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Mutational Analysis of the *cbb* Operon (CO_2 Assimilation) Promoter of *Ralstonia eutropha*

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P_L promoters direct the transcription of the duplicated *cbb* operons from the facultative chemoautotroph *Ralstonia eutropha* H16. The operons encode most enzymes of the Calvin-Benson-Bassham carbon reduction cycle required for CO_2 assimilation. Their transcription depends on the activator protein CbbR. Structure-function relationships in the cloned chromosomal promoter region were analyzed by site-directed mutagenesis. P_L was altered in its presumed hexameric −35 and/or −10 box or in the spacer region between the boxes to achieve a greater or lesser resemblance to the structure of the σ^{70} consensus promoter of *Escherichia coli*. P_L::*lacZ* transcriptional fusions of various promoter variants were assayed in transconjugant strains of *R. eutropha* as well as in corresponding *cbbR* deletion mutants. Mutations increasing the similarity of the −35 and/or −10 box to the consensus sequence stimulated P_L activity to various extents, whereas mutations deviating from the consensus decreased the activity. The length of the spacer region also proved to be critical. The conversion of the boxes, either individually or simultaneously, into the consensus sequences resulted in a highly active P_L. All improved P_L mutants, however, retained the activation under inducing or derepressing growth conditions, although the full-consensus promoter was nearly constitutive. They were also activated in the *cbbR* mutants. The activity of the overlapping, divergently oriented *cbbR* promoter was less affected by the mutations. The half- and full-consensus P_L mutants were comparably active in *E. coli*. Two major conclusions were drawn from the results: (i) the location and function of P_L were verified, and (ii) indirect evidence was obtained for the involvement of another regulator(s), besides CbbR, in the transcriptional control of the *R. eutropha cbb* operons.

Autotrophy denotes the ability of an organism to gain the majority of its cell carbon by the assimilation of CO_2. The Calvin-Benson-Bassham carbon reduction cycle (2) is the quantitatively predominating route of CO_2 fixation among autotrophs (28). *Ralstonia eutropha* (*Alcaligenes eutrophus*) is an aerobic, facultatively chemoautotrophic β-proteobacterium, which fixes CO_2 via the Calvin cycle, by using either hydrogen or formate as an energy source for litho- or organoautotrophic growth, respectively (5). Most genes of Calvin cycle enzymes in *R. eutropha*, including those of ribulose-1,5-bisphosphate carboxylase oxygenase (RubisCO)—the actual CO_2-fixing enzyme of the cycle—are encoded within a chromosomal operon (cbb_c operon) of about 15 kbp. Strain H16 harbors a second, highly homologous, and identically organized copy of the operon (cbb_p operon) located on the megaplasmid pHG1 (20). The cbb_p operon lacks, however, the 3′-terminal $cbbB_c$ gene present in the cbb_c operon (4), whereas the large subunit gene *cbbL* of RubisCO forms the 5′ ends of both operons. Although the organizations and sizes of *cbb* operons vary considerably among autotrophic bacteria, they also show some common features (11, 20, 33).

The cbb_c operon of *R. eutropha* H16 is separated by 167 bp from the divergently oriented regulatory gene *cbbR*, whose product is required for transcription activation of the duplicate *cbb* operons. CbbR belongs to the LysR family of transcriptional regulator proteins (32). A deficient *cbbR* gene (*cbbR′*) is situated exactly in the position corresponding to the cbb_p operon on pHG1 (37). The intergenic segments between *cbbR* or *cbbR′* and $cbbL_c$ or $cbbL_p$ constitute the control regions of the operons, as they contain the operators and promoters of the system (19, 21). CbbR has been shown to bind preferentially between positions −29 and −74 relative to the transcriptional start point of the cbb_c operon (19). Consistent with metabolic economy, the heterotrophic growth of the organism on most organic substrates completely represses the transcription of the *cbb* operons, to avoid wasting of energy that would be caused by CO_2 fixation under these conditions. Partial derepression occurs during growth on a few substrates, such as fructose and gluconate, but only autotrophic growth results in the full derepression or induction of the operons (6, 10, 21). The transcriptional control is postulated to involve the transduction of a still unknown metabolic signal sensed by CbbR, which modulates the activity of the cbb_c and cbb_p operon promoter P_L (20). No significant subpromoter activity was detected within the operons (31). Structural motifs tentatively assigned to P_L resemble those of σ^{70}-dependent promoters of *Escherichia coli* (12), and this applies also, although in a less pronounced way, to the presumed *cbbR* and *cbbR′* promoter P_R. The divergent promoters P_L and P_R are arranged in a back-to-back configuration, according to Beck and Warren (3), with overlapping −35 boxes (21).

