with overlapping specificities (6), it is possible that *C. crescentus* also has several general transaminases, so that a mutation in *ilvE* would not result in an isoleucine and valine requirement.

It is unusual that the *ilvA* strains grow when supplemented with either isoleucine or methionine, since no similar *ilvA* mutants have been reported in other bacteria. A common element which could link isoleucine and methionine metabolism is α-ketobutyrate. Homocysteine, a product of methionine metabolism, and serine condense to form cystathionine, which may be irreversibly cleaved by cystathioninase to yield cysteine and α-ketobutyrate. Thus, it is possible that the metabolism of methionine in *C. crescentus* provides a source of α-ketobutyrate for isoleucine synthesis in *ilvA* mutants.

With only one AHAS, regulation of *ilv* biosynthesis may be less finely tuned in *C. crescentus* than in the enteric bacteria. The presence of at least three AHAS isozymes in the members of the *Enterobacteriaceae* allows species from that family to be responsive to a large variety of physiological conditions. Experiments which have measured the specificities of each isozyme from *E. coli* for the two possible AHAS substrates, α-ketobutyrate and pyruvate, have indicated that AHAS I should not be able to support normal growth (2). AHAS I was found to have a similar affinity for both substrates, and since intracellular concentrations of pyruvate are typically 100-fold higher than those of α-ketobutyrate (2), synthesis of valine by AHAS I would be greatly favored, to the detriment of isoleucine synthesis. However, in situations in which the pyruvate concentrations are much lower as a result of growth on a poor carbon source, AHAS I may provide the majority of both valine and isoleucine biosynthesis. In contrast, AHAS II and AHAS III have greatly increased specificity for α-ketobutyrate, which compensates for its lower intracellular concentrations. Normal growth can be supported by either. If a single *C. crescentus* AHAS has a high affinity for α-ketobutyrate similar to the *E. coli* AHAS II and AHAS III, then carbon flow through the

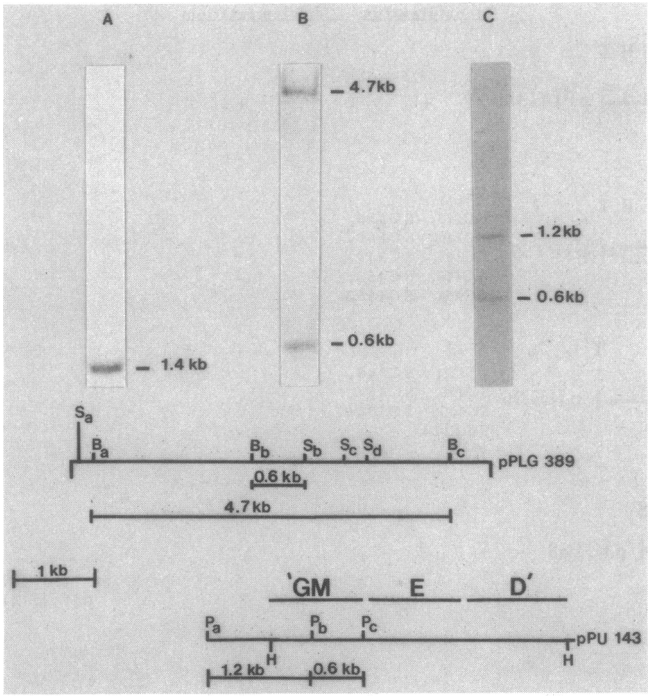

FIG. 5. DNA hybridization between *C. crescentus* and enterobacterial *ilv* genes. DNA fragments were detected after Southern hybridization and subsequent autoradiography. (A) A 1.4-kb *Sal*I-*Sal*I fragment detected by hybridization of an 817-bp internal fragment of the *E. coli ilvD* gene to pJCT200 containing *C. crescentus ilvD* DNA. (B) A 0.6-kb *Bam*HI-*Sal*I fragment generated by digestion of pPLG389 and subsequent hybridization with *S. marcescens ilv* DNA contained in pPU143. The 4.7-kb fragment results from partial digestion of a *Bam*HI site (B_b) and was present on other occasions when pPLG389 was digested with *Bam*HI. This fragment also contains the 0.6-kb *Bam*HI-*Sal*I region. A partial restriction map of pPLG389 illustrating the locations of both fragments is presented. (C) 1.2- and 0.6-kb *Pst*I-*Pst*I fragments detected by hybridization of *Pst*I-digested pPU143 with pPLG389. The *S. marcescens* DNA contained in pPU143 resides on a 3.7-kb *Hin*dIII fragment cloned into pBR322. Digestion with *Pst*I produces the 1.2-kb fragment spanning the *Hin*dIII site. This fragment contains a portion of pBR322 DNA as well as a portion of the *S. marcescens ilvG* DNA as described above. Abbreviations for restriction enzymes: B, *Bam*HI; H, *Hin*dIII; P, *Pst*I; S, *Sal*I.

ilv pathway in *C. crescentus* may be analogous to that in *E. coli* strains in which only AHAS II or AHAS III is functional.

Studies of metabolic genes offer an opportunity to compare various classes of genes in *C. crescentus*. The molecular analysis of *ilvBN* and *ilvD* is under way in our laboratory, and preliminary results indicate that *ilvBN* may be regulated by a transcription attenuation mechanism mediated through a leader sequence. The *ilvBN* promoter appears to have similarities to another amino acid biosynthetic operon promoter, the *trpFBA* promoter (40), and appears to be different from the *nif*-like promoters found in genes involved in flagellum biosynthesis. Thus, the continued characterization of the *C. crescentus ilv* genes will allow comparison of promoter elements within *C. crescentus*. Molecular analysis of *ilvBN* and *ilvD* should provide insight into the conservation of these genes in bacteria.

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