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Review

Transcriptional regulation of catabolic pathways for aromatic compounds in *Corynebacterium glutamicum*

K. Brinkrolf[1,2], I. Brune[1] and A. Tauch[1]

[1]Institut für Genomforschung, Centrum für Biotechnologie, Universität Bielefeld, Bielefeld, Germany
[2]International NRW Graduate School in Bioinformatics and Genome Research, Centrum für Biotechnologie, Universität Bielefeld, Bielefeld, Germany
Corresponding author: A. Tauch
E-mail: Andreas.Tauch@Genetik.Uni-Bielefeld.DE

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ABSTRACT. *Corynebacterium glutamicum* is a gram-positive soil microorganism able to utilize a large variety of aromatic compounds as the sole carbon source. The corresponding catabolic routes are associated with multiple ring-fission dioxygenases and among other channeling reactions, include the gentisate pathway, the protocatechuate and catechol branches of the β-ketoadipate pathway and two potential hydroxyquinol pathways. Genes encoding the enzymatic machinery for the bioconversion of aromatic compounds are organized in several clusters in the *C. glutamicum* genome. Expression of the gene clusters is under specific transcriptional control, apparently including eight DNA-binding proteins belonging to the AraC, IclR, LuxR, PadR, and TetR families of transcriptional regulators. Expression of the gentisate pathway involved in the utilization of 3-hydroxybenzoate and gentisate is positively regulated by an IclR-type activator. The metabolic channeling of ferulate, vanillin and vanillate into the protocatechuate branch of the β-ketoadipate pathway is controlled by a PadR-like repressor. Regulatory proteins of

the IclR and LuxR families participate in transcriptional regulation of the branches of the β-ketoadipate pathway that are involved in the utilization of benzoate, 4-hydroxybenzoate and protocatechuate. The channeling of phenol into this pathway may be under positive transcriptional control by an AraC-type activator. One of the potential hydroxyquinol pathways of *C. glutamicum* is apparently repressed by a TetR-type regulator. This global analysis revealed that transcriptional regulation of aromatic compound utilization is mainly controlled by single regulatory proteins sensing the presence of aromatic compounds, thus representing single input motifs within the transcriptional regulatory network of *C. glutamicum*.

Key words: *Corynebacterium glutamicum*, *Corynebacterium efficiens*, Gentisate pathway, β-ketoadipate pathway, Hydroxyquinol pathway, Transcriptional regulation

INTRODUCTION

Microorganisms constantly have to adapt to changing environmental conditions in order to maintain their functional homeostasis and to handle different types of environmental and cellular stresses (Matic et al., 2004). Therefore, they sense alterations within their surrounding and apply complex molecular strategies involving transcriptional regulation of gene expression to overcome unfavorable conditions. DNA-binding transcriptional regulators are the most important components of these regulatory systems since they specifically detect the presence of effector molecules to exert their regulatory role in gene expression (Madan Babu and Teichmann, 2003a; Martinez-Antonio et al., 2006). Depending on the environmental conditions, only certain fractions of the genome of a bacterial cell are expressed, according to the behavior of transcriptional regulators within the complete network of regulatory interactions (Resendis-Antonio et al., 2005). While some regulatory proteins are only responsible for the transcriptional control of single genes or gene clusters, others organize the activation or repression of numerous target genes (Teichmann and Babu, 2004). Nowadays, knowledge of the whole genome sequence of a microorganism provides the possibility to determine the total set of encoded DNA-binding transcriptional regulators (Perez-Rueda and Collado-Vides, 2000; Guedon et al., 2002; Brune et al., 2005; Moreno-Campuzano et al., 2006) and to apply bioinformatics and post-genomic techniques, such as DNA microarray hybridizations, to reconstruct the global connectivity of bacterial regulatory networks (Herrgard et al., 2004).

Corynebacterium glutamicum is a gram-positive soil bacterium that is traditionally applied in biotechnological production processes, particularly in the fermentative production of amino acids (Hermann, 2003). The complete genome sequence of the wild-type strain *C. glutamicum* ATCC 13032 provided the basis to identify and analyze the repertoire of transcriptional regulators in this species (Kalinowski et al., 2003; Brune et al., 2005). The total set of DNA-binding transcriptional regulators of *C. glutamicum* was determined to be 127 proteins

that can be further associated with one of five well-defined and functionally distinct regulatory modules with respect to the physiological role of their target genes (Brune et al., 2005; Baumbach et al., 2006). As a free-living soil bacterium, *C. glutamicum* is exposed to permanent variations in the supply of nutrients specified by its natural habitat so that a fast adaptation to changing environmental conditions is of great importance to allow optimal growth and reproduction. The high demand for flexibility in the consumption of different carbon and energy sources may be the reason why a large proportion of regulatory proteins can be associated with transcriptional control of bacterial carbohydrate metabolism (Guedon et al., 2002; Brune et al., 2005; Resendis-Antonio et al., 2005).