As a prerequisite to understanding the regulatory mechanism acting upon the *cbb* operons of *R. eutropha* H16, critical structural properties of P_L must be known. Therefore, a mutational analysis of chromosomal P_L was performed that aimed at defining sequence elements important for the promoter activity. The activities of altered P_L promoters were determined not only in the homologous host *R. eutropha* but also in *E. coli* and in newly constructed *cbbR* deletion mutants of *R.*

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TABLE 1. List of bacterial strains and plasmids

Strain or plasmid	Relevant characteristic(s)[a]	Reference or source
R. eutropha		
H16	Cfx Hox Fox, pHG1; wild type	ATCC 17699
HF210	Sm^r Cfx Hox^- Fox, $pHG1^-$; derivative of strain HF39, a Sm^r mutant of strain H16	17
HB14	Cfx^- Hox Fox, pHG1; *cbbR*Δ; derivative of strain H16	This study
HB15	Sm^r Cfx^- Hox^- Fox, $pHG1^-$; *cbbR*Δ; derivative of strain HF210	This study
E. coli		
XL1-Blue	Tc^r; *recA1 endA1 gyrA96 thi hsdR17 supE44 relA1 lac*(F′[*proAB lacI*^{q} *lacZ*Δ*M15* Tn*10*])	8
JW1	*ara* Δ(*lac-proAB*) *rpsL thi* Φ80(*lacZ*Δ*M15*) F′(*lacI*^{q} *lacZ*Δ*M15 proAB*$^+$)	16
S17-1	Sm^r Tp^r *mod*$^+$ *res thi pro recA*; integrated RP4 (Tc::Mu-Km::Tn*7*)	34
Plasmids		
pUC18/19	Ap^r *lacPOZ′*	38
pBluescript KS	Ap^r *lacPOZ′*	Stratagene
pBBR1MCS-3	Tc^r *lacPOZ′*	18
pBH2241[b]	pUC19::224-bp *Bgl*II-*Hin*fI fragment containing the intergenic region between *cbbR* and $cbbL_c$	21
pAEC1200	pUC18::1.8-kb *Hin*dIII-*Sal*I fragment containing *cbbR*	This study
pAEC1200Δ	pUC18::0.9-kb *Hin*dIII-*Sal*I fragment, derivative of pAEC1200 with deleted 0.9-kb *Bgl*II-*Sma*I segment in *cbbR*	This study
pLO1	Km^r; *sacB* RP4-*oriT* ColE1-*oriT*	24
pNHG1	Km^r Tc^r; pLO1::1.48-kb *Dde*I-*Vsp*I fragment from pBBR1MCS-3	This study
pNHG110	pNHG1::0.9-kb *Hin*dIII-*Sal*I fragment from pAEC1200Δ	This study
pUW7	Tc^r; pVK101::1,031-bp *Dde*I fragment containing *cbbR*	37
pBK	Tc^r; double operon fusion plasmid with divergently oriented *lacZ* (β-galactosidase) and *gusA* (β-glucuronidase) as reporter genes	21
pBK2241[c]	pBK::236-bp *Xba*I-*Pst*I fragment from pBH2241	21
pBKM1 through pBKM14	pBK::236-bp *Xba*I-*Pst*I fragment with different mutations in P_L (Table 2)	This study

[a] Ap^r, ampicillin resistance; Sm^r, streptomycin resistance; Tc^r, tetracycline resistance; Tp^r, trimethoprim resistance; Cfx, ability for autotrophic CO_2 fixation; Hox, ability for H_2 oxidation; Fox, ability for formate oxidation; pHG1, megaplasmid.
[b] In pBH2241 cbb_c operon promoter P_L is oriented divergently, relative to *lacZ*.
[c] In pBK2241 and pBKM1 through pBKM14, P_L is oriented colinearly, relative to *lacZ*.

eutropha. The results verified the location and function of the promoter and provided a first hint that, in addition to CbbR, another regulator(s) might participate in the transcriptional control of the *cbb* operons in *R. eutropha*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains and plasmids used in this study are shown in Table 1. Strains of *R. eutropha* were grown in a nutrient broth or mineral medium (MM) at 30°C as described previously (36). MM was routinely supplemented with 0.2% (wt/vol) organic substrate for either heterotrophic or organoautotrophic growth on formate. Mixotrophic cultures were initially grown on 0.1% (wt/vol) fructose until reaching an optical density at 436 nm of 1 to 2, before 0.2% (wt/vol) formate was added and the incubation was continued for another 4 h. Lithoautotrophic cultures were gassed with a mixture of H_2, CO_2, and O_2 (8:1:1 [vol/vol/vol]). *E. coli* was propagated in Luria-Bertani medium at 37 or 30°C. If required, the media contained ampicillin (50 μg/ml), kanamycin (50 μg/ml for *E. coli*, 450 μg/ml in MM for *R. eutropha*, or 120 μg/ml in nutrient broth), or tetracycline (15 μg/ml for *E. coli* or 20 μg/ml for *R. eutropha*) as selective antibiotics.

Manipulation and sequencing of DNA. Standard procedures (1, 29) were employed to isolate genomic and plasmid DNA from bacteria, to transform plasmid DNA into *E. coli*, and for general DNA handling. Restriction endonucleases and DNA-modifying enzymes were used under the reaction conditions recommended by the manufacturers. DNA probes to be applied in Southern hybridizations were nonradioactively labelled with peroxidase by means of a special reaction system (ECL direct labelling and detection kit; Amersham, Brunswick, Germany). For the hybridizations, DNA fragments were separated by agarose gel electrophoresis and transferred onto a nylon membrane (Biodyne B; Pall, Dreieich, Germany) by vacuum blotting. DNA sequences were determined by the dideoxy chain termination method (30) and PCR cycle sequencing (SequiTherm cycle sequencing kit; Biozym, Hessisch Oldendorf, Germany) with ^{35}S- or fluorescence-labelled oligonucleotide primers. Plasmids were conjugally transferred from *E. coli* S17-1 to strains of *R. eutropha* by biparental mating (35).

