Identification and Characterization of the ilvR Gene Encoding a LysR-Type Regulator of Caulobacter crescentus

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The ilvR gene was located upstream of and transcribed divergently from the ilvD gene of Caulobacter crescentus. DNA nucleotide analysis determined that the ilvR and ilvD translation initiation codons are 98 bp apart. The promoter activity of the DNA region containing the divergent promoters was analyzed by using transcriptional fusions to promoterless reporter genes and immunoblot assays. The results indicate that the ilvR gene product positively regulates the expression of the ilvD gene while negatively autoregulating its own expression. The ilvR gene codes for a protein of 296 amino acid residues (MW, 37,212). The N-terminal amino acid sequence of the IlvR protein contains a helix-turn-helix motif, suggesting that it is involved in protein-DNA interactions. Protein extracts from both wild-type and merodiploid strains showed specific DNA binding to a 227-bp DNA fragment spanning the ilvD-ilvR promoter region, while no protein-DNA complexes were observed in cell extracts from an ilvR mutant strain. Amino acid sequence comparison revealed that the IlvR protein is a member of the LysR family of transcriptional regulators.

Caulobacter crescentus is a dimorphic bacterium that generates two daughter cells as a consequence of differentiation during its normal cell cycle and subsequent cell division. To understand the mechanisms involved in differentiation, we have begun to compare the regulation of periodically expressed genes with that of genes expressed throughout the cell cycle. Isoleucine-valine-biosynthetic genes in C. crescentus have been mapped to two loci on the chromosome, designated the ilvA and ilvB regions (28). The ilvB region contains the ilvBN genes, and the ilvA region contains the ilvD gene. The ilvBN genes encode the large and small subunits of acetohydroxy acid synthase I, which seems to be the only acetohydroxy acid synthase in C. crescentus, while the ilvD gene encodes dihydroxy acid dehydrase (28). Expression of the ilvBN genes is regulated by attenuation (29), which functions in an analogous manner to the other systems controlled by this process (30). Expression of the ilvGMEDA operon in enteric bacteria is controlled primarily by attenuation in response to the intracellular levels of the cognate aminoacylated tRNAs (16). However, in C. crescentus the expression of the ilvD gene, which is a monocistronic transcriptional unit (17a), seems to be controlled by a different mechanism.

During the course of this study, we determined that the deduced amino acid sequence of an open reading frame upstream and divergent from the ilvD promoter, designated ilvR, exhibited significant identity to proteins of the LysR family of transcriptional regulatory proteins (12). In this report, we present evidence that the product of the ilvR regulatory locus positively regulates ilvD gene expression and autoregulates its own expression.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains, plasmids, and bacteriophages used in this study are listed in Table 1. The growth conditions, medium composition, and antibiotics used for Escherichia coli and C. crescentus cells have been described previously (7).

DNA manipulations. Plasmid DNA extraction, DNA digestion and ligation, and transformation of E. coli host cells were performed as described by Sambrook et al. (22). Plasmid DNAs were introduced into the C. crescentus strains either by conjugation (13) or by electroporation. For electroporation, a Gene Pulser device (Bio-Rad, Hercules, Calif.) was used at settings of 1,750 V, 200 μF, and 25 μF. Transformants were selected and screened on solid media supplemented with the appropriate antibiotics. DNA sequence analysis was carried out by the dideoxy-chain termination method of Sanger et al. (23), with Sequenase Version 2.0 (U.S. Biochemical Corp.). The complete nucleotide sequence of each strand was determined. Computer analyses of the DNA sequences were performed by the University of Wisconsin Genetics Computer Group package (6).

