Microorganisms are often confronted with continuously changing environmental conditions to which they have to adapt in an appropriate way in order to survive. The regulation of bacterial metabolism in response to environmental changes, in particular in nutrient availability, is the main focus of research in our laboratory. Important questions studied are how microorganisms sense environmental changes, translate these into cellular signals, and induce the proper responses. One of the model systems used to address these questions is the gram-negative soil bacterium *Xanthobacter flavus* (Meijer W.G., 1990). This bacterium can grow under both heterotrophic and autotrophic conditions. During autotrophic growth, *X. flavus* employs the Calvin cycle for the assimilation of CO_2. This results in the conversion of $3CO_2$ into 3-phosphoglycerate at the expense of 9 ATP and 6 NADH. The energy required for the assimilation of CO_2 is derived from the oxidation of methanol, formate, or hydrogen.

Detailed physiological studies carried out to date have revealed that two main physiological conditions stimulate autotrophic CO_2 fixation in *X. flavus* (Meijer W.G., 1990):

1. a limiting supply of organic carbon sources that support heterotrophic growth
2. a sufficient availability of energy sources.

Elucidation of the underlying molecular mechanisms controlling autotrophic and heterotrophic metabolism necessitates identification of the components which play a role in this process and an analysis of their genetic organization and function. This requires a multidisciplinary study of the physiology, biochemistry and molecular biology of such systems.

Prior to the start of the study described in this thesis, the genes encoding RuBisCO (*cbbLS*), fructosebisphosphatase (*cbbF*), phosphoribulokinase (*cbbP*) and a gene of unknown function (*cbbX*) had already been identified in *X. flavus*. Analysis of mRNA isolated from autotrophically grown cells and gene disruption studies showed that these genes are under the control of a single promoter located upstream from the *cbbL* gene (Meijer *et al.* 1991, 1990). In addition, *in vivo* studies with fusions between the *cbb* promoter and the reporter gene *lacZ* demonstrated that the regulation of the *cbb* genes is exerted at the level of transcription (Meijer *et al.* 1991). One of the aims of the research described in this thesis was to elucidate the organization and regulation of other genes encoding enzymes of the Calvin cycle in *X. flavus*. Analysis of the downstream region of the *cbb* operon revealed the presence of three genes transcribed in the same direction as the other structural *cbb* genes: *cbbT* and *cbbA*, encoding transketolase and FBP aldolase, respectively, and the 5' end of a gene encoding pentose-5-phosphate-3-epimerase (*cbbE*) (chapters II, V). The close linkage between the *cbbLSXFP* genes and the newly identified

cbbTAE genes strongly suggests that they are organized in a single operon, as is the case in *A. eutrophus* and *R. sphaeroides* (Gibson *et al.* 1991, 1990; Hallenbeck *et al.* 1990a,b). However, the gene spacing in the *cbbPTAE* cluster varies between 255 and 267 base pairs, which is much larger than that observed within the *cbbLSXFP* cluster. It therefore cannot be excluded that the *cbbTAE* genes have individual promoters and are not dependent on the promoter of the *cbbLSXFP* operon. Interestingly, the genes encoding components of the photosystem in purple photosynthetic bacteria are organized in superoperons in which overlapping transcriptional units allow a balanced synthesis of pigments and structural components of the photosystem (Bauer and Bird, 1996). It is conceivable that the *cbb* genes are also organized in a superoperon.

A salient characteristic of the *cbb* operons in facultatively autotrophic bacteria is that they are generally not expressed during heterotrophic growth conditions (Bowien *et al.* 1996; Gibson, 1995; Tabita, 1995). Transketolase, FBPase, FBP aldolase and pentosephosphate epimerase activities are required for both heterotrophic and autotrophic growth. This indicates that in addition to the *cbbFTAE* genes expressed during autotrophic growth, genes encoding isoenzyme forms which are expressed during heterotrophic growth may exist. Evidence supporting this is presented in chapters II, III and V. Biochemical and genetic evidence shows that *X. flavus* possesses two FBPase enzyme forms (chapter III). The cloning and characterization of two transketolase genes (*tkt*, *cbbT*) is described in chapters II and V. Since the *tkt* gene is located outside the *cbb* operon, it is likely that the transketolase encoded by this gene participates in the pentosephosphate cycle. Two forms of FBP aldolase were detected in extracts of methanol grown cells (chapter II). One of these is Fe^{2+} dependent, which defines it as a class II FBP aldolase. This form is only present following autotrophic growth, whereas a class I FBP aldolase, which is Fe^{2+} independent, is constitutively expressed.