Generation of mutations in P_L and construction of transcriptional fusions. Oligonucleotide-directed mutagenesis carried out in a two-stage PCR (9) was employed to introduce mutations into the cbb_c operon promoter P_L. The specifically designed oligonucleotides M1 through M14 (24-mers; not shown) contained the desired mutations close to their centers. Together with a reverse primer (24-mer) they served individually to perform the first amplification (30 cycles at 95°C for 30 s, 59°C for 30 s, and 72°C for 60 s) of a segment (121 to 147 bp) of the 224-bp *Bgl*II-*Hin*fI DNA fragment inserted in pBH2241. The reaction mixtures (100 μl) included 200 ng of pBH2241 as a template, a 2 μM concentration of mutagenesis primer, a 2 μM concentration of reverse primer, 2 mM concentrations of dATP, dGTP, dCTP, and dTTP, 25 mM $MgCl_2$, and 2 U of *Tfl* DNA polymerase in a buffer system formulated by the supplier of the polymerase (Biozym) and were placed in a thermal cycler (model PTC-100; MJ Research, Watertown, Mass.). The mutagenized PCR product was purified by electrophoresis in low-melting-point agarose. It was used as the primer in a second PCR (30 cycles at 95°C for 30 s, 50°C for 60 s, and 72°C for 60 s) together with a universal primer (24-mer) to accomplish the amplification of the complete *Bgl*II-*Hin*fI fragment. If insufficient amounts of the product (330 bp) were obtained in the second PCR, it was reamplified in a third PCR (the same conditions as in the first PCR) by means of the universal and reverse primers. The product of the final PCR was digested with either *Eco*RI and *Hin*dIII or *Xba*I and *Pst*I prior to being cloned into correspondingly cleaved pUC19 or pBluescript KS, respectively. DNA sequencing of the respective plasmids pM1 through pM14 (not listed) confirmed the specific mutations. Finally, the inserts of these plasmids were excised with *Xba*I and *Pst*I and recloned into correspondingly digested pBK. The resulting plasmids, pBKM1 through pBKM14, carried the cbb_c::*lacZ* transcriptional fusions with differently modified P_L promoters.

Construction of suicide plasmid pNHG1. The suicide vector pLO1 (7,322 bp [23]) has been designed as a tool for gene replacement mutagenesis in gram-negative bacteria, especially in *R. eutropha* (24). However, because of the inherently low sensitivities of *R. eutropha* H16 and particularly of strain HF210 to kanamycin, an additional, easily selectable marker in these strains was incorporated into pLO1. For this purpose, the tetracycline resistance gene *tet* was excised from pBBR1MCS-3 as a 1,484-bp *Dde*I-*Vsp*I DNA fragment. Plasmid pLO1 was partially digested with *Bam*HI, and the recessed 3′ ends of the linearized plasmid and of the *Dde*I-*Vsp*I fragment were filled in by treatment with the Klenow fragment of DNA polymerase I. The ligation of the two DNA fragments resulted eventually in the isolation of the new suicide vector pNHG1 (Fig. 1).

Construction of *cbbR* deletion mutants. To construct isogenic *cbbR* deletion mutants of *R. eutropha* H16 and HF210, a 1.8-kb *Hin*dIII-*Sal*I fragment containing *cbbR* was first cloned into pUC18, yielding pAEC1200. The plasmid was

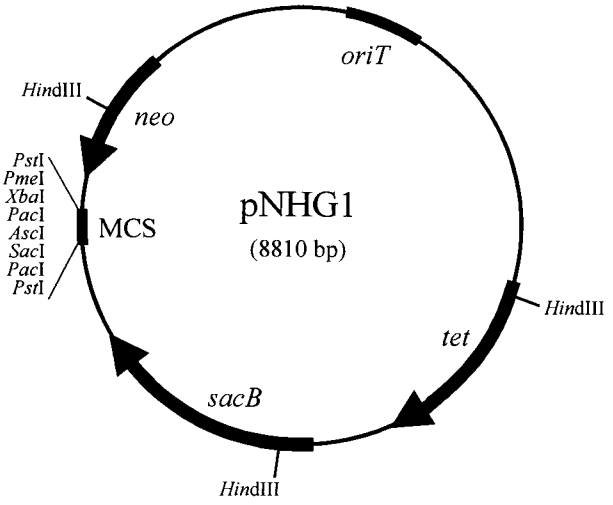

FIG. 1. Map of suicide vector pNHG1. The plasmid encompasses 8,810 bp and was constructed by inserting a fragment carrying the tetracycline resistance gene *tet* from pBBR1MCS-3 into pLO1. Only unique cleavage sites of restriction endonucleases are indicated within the multiple cloning site (MCS). *neo*, the gene encoding kanamycin resistance; *oriT*, the origin of transfer replication; *sacB*, the gene encoding levansucrase.