Construction of the ilvR mutant strain. Plasmids with the CoE1-type origin are not replicated in C. crescentus. Therefore, they are maintained in these cells only if the plasmid is integrated into the chromosome. The integration takes place via homologous recombination between cloned regions of chromosomal DNA and the chromosome. Plasmid pJM2-165, carrying the transcriptional fusion ilvR::cat, was introduced into SC3844 by electroporation. pJM2-165, a pUC18 derivative, contains a 5.2-kb fragment of C. crescentus DNA which includes the ilvD gene and an interrupted ilvR gene. The ilvR interruption was done by inserting a 0.8-kb promoterless cat gene in place of a 0.5-kb SalI fragment of the ilvR coding region. Thus, the insertion increases the size of the region by about 0.3 kb. The transformants were screened for occurrence of double-crossover recombination by selecting for CmR AmpR isolates. The double-crossover event resulted in interruption of the chromosomal copy of the ilvR gene. One such isolate was designated SC3950. A single-crossover recombination resulted in insertion of the entire plasmid into the target gene (ilvR), causing interruption of the chromosomal copy of ilvR and reconstruction of another wild-type copy of the target gene. This produced a merodiploid strain containing an ilvR::cat fusion (SC3951). The identities of the recombinations were confirmed by Southern blot analysis (26).

Chromosomal DNA isolation. Five-milliliter overnight cultures of C. crescentus were collected by centrifugation, washed in TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]),
suspended in 2 ml of TE, and treated with lysozyme (2 mg/ml) for 30 min at room temperature. RNase A (50 µg/ml) was added and incubated for 15 min at 42°C. Sodium dodecyl sulfate (SDS) (1%) and proteinase K (100 µg/ml) were added to the cell suspension, and the mixture was incubated at 55°C overnight. Chromosomal DNA was then prepared by phenol-chloroform extraction and ethanol precipitation.

**Gel mobility shift assay.** Cells from 10 ml of exponentially growing cultures were harvested, washed with 0.1 X TE buffer (1 mM Tris-HCl [pH 8.0], 0.1 mM EDTA [pH 8.0]), suspended in 1 ml of binding buffer (50 mM Tris-HCl, 75 mM KCl, 10% glycerol, 1 mM EDTA [pH 8.0]), and sonicated. The cell debris was collected by microcentrifuge, and the supernatant was stored at −70°C. The protein-binding mixture contained 1× binding buffer, 0.3 to 10 µg of total cell protein, 4 µg of sonicated salmon sperm DNA, 3 µg of bovine serum albumin, and 30,000 cpm of a 32P-labeled DNA fragment end labeled with polynucleotide kinase in a total volume of 20 µl. The mixture was incubated at room temperature for 20 min prior to electrophoresis. Gel electrophoresis was carried out in a 4% nondenaturing polyacrylamide gel at 4°C. Gels were then dried and visualized by autoradiography. Competition experiments with unlabelled specific DNA were performed to demonstrate the specificity of Ivr binding.

**Western blot (immunoblot) analysis.** A 200-µl aliquot of each culture was harvested during the mid-logarithmic phase of growth (10^8 cells per ml) and suspended in 2 x sample loading buffer (22). Following SDS-polyacrylamide gel electrophoresis (15), fractionated proteins were transferred to an Immobilon-P membrane (Millipore) with the Trans-Blot system (Bio-Rad Laboratories, Richmond, Calif.). BLOTTO (5% nonfat dry milk in phosphate-buffered saline) was used as a blocking agent. Rabbit anti-chloramphenical acetyltransferase (anti-CAT) or anti-neomycin phosphotransferase (anti-NptII) (5'-3' Inc.) was used as the primary antibody, and peroxidase-conjugated goat anti-rabbit immunoglobulin G was used as the secondary antibody. Staining was performed with H2O2 and 4-chloronaphthol in accordance with standard procedures (11).

**Nucleotide sequence accession number.** The DNA nucleotide sequence in Figure 2 has been deposited in GenBank under accession number L24392.