Besides being able to degrade sugars, *C. glutamicum* also shows the ability to utilize a large variety of aromatic compounds for growth (Shen and Liu, 2005; Shen et al., 2005a,b; Merkens et al., 2005). Aromatic compounds, such as the complex aromatic polymer lignin, are natural components of the environment of soil bacteria and represent an alternative source to sugars for carbon and energy (Harwood and Parales, 1996). The presence of oxygen is a precondition for a fast and effective assimilation of aromatic compounds, although anaerobic decomposition by some bacteria is also possible. The catabolic reactions take place in two principle steps comprising the preparation of enzymatic ring cleavage, termed the peripheral pathway, and the ring-fission itself (Diaz, 2004). To prepare aromatic compounds for ring cleavage, a variety of chemical modifications involving mono- or dioxygenation are carried out. The ring modification reactions of the peripheral pathways result in the formation of dihydroxylated benzene derivatives, such as gentisate, protocatechuate and catechol. These intermediate compounds are the substrates of specific ring-fission enzymes that integrate oxygen to open the aromatic ring in the second phase of degradation (Harwood and Parales, 1996). Fission takes place either between two adjacent hydroxyl groups (*ortho* cleavage) or proximal to one of the two hydroxyl groups (*meta* cleavage) of the aromatic compound. A third ring cleavage pathway processes aromatic compounds, such as gentisate, whose two hydroxyl groups on the aromatic ring are *para* to each other (Harwood and Parales, 1996). In principle, structurally diverse aromatic compounds are degraded through different peripheral pathways to only few intermediates that are further channeled via common reactions into the central carbohydrate metabolism of the bacterial cell (Diaz, 2004).

In this review, we summarize the current knowledge of the ability of *C. glutamicum* to utilize various aromatic compounds as the sole source of carbon and energy for growth. We will present the structure of the different catabolic pathways of aromatic compounds identified in the *C. glutamicum* genome sequence and the specific ring-fission dioxygenases they involve. Furthermore, we will connect these catabolic pathways to transcriptional regulation carried out by different types of regulatory proteins.

TRANSCRIPTIONAL REGULATION OF THE GENTISATE PATHWAY OF *CORYNEBACTERIUM GLUTAMICUM*

C. glutamicum can utilize 3-hydroxybenzoate and gentisate as the sole carbon source for growth (Shen et al., 2005a). Gentisate is degraded to pyruvate and fumarate, whereas 3-hydroxybenzoate can be converted into gentisate by 3-hydroxybenzoate 6-hydroxylase activity (Figure 1A). The relevant ring-cleavage activity of this pathway is provided by gentisate 1,2-dioxygenase (Harwood and Parales, 1996). A cluster of six genes (*cg3349* to *cg3354*) was

recently shown to encode the enzymes involved in the gentisate pathway of *C. glutamicum* (Figure 1B; Shen et al., 2005a). A homologous gene cluster (*ce2858* to *ce2863*) is present in the genome of *C. efficiens* (Figure 1B; Nishio et al., 2003). When *C. glutamicum* cells were cultivated with 3-hydroxybenzoate and gentisate as the sole carbon source, gentisate 1,2-dioxygenase activity was detected, suggesting that both aromatic compounds are assimilated by the gentisate pathway (Shen et al., 2005a). The respective enzymatic functions were deduced from enzyme assays with purified proteins and from genetic studies using defined deletion mutants of *C. glutamicum*, providing evidence that *cg3349* (*nagL*), *cg3350* (*nagK*) and *cg3351* (*nagI*) encode maleylpyruvate isomerase, fumarylpyruvate hydrolase and gentisate 1,2-dioxygenase, respectively (Hintner et al., 2004; Shen et al., 2005a). The Cg3349 protein represents a new type of maleylpyruvate isomerase since it is independent of glutathione and requires mycothiol as co-factor (Shen et al., 2005a; Feng et al., 2006). Furthermore, deletion of the *cg3353* (*nagT*) gene encoding a transport protein resulted in the loss of the ability to utilize gentisate, but did not affect the growth of *C. glutamicum* with 3-hydroxybenzoate, suggesting that *cg3353* codes for a gentisate transporter (Shen et al., 2005a). This result was substantiated very recently by the heterologous expression and analysis of a Cg3353-GFP fusion protein in *Ralstonia* sp strain U2 (Xu et al., 2006). On the other hand, deletion of *cg3354* negatively affected the growth of *C. glutamicum* with 3-hydroxybenzoate. *Escherichia coli* cells expressing the Cg3354 protein converted 3-hydroxybenzoate into gentisate, indicating that the protein has 3-hydroxybenzoate hydroxylase activity (Figure 1A).

A regulatory gene encoding a transcriptional regulator of the IclR family (Table 1; Brune et al., 2005) is located upstream of the gentisate 1,2-dioxygenase gene (Figure 1B). Deletion of the *cg3352* (*nagR*) gene resulted in significant loss of the enzyme activities encoded by *cg3351*, *cg3350* and *cg3349* (Figure 1B) and of the ability to grow with 3-hydroxybenzoate and gentisate as the sole carbon source (Shen et al., 2005a). These data demonstrated that at least the genes *cg3349* to *cg3351* are under positive transcriptional control and that *cg3352* encodes an activator protein of the gentisate pathway (Table 1). Activation of expression of the gentisate pathway thus requires both a functional Cg3352 (NagR) protein and the presence of either gentisate or 3-hydroxybenzoate that could be converted into gentisate by hydroxylation.