linearized with *Bgl*II and further digested with *Hin*dIII after filling in the recessed 3′ ends of the *Bgl*II site by treatment with Klenow fragment. In a separate reaction pAEC1200 was cut with *Hin*dIII and *Sma*I. The resulting 3.2-kb *Hin*dIII-*Bgl*II and 0.4-kb *Hin*dIII-*Sma*I fragments, respectively, were ligated to produce pAEC1200Δ, which lacked the internal 891 bp of *cbbR* (total length of the gene, 954 bp). To reclone the 0.9-kb *Hin*dIII-*Sal*I fragment, pAEC1200Δ was digested with *Hin*dIII, treated with Klenow fragment to fill in the 3′ ends of the site, and cleaved with *Xba*I. The 0.9-kb fragment carrying *cbbR*Δ was finally ligated to pNHG1 digested with *Xba*I and *Pme*I, generating pNHG110.

An allelic exchange of *cbbR*Δ for *cbbR* was achieved by two consecutively selected recombination events. Single recombinants (heterogenotes) of H16 and HF210 were characterized by simultaneously acquired tetracycline and kanamycin resistance after the conjugal transfer of pNHG1 from *E. coli* S17-1. Double recombinants (homogenotes), which gained sucrose resistance and concomitantly lost both antibiotic resistances, were obtained from heterogenotes as described by Lenz et al. (24).

Enzyme assays. The activity of RubisCO in *R. eutropha* was determined in a radiometric, whole-cell assay based on the fixation of $^{14}CO_2$ as described previously (22). A colorimetric assay was used for β-galactosidase (25), as well as a fluorimetric assay for β-glucuronidase (14), employing crude cell extracts prepared from *R. eutropha* or *E. coli*. One unit of activity represents the amount of enzyme catalyzing the formation of 1 μmol of product per min. Cultures of the strains were grown to an optical density at 436 nm of 2 to 3. The cells were harvested, resuspended in the appropriate buffer, and disrupted by sonication. Cell extracts were obtained after centrifugation at 14,000 × *g* for 20 min to remove unbroken cells and cell debris. Protein concentrations in the extracts were estimated by the method of Bradford (7).

RESULTS

Mutational modification of P_L. The proposed σ^{70}-type P_L promoters of the two *cbb* operons of *R. eutropha* H16 are believed to have the following structure on the nontemplate DNA strand: [−35] TTTACC-N_{17}-TATACC [−10] (Table 2). The resemblance of P_L to the canonical σ^{70}-dependent promoter of *E. coli* (12) is evident for the hexameric −35 and −10 boxes as well as the length (17 bp) of the spacer region. In order to study the functional significance of the P_L substructures, various mutations were introduced into the chromosomal wild-type promoter cloned within plasmid pBH2241. Specifically designed oligonucleotides were used to direct the PCR-based mutageneses. Fourteen different mutant P_L promoters were produced that carried single or multiple sequence alterations (Table 2). The −10 and −35 boxes of mutants M1 and M6, respectively, matched the corresponding elements of the *E. coli* consensus promoter. Mutant M9, which was generated from M1, contains the consensus sequence in both regions. The spacer mutants included clones with either a single nucleotide deletion (M12), an insertion (M13), or a double substitution (M14). P_L::*lacZ* transcriptional fusions were constructed for each mutant (pM1 through pM14) to enable the subsequent determination of the different promoter activities in *R. eutropha* as well as in *E. coli*.

Isolation and phenotype of *cbbR* deletion mutants. Because the *cbb* promoter activities were intended to be determined in both the wild-type and a CbbR-free background, *cbbR* null mutants of *R. eutropha* wild-type H16 and pHG1-cured HF210 were constructed by gene replacement mutagenesis. The derived mutants, HB14 and HB15, respectively, carried an 891-bp in-frame deletion within *cbbR*, as verified by the sequencing of corresponding PCR-generated fragments and by Southern hy-

TABLE 2. Sequences of mutant P_L promoters of the cbb_c operon from *R. eutropha* H16

Promoter	DNA sequence of P_L[a] −35 —spacer region— −10
Wild type	*cbbR*-TTTACCTTATGTGGGTGGGCTTATATCTT-$cbbL_c$
M1	TTTACC -N_{17}- TAT**AA**T[b]
M2	TTTACC -N_{17}- TATC**A**T
M3	TTTACC -N_{17}- TAT**A**TT
M4	TTTACC -N_{17}- **CC**TCTT
M5	TT**G**ACC -N_{17}- TATCTT
M6	TT**G**AC**A** -N_{17}- TATCTT
M7	TTTAC**A** -N_{17}- TATCTT
M8	**CC**TACC -N_{17}- TATCTT
M9	TT**G**AC**A** -N_{17}- TAT**AA**T
M10	**C**T**G**AC**A** -N_{17}- TAT**AA**T
M11	TTTAC**A** -N_{17}- TA**CC**TT
M12	TTTACCTTATGTGGG-GGGCTTATATCTT
M13	TTTACCTTATGTGGGT**G**GGGCTTATATCTT
M14	TTTACCTTAT**CA**GGGTGGGCTTATATCTT
σ^{70}-dependent consensus of *E. coli*	TTGACA -N_{17}- TATAAT

[a] The −35 and −10 boxes of wild-type P_L are boxed; N_{17} indicates an unmutated spacer region in P_L and an unspecified spacer sequence in the *E. coli* consensus promoter.