### RESULTS

**Localization of the ilvD gene and identification of a novel regulatory locus.** The ilvD gene was initially localized to a C. crescentus genomic clone (pJC200) by complementation of an ilvD mutant (28). When the 30-kbp chromosomal DNA insert in pJC200 was cleaved by the BamHI restriction enzyme, fragments were obtained that were approximately 60, 5.3, 5.2, 5.0, 4.0, 3.7, and 1.0 kb long. To localize the ilvD gene, BamHI-digested pJC200 was subjected to Southern blot hybridization with a 1.3-kb Smal-XhoI internal DNA fragment from the ilvD gene of E. coli (17) as the hybridization probe. This experiment revealed that the ilvD gene was located on the 5.2-kb BamHI fragment (data not shown). To define the ilvD

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**Table 1. Bacterial strains and plasmids used in this study**

<table>
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<th>Strain or plasmid</th>
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locus within this fragment, the 3.1- and 2.1-kb XhoI-BamHI subfragments were cloned in plasmid pJM2-33 (29) and introduced into the ilvD mutants SC3428, SC3429, SC3430, and SC3431 by electroporation and plasmid-containing strains were selected on minimal medium supplemented with gentamicin (25 mg/liter). Recombinant plasmid pJM2-34, carrying the 3.1-kb XhoI-BamHI fragment (Fig. 1), complemented the ilvD deficiency of the mutant strains. Moreover, transformants carrying plasmid pJM2-34 were chloramphenicol resistant. This appeared to be the consequence of readthrough transcription in the BamHI-to-XhoI orientation, resulting in expression of the promoterless cat gene of pJM2-33. However, insertion of a promoterless neo gene in the opposite orientation (Fig. 1, pJM2-171) also resulted in expression of the kanamycin resistance gene. This result suggested that two transcriptional units were transcribed in opposite orientations from promoters contained within the 1.2-kb XhoI-EcoRV DNA region. The locations of the two promoters were confirmed by nuclease S1 protection assays (data not shown).

The XhoI-EcoRV DNA fragment was cloned in pBluescript plasmids, and the DNA nucleotide sequence of this region was determined. DNA nucleotide sequence analysis revealed two potential start codons and two partial open reading frames (ORFs) in opposite orientations from each other. Comparison of these partial ORFs with the E. coli ilvD gene product (17) revealed that the polypeptide encoded by ORF\(_\text{L}\) was 87% identical to the E. coli IlvD amino acid sequence (data not shown). Therefore, ORF\(_\text{L}\) was designated the ilvD gene.

Homology of ORF\(_\text{R}\) with proteins of the LysR family of transcriptional regulators. To identify the role of ORF\(_\text{R}\), the entire nucleotide sequence of ORF\(_\text{R}\) was sequenced across the region beyond the XhoI restriction site (Fig. 2). The ORF\(_\text{R}\) gene is 891 nucleotides long and is preceded by a DNA region, AGA, which has homology to the consensus ribosome-binding site (8, 25). The deduced amino acid sequence of ORF\(_\text{R}\) was 296 residues, which would have a calculated molecular mass of 37,212 Da. The codon usage of ORF\(_\text{R}\) was characteristic of other C. crescentus genes (24). The Swiss-Prot data bank was searched to find homology to the deduced amino acid sequence of ORF\(_\text{R}\). We found significant identities to a large number of bacterial proteins known as LysR-type transcriptional regulators, including Pseudomonas putida CatR (35% identity) (21), Alcaligenes eutrophus TfdS (33% identity) (14), Pseudomonas sp. strain p51 TcbR (31% identity) (32), E. coli OxyR (28% identity) (4), E. coli YfeB (28% identity) (2), E. coli IlvY (25% identity) (35, 36), and Bacillus subtilis Gtc (25% identity) (1). The homology was more pronounced at the N terminus, which contains a helix-turn-helix motif. This was expected, since most bacterial transcriptional regulatory proteins interact with their target sequences through a helix-turn-helix structural motif (12, 19).

FIG. 1. Schematic diagrams of the DNA region containing the ilvD and ilvR genes and plasmid derivatives of this region. The slashed dashed lines represent replacement of the internal EcoRV fragment of ilvD and the internal SalI fragment of ilvR with the promoterless neo and cat genes, respectively. Only the relevant restriction enzyme sites are indicated. Restriction enzyme site abbreviations: B, BamHI; S, SalI; L, SalI; X, XhoI; D, DdeI; N, NcoI; V, EcoRV; R, EcoRI. The symbol Xxxxx indicates the fragment used as a hybridization probe in Southern blot hybridization and gel mobility shift assays. P\(_\text{D}\) and P\(_\text{R}\) indicate the promoter regions of the ilvD and ilvR genes, respectively. Arrows indicate direction of transcription.