TRANSCRIPTIONAL REGULATION OF THE VANILLATE PATHWAY OF *CORYNEBACTERIUM GLUTAMICUM*

Microbiological assays revealed that *C. glutamicum* is able to grow on ferulate, vanillin and vanillate as the sole carbon source (Merkens et al., 2005; Shen et al., 2005b). Bioconversion of ferulate, vanillin and vanillate leads to protocatechuate, thus channeling these aromatic compounds into the protocatechuate branch of the β-ketoadipate pathway (Figure 2A). Thereby, enzymatic conversion of vanillate into protocatechuate is catalyzed by vanillate demethylase (Mason and Cammack, 1992). A cluster of four genes (*cg2615* to *cg2618*) has been associated with vanillate metabolism in *C. glutamicum* (Merkens et al., 2005), including the *vanA*, *vanB* and *vanK* genes and the divergently transcribed *vanR* gene (Figure 2B). A similar gene cluster (*ce0633* to *ce0636*) has been detected in the genome sequence of *C. efficiens* (Figure 2B; Nishio et al., 2003). The deduced proteins of the *vanAB* genes of *C. glutamicum* showed significant similarity to vanillate demethylase subunits A and B from different microorganisms

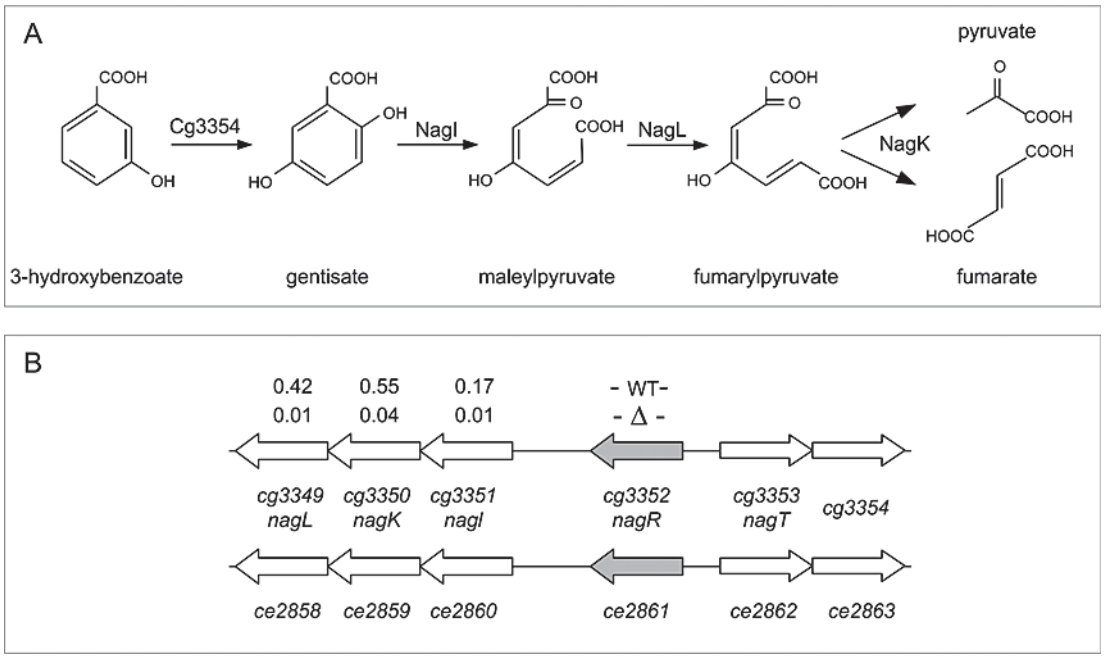

Figure 1. The gentisate pathway of *Corynebacterium glutamicum*. **A.** Metabolic pathway for the utilization of 3-hydroxybenzoate and gentisate as the sole carbon source for growth. Enzymes involved in the corresponding reactions of the pathway include gentisate 1,2-dioxygenase (NagI), maleylpyruvate isomerase (NagL) and fumarylpyruvate hydrolase (NagK). 3-Hydroxybenzoate hydroxylase activity (Cg3354) is necessary for the conversion of 3-hydroxybenzoate into gentisate. **B.** Genetic organization of the genes involved in the gentisate pathway of *C. glutamicum* and *C. efficiens*. Genes encoding transcriptional regulators are shown as gray arrows. The *nagT* gene encodes a membrane protein that may be involved in gentisate uptake (Shen et al., 2005a). Specific enzyme activities (units/mg protein) of NagL, NagK and NagI measured in the wild-type (-WT-) and a *cg3352* deletion mutant (-Δ-) of *C. glutamicum* upon growth with 3-hydroxybenzoate and acetate as carbon sources are indicated above the arrows (Shen et al., 2005a).

(Kalinowski et al., 2003). A *C. glutamicum* mutant carrying a disrupted *vanA* gene was unable to grow on ferulate and vanillate, whereas growth on protocatechuate was not impaired (Merkens et al., 2005). The *vanK* gene has been suggested to encode a protocatechuate transporter since the metabolism of ferulate and vanillate was not impaired in a *vanK* disruption mutant, while growth on protocatechuate was biphasic. Further growth experiments showed that glucose and vanillate were co-metabolized by *C. glutamicum*, revealing no indication for the participation of glucose-mediated carbon catabolite response in the expression of the *van* genes. Induction of expression of the *vanABK* genes was observed with ferulate and vanillate, while protocatechuate had no positive effect on *vanA* gene expression (Merkens et al., 2005). Reverse transcription-PCR assays revealed a common transcript of the *vanABK* cluster, suggesting that the genes are transcriptionally organized as an operon. The common transcript of *vanABK* starts 59 nucleotides upstream of the *vanA* start codon as has been determined by 5' RACE experiments, resulting in the mapping of a potential promoter region (Figure 2C).