[b] Mutated, deleted (M12), and inserted (M13) bases are in boldface type.

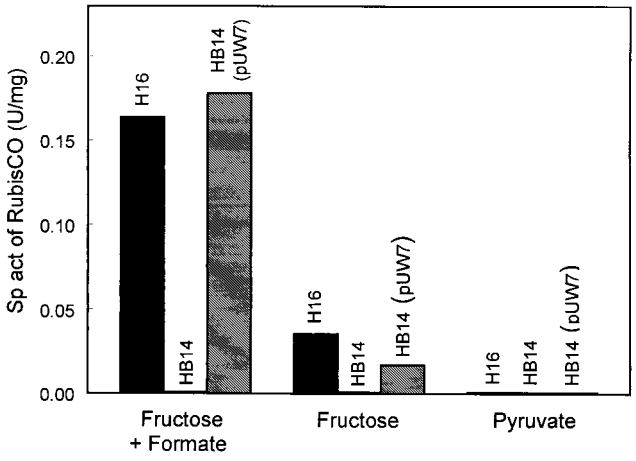

FIG. 2. Activities of RubisCO in *R. eutropha* H16, HB14, and HB14(pUW7) grown on fructose plus formate, fructose, or pyruvate. The enzyme activity was determined by means of a whole-cell assay. Sp act, specific activity.

bridizations (data not shown). The potential product of *cbbR*Δ is a small protein of 20 amino acid residues that lacks 297 residues, including the helix-turn-helix structural motif in the NH_2-terminal domain, of the authentic CbbR. A *cbbR*Δ-derived product with binding capacity to the *cbb* operator region could therefore not be formed in the mutants. Both strains concurrently lost the ability to grow autotrophically and to derepress or induce the *cbb* operons. They regained these properties by in *trans* complementation with *cbbR* present on plasmid pUW7. The transconjugant HB14(pUW7) showed RubisCO activities similar to that of wild-type H16 after mixotrophic growth on fructose plus formate or heterotrophic growth on fructose, whereas no activity was detected in cells grown on pyruvate (Fig. 2). These data corroborated the function of CbbR as an activator of the *cbb* operons and the suitability of the mutants for the subsequent promoter assays.

Activities of mutant P_L in *R. eutropha*. The β-galactosidase reporter activities originating from the various mutant P_L::*lacZ* fusions were first determined in transconjugants of wild-type *R. eutropha* H16 grown under lithoautotrophic (H_2-CO_2) or heterotrophic (fructose or pyruvate) conditions. Confidence in the significance of the individual data on promoter activities was ensured by obtaining them from at least three independent cultures. Promoters with a closer match of the −10 (M1, M2, and M3) or −35 (M5, M6, and M7) box of P_L to those of the σ^{70} consensus promoter of *E. coli* showed increased activity relative to that of the wild-type promoter present in pBK2241 (Table 3). The increase depended on the position and number of base substitutions but occurred independently of the growth substrate. This was particularly evident for mutants M1 (−10 consensus), M6 (−35 consensus), and M9 (full consensus), whose activities in autotrophic cells reached a level about twofold that of the wild type. More significant, however, was the drastic increase in heterotrophic cells, even after growth on the normally strongly repressing pyruvate. The basal activity of P_L M9 in pyruvate-grown cells was actually higher than that of the wild-type promoter under autotrophic conditions. At least M9 can thus be considered to be nearly constitutive. Nevertheless, all improved variants of P_L still exhibited the principal regulatory pattern of the wild-type promoter characterized by derepression or induction in autotrophically or fructose-grown cells. Diminishing the resemblance of either the −10 (M4) or −35 (M8) box to the consensus by replacing the conserved distal bases TA or TT with CC resulted in a nearly total loss of promoter activity. The substitution of the distal T of the −35 box in the full-consensus M9 converted the constitutive into a repressible promoter (M10). Replacing one consensus base in the −10 box of wild-type P_L and simultaneously introducing a 1-base match in the −35 box gave a promoter (M11) which was slightly more active than the wild type and also retained the activation. In contrast, the deletion of one base within the spacer region ($N_{17}\rightarrow N_{16}$) largely inactivated P_L (M12), whereas the insertion of one base ($N_{17}\rightarrow N_{18}$) caused only a partial loss of activity (M13). An exchange of two adjacent bases (GT→TA) within the spacer had no significant effect on the promoter (M14).