The genes encoding glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase are not located within the *cbb* operon, but were found in a separate *gap-pgk* operon (chapter V; Meijer, 1994). In contrast to the *cbb* operon, the *gap-pgk* operon is required for both autotrophic and heterotrophic growth and is constitutively expressed. However, the expression of this operon is superinduced following a transition from heterotrophic to autotrophic growth conditions (Meijer *et al.* 1996; Meijer, 1994).

The dramatic increase in the activities of enzymes normally participating in gluconeogenesis and the pentose-phosphate cycle following a transition from heterotrophic to autotrophic growth conditions is required to allow a high flux of carbon through the Calvin cycle. A possible role for the *cbbF, cbbT* and *cbbA* genes could be that they merely serve to increase the activities of FBPase, transketolase and FBP aldolase during autotrophic growth. However, as is the case for the *gap-pgk* operon, this could

have been achieved by a superinduction of the genes encoding the heterotrophic counterparts of these enzymes. It is therefore more likely that the enzymes encoded by *cbbF*, *cbbT*, and *cbbA* encode enzymes with regulatory or kinetic properties especially suited for a role in the Calvin cycle.

The biochemical characterization of FBPase (chapter III) shows that this is indeed the case. Although both the heterotrophic and autotrophic enzymes have SBPase activity which is required for functioning of the Calvin cycle, the SBPase activity of the enzyme encoded by *cbbF* is twice as high as its FBPase activity. The *in vivo* role of CbbF may therefore be that of a SBPase, which is a characteristic enzyme of the Calvin cycle. In fact, chloroplasts contain a specific SBPase, which has no reactivity towards FBP. The activities of both the chloroplast SBPase and the enzyme encoded by *cbbF* respond to the energy status of the cell. The plant enzyme is activated by light via a thioredoxin mechanism (Chardot and Meunier, 1991; Schimkat *et al.* 1990; Gardeman *et al.* 1986), and the CbbF protein is activated by ATP (chapter III). Although the mechanism is different, the regulation of the activity of these enzymes is based on the same principles. Whether the enzymes encoded by *cbbT, cbbA* and *cbbE* also possess properties which are characteristic for a role in autotrophic metabolism awaits their biochemical characterization.

The unique Calvin cycle enzymes phosphoribulokinase and RuBisCO encountered in various autotrophic bacteria probably have a common ancestor. However, the origin of the other Calvin cycle enzymes is less clear. It is possible that following the acquisition of *cbbLS* and *cbbP* by a heterotrophic bacterium, genes encoding other Calvin cycle enzymes arose via duplication of genes encoding enzymes involved in heterotrophic metabolism. Inspection of the *cbb* gene clusters in various bacteria shows that not only the organization of the *cbbLS* genes, but also of the *cbbFPT(A)* genes are highly conserved (Gibson, 1995). This may reflect a common origin for these *cbb* genes. A comparison of the *cbbT* and *tkt* genes of *X. flavus* shows that the transketolase proteins encoded by these genes are not more related to each other than to transketolase proteins of other bacteria, which strongly argues against the possibility that the *cbbT* gene arose from a *tkt* gene duplication event (chapter V). A phylogenetic analysis of FBP aldolase shows that the autotrophic enzymes have a common ancestry and were probably obtained via lateral gene transfer from a gram-positive bacterium (chapter II). This scenario is not unlikely since *cbb* genes are frequently encountered on mobile genetic elements which have been shown to be able to cross the species barrier (Ecker *et al.* 1986; Friedrich *et al.* 1981).