The *vanR* gene (*cg2615*) codes for a DNA-binding protein that has been classified in the PadR family of transcriptional regulators (Table 1; Brune et al., 2005). This protein family includes, for instance, functionally characterized members that are involved in the negative transcriptional regulation of phenolic acid metabolism (Bateman et al., 2002). The regulatory

Table 1. Transcriptional regulators involved in aromatic compound utilization by *Corynebacterium glutamicum*.

Gene		Protein					Physiological role	
No.	Name	Regulator type	Protein size [aa]	Molecular mass [kDa]	HTH type of DNA-binding domain	Function	Regulated pathway associated with ring-fission activity by	Regulated pathway involved in utilization of
cg3352	*nagR*	IclR	258	28.0	winged helix (N)	Activator[1]	gentisate 1,2-dioxygenase	3-hydroxybenzoate, gentisate
cg2615	*vanR*	PadR	192	21.5	winged helix (N)	Repressor[1]	protocatechuate 3,4-dioxygenase	ferulate, vanillin, vanillate
cg2624	*pcaR*	IclR	255	27.7	winged helix (N)	Repressor[1]	protocatechuate 3,4-dioxygenase	4-hydroxybenzoate, protocatechuate, *p*-cresol
cg2627	*pcaO*	LuxR	687	75.6	C-terminal effector domain (C)	Activator	protocatechuate 3,4-dioxygenase	4-hydroxybenzoate, protocatechuate, *p*-cresol
cg2641	*benR*	LuxR	895	99.8	C-terminal effector domain (C)	Activator	catechol 1,2-dioxygenase	benzoate, benzyl alcohol
cg2965	-	AraC	337	37.7	homeodomain-like (C)	Activator	catechol 1,2-dioxygenase	phenol
cg1308	-	TetR	229	25.1	homeodomain-like (N)	Repressor[1]	hydroxyquinol 1,2-dioxygenase	resorcinol, 2,4-dihydroxybenzoate[2]
cg3388	-	IclR	494	53.2	winged helix (N + C)	Activator	hydroxyquinol 1,2-dioxygenase	resorcinol, 2,4-dihydroxybenzoate[2]

[1]Predicted regulatory function experimentally verified; [2]Probable aromatic compounds degraded by the respective pathway (Shen et al., 2005b). N = amino-terminal; C = carboxy-terminal.