TABLE 3. Activities of mutant P_L promoters determined by P_L::*lacZ* transcriptional fusions in transconjugants of *R. eutropha* H16

Transconjugant	Sp act of β-galactosidase (mU/mg of protein)[a] grown on:		
	H_2-CO_2	Fructose	Pyruvate
pBK[b]	4	3	3
pBK2241[c]	930	100	6
pBKM1[d]	1,590	1,200	90
pBKM2	1,120	560	16
pBKM3	930	360	8
pBKM4	5	5	6
pBKM5	1,770	300	16
pBKM6	1,940	1,440	90
pBKM7	1,580	240	12
pBKM8	15	7	5
pBKM9	2,200	1,610	1,270
pBKM10	1,300	320	24
pBKM11	1,580	120	6
pBKM12	40	9	6
pBKM13	320	70	6
pBKM14	1,140	100	6

[a] The activity values represent mean values obtained from at least three independent determinations. They varied up to ±5 mU/mg in the low-level activity range (3 to 40 mU/mg), up to ±20 mU/mg in the intermediate range (70 to 360 mU/mg), and up to ±100 mU/mg in the high range (930 to 2,200 mU/mg).
[b] Transconjugant H16(pBK) served as a background reference.
[c] pBK2241 contains wild-type P_L fused to *lacZ* and P_R fused to *gusA*.
[d] pBKM1 through pBKM14 contain mutant P_L M1 through M14, respectively.

The activities of M1, M6, and M9 were also monitored in the *cbbR*Δ mutants HB14 and HB15, to evaluate the effect of the CbbR activator protein on these most active promoters. HB15 was included in these studies to examine the possible influence of plasmid pHG1 on the activity of the promoters. As expected, wild-type P_L was inactive in the CbbR-less background of the HB14 and HB15 transconjugants (Table 4). The modified P_L promoters, however, were active in the mutants, although at levels reduced by one-quarter to one-third compared to those of the parent strains H16 and HF210, respectively, when grown on pyruvate. Since the transconjugants of both mutants displayed a similar regulatory pattern, the presence of pHG1 did not principally affect the activities of these altered promoters. The activities appeared to be largely independent of CbbR yet were enhanced 1.3- to 3.2-fold during mixotrophic growth. Therefore, a basal activation or derepression mechanism is proposed to affect P_L even in the absence of CbbR.

Activities of mutant P_L promoters in *E. coli*. Because the rationale to mutagenize P_L of *R. eutropha* was based on the consensus structure of an *E. coli* σ^{70} promoter, the activities of the various P_L::*lacZ* fusions were also determined in *E. coli* transformants. Wild-type P_L showed very low but reproducible

TABLE 4. Activities of mutant P_L promoters determined by P_L::*lacZ* transcriptional fusions in transconjugants of *R. eutropha*

Transconjugant	Sp act of β-galactosidase (mU/mg of protein)[a] for:							
	Fructose + formate				Pyruvate			
	H16	HB14	HF210	HB15	H16	HB14	HF210	HB15
pBK[b]	4	3	3	2	3	3	3	4
pBK2241[c]	780	6	490	5	6	4	6	2
pBKM1[d]	1,980	160	1,100	130	90	60	60	40
pBKM6	1,570	170	1,590	70	90	60	40	30
pBKM9	1,800	1,150	1,630	790	1,270	860	730	480

[a] The activity values represent mean values obtained from at least three independent determinations. They varied up to ±5 mU/mg in the low-level activity range (2 to 40 mU/mg), up to ±10 mU/mg in the intermediate range (60 to 170 mU/mg), and up to ±100 mU/mg in the high range (480 to 1,980 mU/mg).
[b] Transconjugant H16(pBK) served as a background reference.
[c] pBK2241 contains wild-type P_L fused to *lacZ* and P_R fused to *gusA*.
[d] pBKM1, pBKM6, and pBKM9 contain P_L M1, M6, and M9, respectively.

activity in the foreign host as did most of the mutant promoters (Fig. 3). A significant increase was observed with mutant M5, but only M1, M6, and M9 yielded high P_L activities. The full-consensus mutant M9 was most active and reached the same level as the activated wild-type P_L in autotrophically grown *R. eutropha*. These data basically supported the findings made on the activities of mutant P_L promoters in the authentic host.

P_R activities associated with mutant P_L. The presumed *cbbR* promoter P_{Ra} active in autotrophically growing *R. eutropha* overlaps P_L. Its −35 box is located within the spacer region of the *cbb* operon promoter (21). Mutations in P_L were thus thought to potentially influence P_R activity. The β-glucuronidase reporter activities of the P_R::*gusA* fusions were determined for P_L mutants M1, M6, and M9 in transconjugants of *R. eutropha* H16 and HF210 as well as of the corresponding *cbbR*Δ mutants HB14 and HB15 grown under heterotrophic or mixotrophic conditions. The previously observed, relatively low P_R activity in pyruvate-grown cells (19, 21) was confirmed for the wild-type P_L region and for mutants M6 and M9 (Table 5). Surprisingly, mutant M1 showed a strongly enhanced activity in all four transconjugants. Determinations with the remaining P_L variants revealed no significant influence of the mutations on P_R activity (data not shown). Growth on fructose plus formate led to significantly higher P_R activities, although the increase was less pronounced in HF210 and HB15 (Table 5). This putative activation or derepression of P_R, which apparently occurs in the wild type as well as in a CbbR-free background, parallels the findings obtained for P_L. P_R activities were not detected in transformants of *E. coli*.