FIG. 2. Nucleotide and deduced amino acid sequences of the ilvR gene. The DNA nucleotides at bp 1 to 253 on the complementary strand correspond to the 5' end of the ilvD gene. The start codons of the ilvD gene at bp 253 on the bottom strand and of the ilvR gene at bp 352 are indicated. The potential ribosome-binding sites (RBS) are shown. The asterisk indicates the ilvR stop codon. The region corresponding to the helix-turn-helix motif is double underlined. Inverted repeats of a potential binding site for the IlvR protein spanning the LysR motif, T-N\(_\text{Nt}X\)-A, on the complementary strand are shown by solid lines.
Extracts and those media, the protein the mutation had for the product medium. Shift yeast (peptone (ilvR mutant) to extracts protein-DNA complex and 2 in three of formation apparent 2 3). Slowly Binding extracts of the SC3844 the chromosome contained the ilvR locus. To investigate the function of the IlvR protein, we inactivated the IlvR protein by replacing the ilvR gene on the chromosome with an in vitro-mutated ilvR gene that contained an insertion of the promoterless cat reporter gene. Interestingly, the presence of the IlvR null mutation had no effect on the growth of the bacteria in rich (peptone yeast extract) or defined (minimal salts-glucose) medium. Therefore, the ilvR gene seems to be a nonessential gene for the growth conditions examined.

**Binding of the IlvR protein to DNA containing the ilvR-ilvD intercistronic region.** To determine whether the ilvR gene product binds to the ilvR-ilvD intercistronic region, a gel mobility shift assay was utilized. Crude cell extracts from SC3844 (ilvR\(^{+}\)), SC3951 (a merodiploid ilvR\(^{+}\) strain), and SC3950 (ilvR mutant) were tested for the ability to bind to an end-labeled 227-bp NcoI-DdeI fragment that contained the ilvR and ilvD control regions (Fig. 1). The results of IlvR binding to the labeled DNA are shown in Fig. 3. When cell extracts from SC3844 and SC3951 were used, DNA was found in three positions: the unbound DNA and two more slowly migrating protein-DNA complexes, C1 and C2 (Fig. 3, lanes 1 and 2 of the SC3844 and SC3951 samples). There was no protein-DNA complex formation in the ilvR mutant cell extract (Fig. 3). The growth media used for these bacterial strains had no apparent effect on protein-DNA binding (Fig. 3). Use of increasing concentrations of cell extract resulted in initial formation of a protein-DNA complex at position C1 (Fig. 4B, lanes 2 to 4). At higher cell extract concentrations, the more slowly migrating band, C2, was formed (Fig. 4B, lanes 5 to 8).

The intensity of band C2 increased drastically at higher protein concentrations. Therefore, the C1 and C2 complexes may be the result of IlvR interactions with two different sites on the DNA. Alternatively, they may represent a single site that is partially or fully saturated by the IlvR protein, respectively. Although the biochemical mechanism that underlies gene expression by LysR-type proteins is not fully understood, activation of gene expression is mediated through binding of the regulatory proteins to the promoter regions of the genes they regulate (9, 10, 21, 32). The DNA nucleotide sequence between the translation initiation codons of the ilvD and ilvR genes contains a T-N11-A LysR motif (Fig. 4A). This cis-acting sequence motif is implicated in binding to LysR-type regulatory proteins (10) and is a candidate for IlvR binding. An AseI restriction enzyme site (ATTAAT) is contained in the LysR motif of the Neot-DdeI DNA region (Fig. 4A). Therefore, two subfragments were generated by digestion of the 227-bp NcoI-DdeI DNA fragment with restriction enzyme AseI and tested for the ability to form DNA-protein complexes. Neither subfragment was capable of forming IlvR-specific DNA-protein complexes (data not shown), a finding consistent with the notion that the T-N11-A motif is involved in IlvR binding.