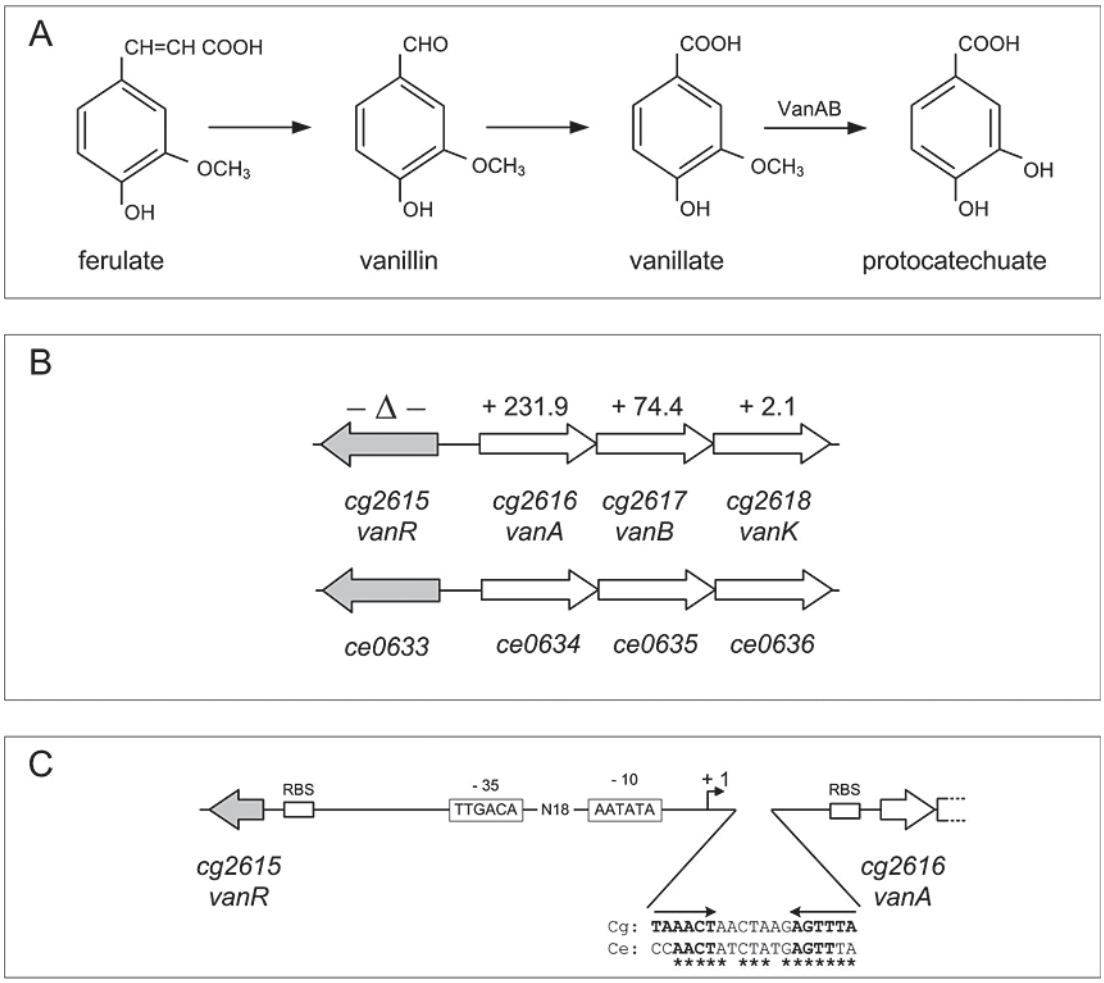

Figure 2. The vanillate pathway of *Corynebacterium glutamicum*. **A.** The conversion of ferulate, vanillin and vanillate to protocatechuate is shown. The final step is catalyzed by vanillate demethylase, VanAB. **B.** Genetic organization of the *van* gene clusters in *C. glutamicum* and *C. efficiens*. The regulatory genes are depicted as gray arrows. Deletion of the *vanR* gene in *C. glutamicum* is indicated (-Δ-). Relative expression of the *vanABK* genes in the deletion mutant compared to that of the *C. glutamicum* wild-type is shown above the arrows. Values were determined by real-time reverse transcription-PCR assays. **C.** Detailed view of the regulatory region in front of the *vanA* gene of *C. glutamicum*. The ribosome-binding site (RBS) and the mapped transcriptional start (+ 1) are specifically marked. The deduced -10 and -35 regions of the mapped *vanABK* promoter are shown along with a predicted operator sequence that was identified at the same positions in the *C. glutamicum* (Cg) and *C. efficiens* (Ce) genome sequence. Identical nucleotides are marked by asterisks, palindromic bases are shown in bold and the inverted repeat is indicated by arrows.

role of *vanR* in *vanABK* gene expression was deduced from a defined deletion mutant and comparison of expression of the *vanABK* genes with that of the *C. glutamicum* wild-type strain. Deletion of *vanR* resulted in enhanced expression of the *vanABK* genes, demonstrating that VanR control of *vanABK* expression is exerted negatively at the level of transcription (Figure 2B). A region of short dyad symmetry was identified downstream of the mapped *vanABK* promoter, indicating a putative operator sequence of a repressor protein (Figure 2C; Madan Babu and Teichmann, 2003b). A similar stretch of DNA was identified in the regulatory region

of the *vanABK* genes of *C. efficiens*. Likewise, predicted operator sequences of related PadR transcriptional regulators identified in *Pediococcus pentosaceus* (Barthelmebs et al., 2000) and *Lactobacillus plantarum* (Gury et al., 2004) are apparently organized in a similar manner, consisting of a short stretch of dyad symmetry along with a non-palindromic center.

TRANSCRIPTIONAL REGULATION OF THE β-KETOADIPATE PATHWAY OF *CORYNEBACTERIUM GLUTAMICUM*

The spectrum of aromatic compound utilization by *C. glutamicum* also includes 4-hydroxybenzoate, protocatechuate and *p*-cresol (Shen and Liu, 2005). Assimilation of 4-hydroxybenzoate and protocatechuate apparently occurs by the β-ketoadipate pathway, consisting of the protocatechuate and catechol branches that converge at β-ketoadipate enol-lactone and a central pathway of three additional steps leading to the final products acetyl-CoA and succinyl-CoA (Figure 3A; Harwood and Parales, 1996). *p*-Cresol may be channeled into the β-ketoadipate pathway by conversion into 4-hydroxybenzoate (Shen et al., 2005b). The key activity of the protocatechuate branch of the β-ketoadipate pathway is represented by protocatechuate 3,4-dioxygenase which is responsible for aromatic ring cleavage. Cells grown in the presence of 4-hydroxybenzoate, protocatechuate and *p*-cresol exhibited significant protocatechuate 3,4-dioxygenase activity (Shen and Liu, 2005), indicating that expression of the protocatechuate branch of the β-ketoadipate pathway is strictly regulated and dependent on the corresponding substrates as inducers. Furthermore, cultivation of *C. glutamicum* in the presence of protocatechuate and glucose revealed no indication for a superior glucose-mediated carbon catabolite control of the β-ketoadipate pathway (Merkens et al., 2005).

The protocatechuate branch of the β-ketoadipate pathway is encoded by a supraoperonic cluster of ten genes (*cg2622* to *cg2631* and *ce2292* to *ce2301*) in the genomes of *C. glutamicum* and *C. efficiens* (Figure 3B; Kalinowski et al., 2003; Nishio et al., 2003). The subunits of protocatechuate 3,4-dioxygenase are obviously encoded by *cg2630* (*pcaG*) and *cg2631* (*pcaH*) since deletion of the *pcaGH* genes in the *C. glutamicum* genome resulted in a mutant strain that had lost protocatechuate 3,4-dioxygenase activity and that was unable to utilize 4-hydroxybenzoate, protocatechuate and *p*-cresol as the sole carbon source for growth (Shen and Liu, 2005). The physiological function of additional genes involved in the protocatechuate branch of the β-ketoadipate pathway was deduced from sequence similarity searches (Figure 3A; Shen et al., 2005b). 4-Hydroxybenzoate hydroxylase activity, necessary for the conversion of 4-hydroxybenzoate into protocatechuate, is most likely encoded by the *pobA* (*cg1226*) gene which is located in a separate transcription unit along with the *pcaK* (*cg1225*) gene encoding a putative 4-hydroxybenzoate transporter (Figure 3B; Shen et al., 2005b).