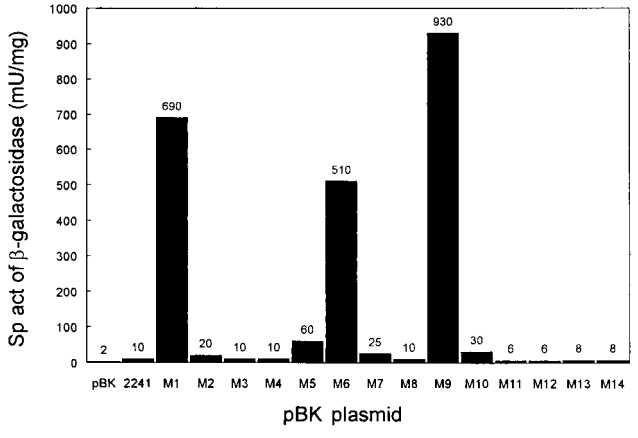

FIG. 3. Activities of mutant P_L promoters determined by P_L::*lacZ* fusions (pBKM1 through pBKM14) in transformants of *E. coli* JW1 grown in Luria-Bertani medium. The strains harboring pBK and pBK2241 served as background and wild-type references, respectively. The specific activity (sp act) values represent mean values obtained from at least three independent determinations. They varied up to ±5 mU/mg in the low-level activity range (2 to 60 mU/mg) and up to ±30 mU/mg in the high range (510 to 930 mU/mg).

TABLE 5. P_R promoter activities associated with the mutant P_L promoter M1, M6, or M9 and determined by P_R::*gusA* transcriptional fusions in transconjugants of *R. eutropha*

Transconjugant	Sp act of β-glucuronidase (μU/mg of protein)[a] for:							
	Fructose + formate				Pyruvate			
	H16	HB14	HF210	HB15	H16	HB14	HF210	HB15
pBK[b]	4	4	6	4	6	9	3	9
pBK2241[c]	480	2,990	40	50	18	240	30	8
pBKM1[d]	2,750	3,430	7,020	7,170	910	900	990	990
pBKM6	160	290	70	120	6	10	35	12
pBKM9	150	390	80	170	20	10	50	17

[a] The activity values represent mean values obtained from at least three independent determinations. They varied up to ±5 μU/mg in the low-level activity range (3 to 40 μU/mg), up to ±50 μU/mg in the intermediate range (50 to 480 μU/mg), and up to ±300 μU/mg in the high range (900 to 7,170 μU/mg).
[b] Transconjugant H16(pBK) served as a background reference.
[c] pBK2241 contains wild-type P_L fused to *lacZ* and P_R fused to *gusA*.
[d] pBKM1, pBKM6, and pBKM9 contain P_L M1, M6, and M9, respectively, fused to *lacZ* and P_R fused to *gusA*.

DISCUSSION

***cbb* promoter activities in a wild-type background.** The present work reports on a detailed mutational study of the *cbb* operon promoter of *R. eutropha*. Its intention was to verify the proposed location of P_L and identify structural attributes which are of importance for the activity and regulation of the promoter. Our previous supposition of P_L as a σ^{70}-type promoter located at the predicted position within the *cbb* control region of *R. eutropha* H16 was confirmed by the data. The resemblance of P_L to the *E. coli* σ^{70} consensus promoter is supported by the low but significant level of expression of the RubisCO genes *cbbLS* in the heterologous host (13). In order to investigate structure-function relationships of P_L, alterations were introduced into the presumed promoter elements to either increase or diminish their similarity to the consensus promoter. Mutations that strongly reduced or abolished the activity of P_L were due to changes deviating from the consensus nucleotides within the −10 or −35 box. The modification of the length of the spacer region between the boxes by a 1-bp deletion or insertion also resulted in drastic decreases of activated promoter activity, corroborating both the structure and location of P_L. The optimal length of the spacer (N_{17}) rather than its sequence is a critical parameter because the exchange of two base pairs in P_L M14 had no effect on the promoter. This finding is consistent with the equivalent activities of the chromosomal and pHG1-borne P_L promoters of *R. eutropha* H16, which differ only in the two spacer bases (GG versus TA) located directly downstream of the mutated bases in M14 (21).

Mutations resulting in a closer match of the −35 and/or −10 box to the consensus stimulated P_L activity. A partial derepression was observed with the half-consensus promoters M1 and M6, and the full-consensus promoter M9 exhibited a strong derepression even in pyruvate-grown cells. Although the basal