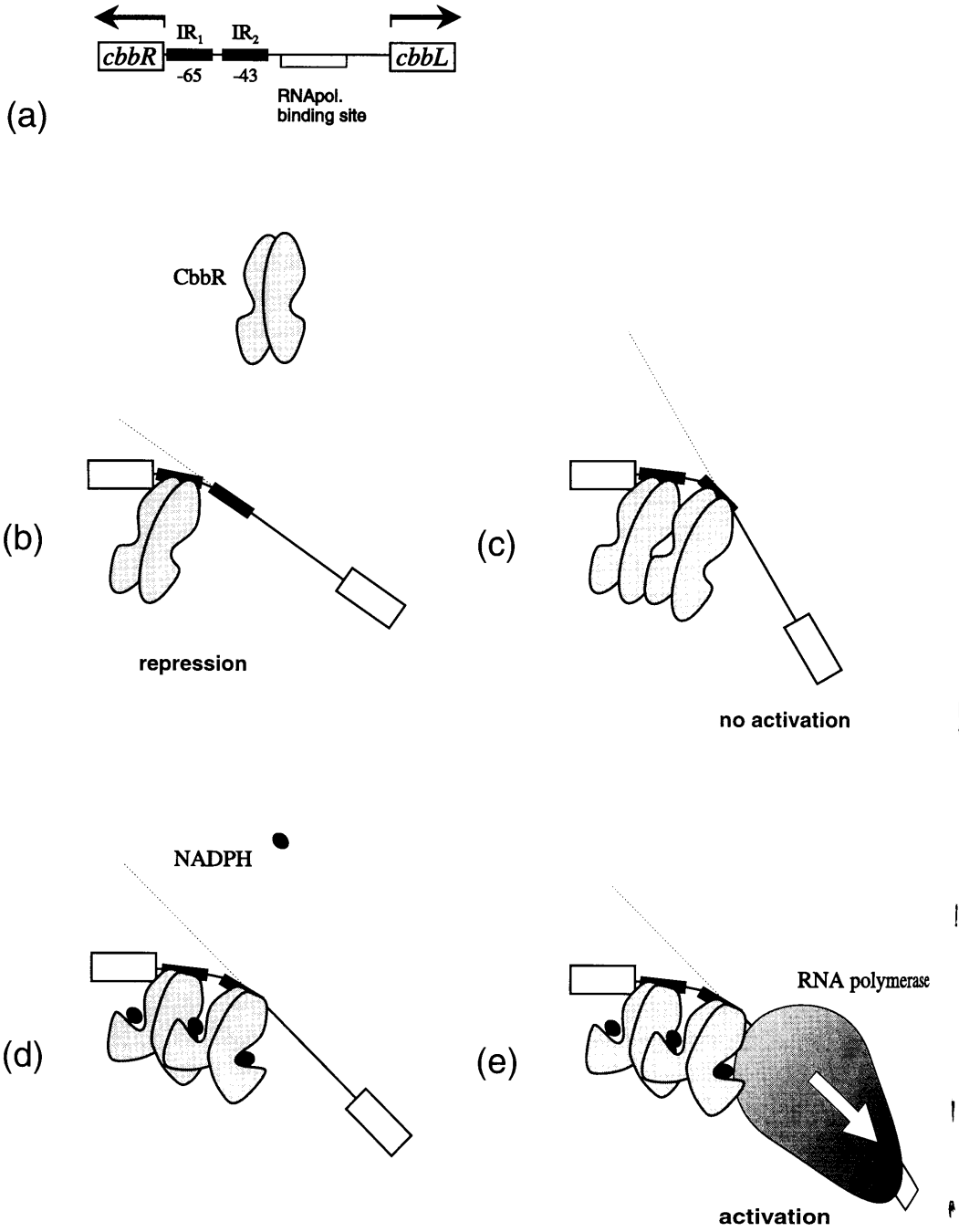
At the onset of the research described in this thesis, the 5' end of an open reading frame displaying similarity to proteins belonging to the LysR family of transcriptional regulators had been identified upstream (transcribed divergently) from the *cbb* operon