**Construction and analysis of chromosomal ilvD::neo and ilvR::cat transcriptional fusions.** To investigate the regulation of the ilvD and ilvR promoters, chromosomal ilvD::neo and ilvR::cat transcriptional fusions were constructed by introducing plasmid pJM2-174 into wild-type _C. crescentus_. Plasmid pJM2-174 carries a double gene fusion in which the promoterless neo and cat genes are inserted into the ilvD and ilvR genes, respectively (Fig. 1 and 5). Expression of these reporter genes allowed us to monitor ilvD and ilvR gene expression by measuring the levels of NptII expression and CAT expression, respectively. Since pJM2-174 contains three regions of homology with the chromosome, multiple integration pathways may occur. As shown in Fig. 5, the insertion may take place by a
single Campbell-like insertion in region 1, 2, or 3 and double crossovers may occur either between homologous regions 1 and 2, 1 and 3, or 2 and 3. In some isolates, a Campbell-like insertion resulted in integration of the entire plasmid into the chromosome at position 3, creating merodiploid strain SC3967. Thus, SC3967 contained a duplication of the region between the two BamHI sites. One copy of the region contained the wild-type ilvD-ilvR genes located at their normal positions on the chromosome, and the second copy contained the neo and cat fusions (Fig. 5). Recombination by double crossovers resulted in replacement of the ilvD and/or ilvR genes by in vitro-engineered transcriptional fusions, and the following strains were constructed: SC3964, ilvD::neo ilvR::cat; SC3965, ilvD + ilvR::cat; SC3966, ilvD::neo ilvR +. The predicted phenotypes of the resultant mutants with regard to antibiotic resistance and auxotrophy were tested on appropriate media, and integrations at the desired regions were confirmed by Southern hybridizations (Fig. 6). When digested with restriction enzyme Smal, the wild-type DNA exhibited only one band (1.4 kb) that hybridized with the 227-bp NcoI-DdeI probe (Fig. 6, lane 1). When the cat gene was inserted in place of an internal SalI fragment in the ilvR gene, Smal digestion resulted in a 1.6-kb band instead of the 1.4-kb fragment. SC3966, which is a representative of crossovers in regions 1 and 2, contained only the 1.4-kb band (Fig. 6, lane 4). To distinguish between strains SC3964 and SC3965, which both generated a 1.6-kb Smal fragment (Fig. 6, lanes 2 and 3 and 6 and 7), Southern analysis was performed with EcoRI-digested DNAs and the same NcoI-DdeI probe. The results of this experiment indicated that SC3964 resulted from crossovers in regions 1 and 3 and SC3965 resulted from crossovers in regions 2 and 3 (data not shown). When the same membranes were hybridized with a pUC18 DNA probe, only SC3967 DNA hybridized to the probe (data not shown), indicating that SC3967 was the only strain with the entire plasmid inserted in the chromosome. As shown in Fig. 6, lanes 5, 8, and 9, Smal digestion of SC3967 DNA yielded both the wild-type (1.4-kb) and mutated (1.6-kb) fragments.

The proteins synthesized by these recombinant strains were subjected to SDS-polyacrylamide gel electrophoresis and Western blot analysis. Expression of ilvD- and ilvR-derived neo