activity of P_L increased dramatically upon closer matching to the consensus, the principal property of activation during autotrophic growth or growth on fructose was retained by all improved promoter mutants. The position and identity of P_L are further confirmed by comparing the *cbb* control regions of various chemo- and photoautotrophic bacteria. Particularly the TTTAC pentamer in the −35 box of the potential σ^{70}-type *cbb* operon promoters seems to be conserved among the organisms (11, 20, 33). In accordance with this suggestion, the substitution of the C for the distal T in the −35 box of the full-consensus mutant promoter M9 caused a severe decrease of the high-level basal activity, again without affecting the activation of mutant M10. It is of interest that the exchange of this strongly conserved base also resulted in a drastic drop of P_L activity in the heterologous host, *E. coli*. The results suggest that both the −35 and the −10 boxes are required for the activity of the promoter. Moreover, a promoter structure resembling or matching that of the *E. coli* σ^{70} consensus appears also to be favorable for activity in *R. eutropha*, although additional promoters from the latter organism need to be characterized to verify this conclusion. The hierarchies of base pair preferences found for the different positions of the boxes in *E. coli* (26) are predicted to be similar in *R. eutropha*. In accordance with these considerations, only P_L mutants M1, M6, and especially M9 displayed high-level activities in *E. coli* that approached those observed in the homologous host and corresponded to that of the autotrophically activated wild-type promoter. Whether P_L and its mutants are also activated in the heterologous host in the presence of CbbR is an open question relating to the overall mechanism of the activation process.

The mutations introduced into P_L were not necessarily expected to affect the activity of the partially overlapping P_R promoter as they changed only sequence segments not part of the presumed −35 and −10 boxes of P_R. In fact, all P_L mutants except M1 did not show large differences in P_R activities between *R. eutropha* H16 and HF210. Why the activity of M1 was vastly enhanced remains unclear. A shift of the P_L transcriptional start point as a conceivable cause of affecting the transcription from P_R was not observed with M1, M6, and M9 (data not shown). The mutations in the −10 box of P_L might have created an alternative P_R promoter structure. However, the low-level P_R activity of M9, which also contains these mutations, is not consistent with this possibility, unless the additional alterations present in the −35 box neutralized the stimulatory effect of the mutations in the −10 box on P_R activity. The observed increase of P_R activities under growth conditions inducing or derepressing P_L confirms earlier results (19, 21). Its explanation must await the detailed elucidation of the *cbb* gene regulation in *R. eutropha*.

***cbb* promoter activities in a CbbR-free background.** A CbbR-deficient mutant, strain HB13, of *R. eutropha* has already been isolated (37). In this mutant *cbbR* is disrupted by a Tn*5* insertion within the 3′ third of the gene (21), potentially permitting the production of a truncated CbbR protein, which might still be able to bind to the operator in the *cbb* control region. Therefore, to provide a noninterfering CbbR-free background for the *cbb* promoter analysis, the *cbbR*Δ mutant strains HB14 and HB15 were constructed.

The comparison of the P_L activities in the transconjugants of the parent strains H16 and HF210 and of the corresponding mutants allows several conclusions to be drawn: (i) CbbR is absolutely required for the transcriptional activation of the *cbb* operon from wild-type P_L; (ii) activator CbbR is also required for the high-level activation of the most-improved P_L mutants, M1, M6, and M9, under both inducing and repressing growth conditions; and (iii) the general activation feature of the promoter, very intriguingly, was not lost in the absence of CbbR, as indicated by the weak but significant induction (1.3- to 3.2-fold) of M1, M6, and M9 in HB14 or HB15 grown under mixotrophic conditions. Because of its high, almost constitutive activity, M9 exhibited only low-level inducibility, even in the parent strains (1.3- to 2.2-fold), in which M1 and M6 were induced considerably more strongly (18- to 36-fold). For unknown reasons all three P_L mutants showed an approximately twofold higher activity level in HB14 than in HB15. It is tempting to speculate that this difference is a reflection of a positive regulatory influence of megaplasmid pHG1 on the activation of P_L.

In contrast to the P_L activities of the tested fusions, the P_R activities were slightly enhanced in HB14 and HB15 compared to those of their parent strains (1.3- to 6.2-fold), when the transconjugants were grown under P_L-inducing conditions. However, there was no principal derepression of P_R due to the lack of CbbR. Considering the observed negative autoregulation of *cbbR* transcription (19), such derepression of P_R would be expected in the absence of CbbR, unless an additional regulatory protein(s) binds to the *cbb* control region.

The mutational increase of the transcriptional competence of P_L resulted in the release of promoters M1, M6, and M9 from complete dependency on CbbR. In these P_L variants the normally essential need for CbbR in the activation of the wild-type promoter seems to be largely compensated for by an improved promoter structure. However, CbbR alone is not thought to be sufficient for the activation of the wild-types, as well as results of promoter. Our experimental concept of the *cbbR* deletion mutants functional studies of the modified promoters in the CbbR-free background, allowed us to obtain an indirect hint at the existence of one or more additional *cbb* regulatory proteins, which are proposed to act in concert with CbbR in the transcriptional control of wild-type P_L and which might be activators or repressors. The occurrence of an additional *cbb* regulator(s) in *R. eutropha* would correspond to the postulated participation of the RegB-RegA two-component signal transduction system, besides CbbR, in the regulation of the *cbb* operons in the phototroph *Rhodobacter sphaeroides* (15, 27). A facultative autotroph does most likely require transcriptional control of its *cbb* operon(s) by more than one regulatory component to adjust to the different modes of energy and carbon metabolism when switching between autotrophic and heterotrophic growth or vice versa. In view of these considerations, the genetic control of autotrophic CO_2 fixation should be part of an integrated regulatory network (15, 21, 33).

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