(Meijer *et al.* 1991). The complete nucleotide sequence of this open reading frame (*cbbR*) is reported in Chapter IV. It is now established that *cbbR* is essential for expression of the Calvin cycle enzymes which are encoded in the *cbb* operon. In addition, CbbR is required for the superinduction of the *gap-pgk* operon which occurs during the transition from heterotrophic to autotrophic growth (chapters IV and V). Following the initial description of an open reading frame encoding a LysR-type transcriptional regulator upstream of the *cbb* operon in *X. flavus*, similar observations were made in various other autotrophic bacteria (Strecker *et al.* 1994; Falcone and Tabita, 1993; Kusano and Sugawara, 1993; Gibson and Tabita, 1993; Kusano *et al.* 1991; Viale *et al.* 1991; Windhövel and Bowien, 1991).

Following the heterologous expression of *cbbR* from *X. flavus* in *Escherichia coli*, binding of the CbbR protein to the *cbb* operon promoter was studied using gel-retardation assays. CbbR binds to two inverted repeats (IR_1 and IR_2) in the *cbbR-cbbL* intergenic region (Fig.1a). Binding of CbbR to IR_1 and IR_2 occurs in a cooperative manner; binding of CbbR to IR_1 is a prerequisite for CbbR binding to IR_2 (chapter VI). In accordance with these *in vitro* studies is the observation that IR_1 is essential for activity of the *cbb* operon promoter *in vivo*. Binding of CbbR to its cognate binding sites introduces a bend in the DNA (chapter VI), which has also been observed for other LysR type proteins (Schell, 1993). The CbbR binding site IR_1 is located immediately upstream from the initiation codon of the *cbbR* gene. It is therefore likely that CbbR negatively controls its own expression in an autoregulatory fashion, as has recently been established for the *cbbR* gene of *A. eutrophus* (Kusian and Bowien, 1995).

A number of metabolites were tested for their ability to influence CbbR binding and hence to fulfil the role of a signal metabolite controlling the expression of the Calvin cycle genes. Only NADPH altered the binding characteristics of CbbR by enhancing binding to IR_1 and IR_2. In addition, the presence of NADPH in the assay mixture also partially relaxed the CbbR-induced bend in the DNA. Analogous ligand-influenced bending has also been observed with CysB and other LysR proteins (Hryniewicz and Kredich, 1995, 1991; Parsek *et al.* 1995,1994; Fisher and Long, 1993; Wang *et al.* 1992).

Based on our current data we propose the following model for the mechanism underlying CbbR induced promoter activation of the *cbb* genes in *X. flavus* (see Fig.1): CbbR, which is a dimer like OxyR (Kullik *et al.* 1995) (van Keulen *et al.* submitted for publication) binds to a high affinity site (IR_1), and induces a bend in the DNA (30°, van Keulen *et al.* submitted for publication). This DNA-protein complex prevents the transcription of *cbbR* (autoregulation). It also facilitates the cooperative binding of a second CbbR dimer to the low affinity site IR_2, which increases the bend in the DNA to 60° (chapter VI). The effector NADPH binds to (free and/or bound) CbbR, which leads to a conformational change of CbbR. This results in an enhanced affinity of CbbR for IR_1