FIG. 6. Southern hybridization. Chromosomal DNAs of ilvD::neo and ilvR::cat insertion mutants were digested with Smal. An end-labeled 227-bp NcoI-DdeI fragment was used as the hybridization probe (Fig. 5). Lanes: 1, CB15 (wild type); 2, 3, and 6, SC3964; 4, SC3966; 7, SC3965; 5, 8, and 9, SC3967 (the repeated strains are independent isolates of the same recombination event). The molecular sizes of fragments are shown on the right.
and cat genes was detected by using anti-NptII and anti-CAT antibodies (Fig. 7). As shown in Fig. 7A, ilvD-derived NptII exhibited residual expression in the absence of IlvR (lane 2), which was increased about 10-fold in the presence of IlvR (strain SC3966, lane 3). Residual expression of the ilvD gene in the absence of IlvR was also demonstrated by growth of SC3950 (ilvD+ ilvR::cat) in minimal media (see above). In contrast, cells carrying an ilvR mutation had increased levels of ilvR::cat expression, as reflected by high levels of CAT expression compared with the same strain containing an IlvR+ plasmid (SC3968) (Fig. 7B, lanes 2 and 3). It is possible that a gene downstream of ilvR is inactivated by a polar insertion in the ilvR gene. However, the simplest interpretation of these results is that the ilvD gene is activated in the presence of a bound IlvR protein, while expression of the ilvR gene is repressed in the presence of its own protein product.

**DISCUSSION**

We have identified a regulatory gene, ilvR, upstream and divergent from the ilvD gene in *C. crescentus*. Our studies led us to conclude that this gene encodes a regulatory factor that probably affects ilvD expression. The deduced amino acid sequence of the ilvR gene product shows that it is a member of a group of procaryotic activator proteins, designated the LysR family. A number of features are shared by all of the LysR-type proteins, and IlvR conforms to all of these features examined to date. (i) They contain a helix-turn-helix motif at the N terminus believed to be involved in DNA-protein interactions (12). (ii) They often regulate the expression of an overlapping promoter on the complementary strand of the DNA (3, 12, 31, 36). (iii) They are often subject to negative autoregulation. (iv) In most cases, the DNA-binding site contains small inverted repeat sequences spanning an invariant T-N1-A motif (9, 10, 31, 36). (v) They are mostly activator proteins (27, 32); however, a few have been shown to act as repressors (18). (vi) They are about 300 amino acid residues long. (vii) They often require the presence of a small inducer molecule for transcripational activation (not identified for IlvR activation). (viii) Almost all members of this family show reduced Lys content and increased Arg content compared with the other procaryotic proteins (33).

The ilvR gene was mutagenized by an in vivo marker replacement method and found to be nonessential. However, expression of the ilvD::neo transcriptional fusion suggested that the IlvR protein stimulates or enhances expression of the ilvD gene. Analogous to the other systems, the ability of the IlvR protein to activate expression of the ilvD gene may depend on the binding of this protein to a specific binding site(s) in the regulatory region. We have shown that a 227-bp DNA fragment encompassing both promoters of the ilvD and ilvR genes specifically interacts with a DNA-binding factor present in the cell extracts from wild-type *C. crescentus*. This binding factor is absent in an ilvR mutant extract.

In *E. coli*, the ilvD gene is contained within the major operon ilvGMEAD, which, together with the downstream ilvY-ilvC genes, codes for all of the enzymes needed for isoleucine synthesis. In *C. crescentus*, however, the ilvD gene is a monocistronic operon. Therefore, the structural organization and also the mode of regulation of ilvD gene expression are different from those of the *E. coli* counterpart. However, the physical organization and the mechanism of gene expression of *C. crescentus* ilvD-ilvR genes are similar to those of the *E. coli* ilvC-ilvY genes. In *E. coli*, the ilvC gene is positively regulated by the product of the ilvY gene (36), which is also a member of the LysR family and has limited homology to the *C. crescentus* ilvR gene. Wek and Hatfield (36) have shown that in the presence of the isomeroreductase substrates, acetohydroxybutyrate, or acetalactate, the ilvY-encoded activator protein binds to the upstream and divergently transcribed ilvC promoter and activates transcription from this promoter. Interestingly, IlvY also interacts with two adjacent operator sites on the promoter region. However, in the presence of the substrate acetohydroxybutyrate and higher concentrations of IlvY, only one DNA-protein complex is formed. By analogy, one or both of the dihydroxy acids may play a similar role in an inducer of *C. crescentus* ilvD expression. The ilvC gene of *C. crescentus* has not been identified (28).

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