Two genes encoding transcriptional regulators are part of the *pca* gene clusters in *C. glutamicum* and *C. efficiens* (Figure 3B). The *pcaR* gene encodes a transcriptional regulator of the IclR family and *pcaO* codes for a transcriptional activator that was grouped into the LuxR protein family (Table 1; Brune et al., 2005). The role of the PcaO protein in the regulation of the transcription of the *pca* genes is currently unknown, whereas the respective regulatory function of the PcaR protein can be deduced from sequence similarity searches. Accordingly, it is likely that PcaR exerts its regulatory role in the expression of the *pca* genes by interacting with 15-bp operator sequences that are located in the *pcaI-pcaR* intergenic region and upstream of *pcaH* and *pobA* (Figure 3C), indicating that the expression of each sub-cluster is

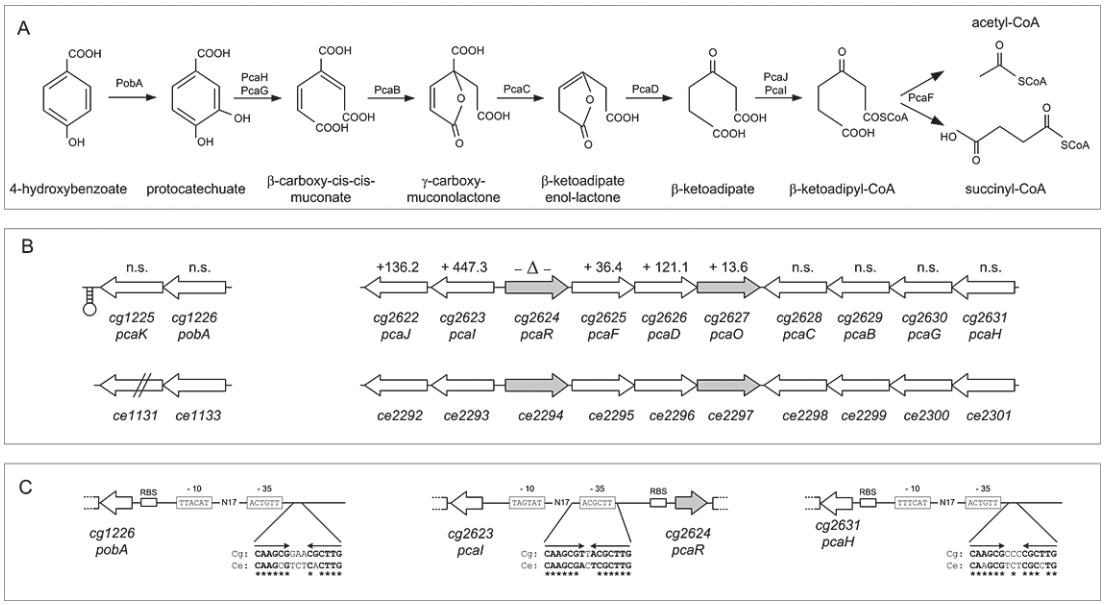

Figure 3. The β-ketoadipate pathway of *Corynebacterium glutamicum*. **A.** Metabolic pathway of the protocatechuate branch of the β-ketoadipate pathway and conversion of β-ketoadipate enol-lactone into acetyl-CoA and succinyl-CoA by enzymatic reactions shared with the catechol branch. Enzymes involved in the pathway include protocatechuate 3,4-dioxygenase (PcaGH), β-carboxy-cis,cis-muconate cycloisomerase (PcaB), γ-carboxymuconolactone decarboxylase (PcaC), β-ketoadipate enol-lactone hydrolase (PcaD), β-ketoadipate succinyl-CoA transferase (PcaI/PcaJ), and β-ketoadipyl-CoA thiolase (PcaF). The 4-hydroxybenzoate hydroxylase PobA is responsible for the conversion of 4-hydroxybenzoate into protocatechuate. **B.** Genetic organization of the *pca* gene clusters in *C. glutamicum* and *C. efficiens*. Regulatory genes are shown as gray arrows. The *pobA* and *pcaK* genes form a separate transcription unit. A predicted transcriptional terminator downstream of *pcaK* is shown by a stem-loop structure. The *pcaK* gene encodes a putative 4-hydroxybenzoate transporter (Shen et al., 2005b). The homologous gene in *C. efficiens* (*ce1131*) apparently contains a frameshift mutation in the 5' part of the coding region, indicated by (//). Deletion of the *pcaR* gene in *C. glutamicum* is marked (-Δ-). Relative expression of the *pcaIJ* and *pcaFDO* genes in the deletion mutant compared to that of the *C. glutamicum* wild-type is shown above the arrows. Values were deduced from real-time reverse transcription-PCR assays. n.s. = not significantly different in expression in the deletion mutant. **C.** Detailed view of the regulatory regions of the *pca* gene clusters of *C. glutamicum*. Predicted -10 and -35 regions of promoters sharing similarity with *C. glutamicum* consensus promoter sequences (Patek et al., 2003) are shown along with a putative 15-bp operator sequence that is conserved in the *C. glutamicum* (Cg) and *C. efficiens* (Ce) genome sequence. Identical nucleotides are marked by asterisks, palindromic bases are shown in bold and the inverted repeat is indicated by arrows. RBS = ribosome-binding site.