(a)
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binding site
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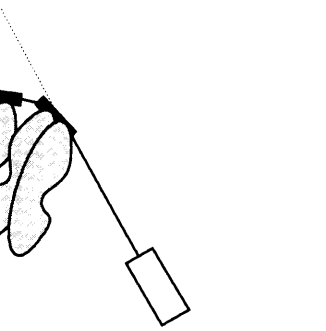
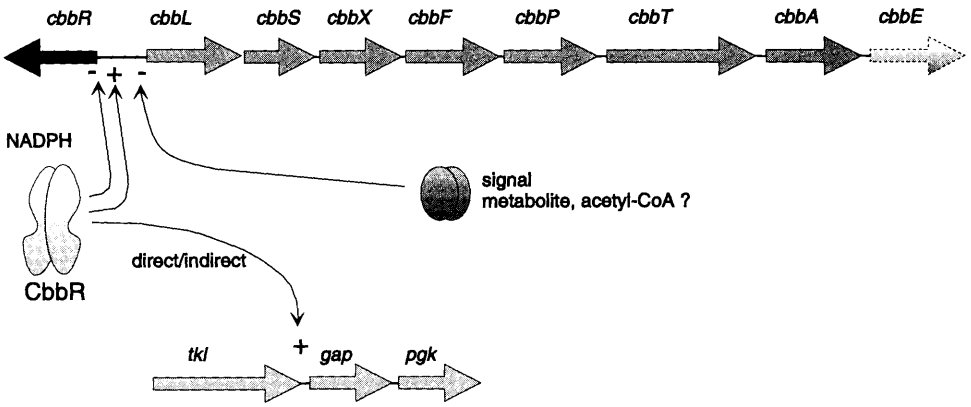
Figure 1. Schematic model of transcriptional activation of the *cbb* promoter by CbbR: (a) The intergenic region between *cbbR* and *cbbL*; the arrows indicate the direction of transcription. (b) CbbR interacts with high affinity site IR_1, and induces a bend in the DNA (30°). (c) Cooperative binding of a second CbbR to IR_2, increasing the angle of the DNA bend (60°). (d) The presence of NADPH results in a conformational change in CbbR. This increases the affinity of CbbR for its binding sites (5-fold), but also leads to a relaxation of the DNA bend (46°). (e) RNA polymerase binds to the *cbb* promoter, and initiates transcription.

and IR_2, and a partial relaxation of the CbbR-induced DNA bend to 46°. Subsequently, the CbbR(NADPH)-DNA complex stimulates RNA polymerase to bind downstream from IR_2, and/or increases open complex formation, finally resulting in transcription of the *cbb* operon.

The LysR-type proteins, CysB and OxyR (Tao *et al.* 1993; Aizawa and Miyachi, 1986) have also been shown to bind to target sites upstream of the promoter, and make direct contact with the C-terminal region of the alpha subunit of RNA polymerase (Ishihama, 1993), which classifies them as Class I transcriptional activators.





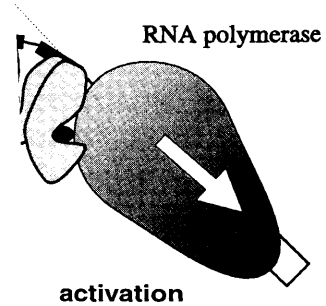


FIG 2. Model representing the regulation of the Calvin cycle enzymes arranged in the *cbb* and the *gap-pgk* operon.

The proposed model for transcriptional regulation of the *cbb* operon explains to a large extent the physiological data, which show that the expression of the Calvin cycle in autotrophic bacteria is sensitive to the redox state of the cell. In *R. sphaeroides* the Calvin cycle is used to dissipate excess reducing equivalents during photoheterotrophic growth in the absence of external electron acceptors such as dimethylsulfoxide (Wang *et*

al. 1993; Falcone and Tabita, 1991). It has been shown for *Xanthobacter* 25a that methanol is a more potent inducer of the Calvin cycle than formate, although both substrates are dissimilated via the same pathway (Croes *et al.* 1991). However, the oxidation of methanol to CO_2 results in the generation of three reducing equivalents, whereas formate oxidation only yields one reducing equivalent. Based on our model we propose that the redox responsive regulation of the *cbb* operon is mediated by CbbR which responds to the intracellular concentration of NADPH. Induction of the Calvin cycle in *X. flavus* only makes sense if the cell has the metabolic energy for biosynthesis, but lacks the required carbon substrates. NADPH, which is primarily used in biosynthetic processes, is an excellent candidate to fulfil a signalling role in this process.

It remains unclear whether CbbR(NADPH) is the only regulator of the *cbb* operon. Physiological studies have shown that organic carbon sources have a negative effect on the expression of the Calvin cycle. Although this effect could be mediated by CbbR(NADPH), it is also possible that another DNA binding factor is responsible for repression of the *cbb* operon (see Fig.2). This suggestion is further supported by the observation that *X. flavus* harbouring multiple plasmid copies containing the *cbb* operon, displays a low level expression of Calvin cycle under non-inducing conditions. This putative repressor protein may be responsive to an intracellular metabolite signalling the carbon status of the cell.

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