under direct transcriptional control by PcaR. Similar partially palindromic sequences are apparently conserved at precisely the same positions in the *pca* gene clusters of *C. efficiens* (Figure 3C). The consensus sequence of the 15-bp operators detected in the *C. glutamicum* genome is represented by the partially palindromic motif 5'-GTTCGC-N_3-GCGAAC-3'. It is worthwhile to note that regulatory proteins of the β-ketoadipate pathway in other bacteria use similar DNA-binding sites for transcriptional regulation. This includes the transcriptional activator PcaR from *Pseudomonas putida* (Romero-Steiner et al., 1994; Guo and Houghton, 1999) and the activators PobR and PcaU from *Acinetobacter calcoaceticus* (DiMarco and Ornston, 1994; Gerischer et al., 1998), indicating that transcriptional control by homologous proteins with the same physiological function is associated with highly similar operator sequences.

However, the PcaR protein of *C. glutamicum* is a member of the IclR family of transcriptional regulators that can also act as repressors of gene expression (Molina-Henares et al.,

2006). When inspecting the very short *pcaI-pcaR* intergenic region of *C. glutamicum*, which is only 64 bp in length, it is apparent that the 15-bp operator of PcaR overlaps the potential promoter of *pcaI* and is located directly adjacent to the ribosome-binding site of the *pcaR* gene (Figure 3C). This genetic organization of the intergenic region is more consistent with a negative regulatory interaction of the PcaR protein (Madan Babu and Teichmann, 2003b). Accordingly, a defined deletion of the *pcaR* gene of *C. glutamicum* resulted in elevated levels of expression of the *pcaIJ* and *pcaFDO* genes as deduced from real-time reverse transcription-PCR assays (Figure 3B). On the other hand, expression of the *pcaHGBC* genes and of the *pobA-pcaK* transcription unit was not significantly affected by the *pcaR* deletion. This data supported the presumption that PcaR acts as a repressor of gene expression, including the regulatory gene *pcaO* (Table 1). This type of hierarchical regulation of *pca* gene expression is reasonable when considering that the β-ketoadipate pathway consists of two specific parallel routes and a common terminal portion (Harwood and Parales, 1996). The *pcaIJ* and *pcaFD* genes are involved in the common part of the β-ketoadipate pathway and expression is negatively regulated only by the PcaR protein (Figure 3A, B). On the other hand, the *pcaHGBC* and *pobA-pcaK* genes, belonging to the protocatechuate branch of the β-ketoadipate pathway, are also under negative transcriptional control by PcaR, but deletion of the *pcaR* gene alone did not result in elevated expression of these genes. This could indicate that a second regulator is involved in triggering the transcriptional response of the specific part of the β-ketoadipate pathway depending on the aromatic compounds present in the environment. This additional regulatory role may be attributed to the LuxR-type transcriptional activator PcaO (Table 1; Brune et al., 2005), which in this way allows a decoupling of the regulation of genes belonging to the specific part of the β-ketoadipate pathway from the more flexible derepression of the common pathway necessary for the utilization of a larger variety of aromatic compounds. However, the precise role of the PcaO regulator in controlling the expression of the protocatechuate branch of the β-ketoadipate pathway remains to be elucidated.

TRANSCRIPTIONAL REGULATION OF THE CATECHOL BRANCH OF THE β-KETOADIPATE PATHWAY OF *CORYNEBACTERIUM GLUTAMICUM*

C. glutamicum can also use benzyl alcohol, benzoate and phenol as the sole carbon and energy source for growth (Shen et al., 2004, 2005b). Benzoate and phenol are representative compounds that are degraded by the catechol branch of the β-ketoadipate pathway (Figure 4A; Harwood and Parales, 1996). Enzyme assays revealed that *C. glutamicum* cells grown in the presence of these aromatic compounds showed catechol 1,2-dioxygenase activity (Shen et al., 2004, 2005b), indicating a tight regulation of the respective catabolic pathway. The genes involved in the bioconversion of benzoate to β-ketoadipate enol-lactone are organized in a single cluster in the genomes of *C. glutamicum* and *C. efficiens* (Kalinowski et al., 2003; Nishio et al., 2003), comprising the divergently oriented *ben* and *cat* sub-clusters (Figure 4B). Benzoate degradation requires the *ben* genes for converting benzoate into catechol and the *cat* genes for degrading the resulting catechol. The physiological role of the respective proteins has been deduced from sequence similarity searches with characterized counterparts from gram-positive and gram-negative bacteria (Figure 4A; Shen et al., 2005b). Additionally, the *cg2636* (*catA*) gene was cloned in *E. coli* and the CatA protein was purified to homogeneity. Subsequent enzyme assays demonstrated that the purified CatA protein possesses catechol 1,2-dioxygenase

activity, providing evidence that both branches of the β-ketoadipate pathway operate in *C. glutamicum* to utilize a wide spectrum of aromatic compounds for growth. Furthermore, phenol may be converted into catechol by phenol hydroxylase activity (Shen et al., 2005b) which is most likely encoded by the *cg2966* and *ce2219* genes of *C. glutamicum* and *C. efficiens*, respectively (Figure 4A, B).

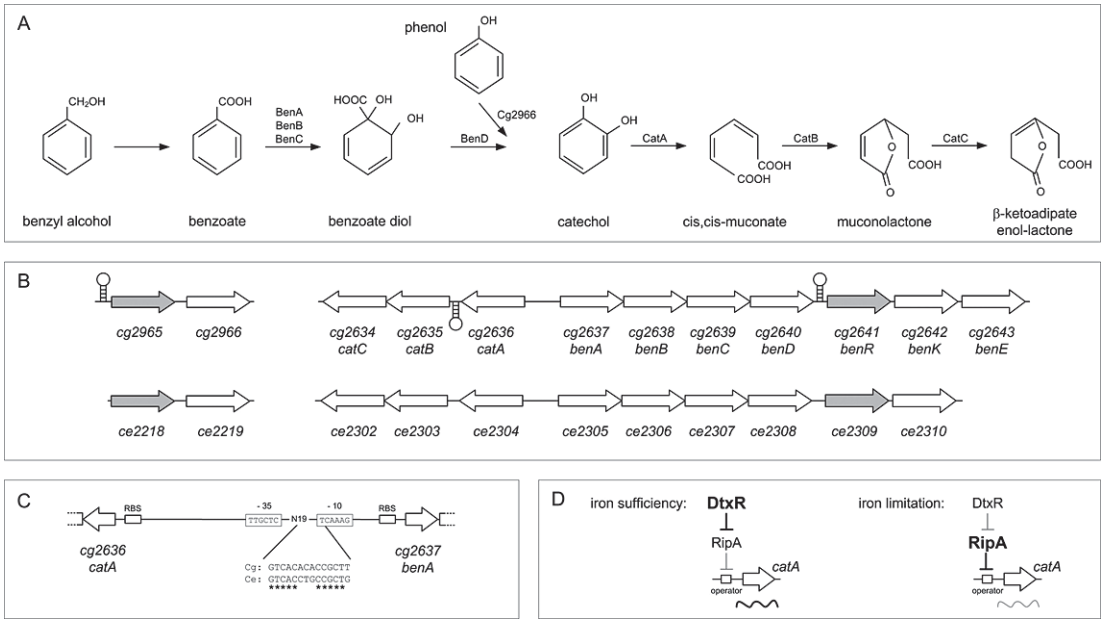

Figure 4. The catechol branch of the β-ketoadipate pathway of *Corynebacterium glutamicum*. **A.** Metabolic pathway for the conversion of benzyl alcohol and benzoate into catechol and reactions of the catechol branch of the β-ketoadipate pathway. The catechol and protocatechuate branches of the β-ketoadipate pathway converge at β-ketoadipate enol-lactone. Enzymes involved in this pathway are: benzoate dioxygenase (BenAB), benzoate dioxygenase reductase (BenC), 2-hydro-1,2-dihydroxybenzoate dehydrogenase (BenD), catechol 1,2-dioxygenase (CatA), cis-cis-muconate cycloisomerase (CatB), and muconolactone isomerase (CatC). Phenol hydroxylase activity (Cg2966) may be involved in the conversion of phenol into catechol. **B.** Genetic organization of the genes involved in assimilation of phenol and benzoate in *C. glutamicum* and *C. efficiens*. Genes encoding transcriptional regulators are marked by gray color. Putative transcriptional terminators are shown by stem-loop structures. The *benK* and *benE* genes encode membrane proteins that may be involved in benzoate transport (Shen et al., 2005b). A *benE* homolog is apparently absent in the *ben* sub-cluster of *C. efficiens*. **C.** Detailed view of the *catA-benA* intergenic region involved in transcriptional regulation of expression of the *catA* gene. The DNA-binding sites of the transcriptional regulator RipA in the *catA-benA* intergenic region of the *C. glutamicum* (Cg) and *C. efficiens* (Ce) genome are shown. Identical nucleotides are marked by asterisks. Potential -10 and -35 regions of a *benA* promoter sharing similarity with the consensus sequence of *C. glutamicum* promoters (Patek et al., 2003) are indicated. RBS = ribosome-binding site. **D.** Model for the hierarchical regulation of *catA* expression by DtxR and RipA. Repressing regulatory interactions are specifically marked (⊥).

Data on the transcriptional regulation of the catechol branch of the β-ketoadipate pathway of *C. glutamicum* are scarce. A putative transcriptional regulator of the LuxR protein family is encoded by the *cg2641* (*benR*) gene (Table 1; Brune et al., 2005). Since LuxR-type regulators are generally regarded to act as transcriptional activators (Bateman et al., 2002; Hansmeier et al., 2006; Cramer et al., 2006), a positive regulatory role of the BenR protein on the transcription of genes belonging to the *ben* and/or *cat* sub-clusters can be assumed. Expression of genes involved in the conversion of benzoate into catechol is also activated in *Acineto-*

bacter sp strain ADP1 and in *P. putida* in response to benzoate, although the regulatory proteins are members of different families and belong to the LysR and AraC/XylS regulators, respectively (Collier et al., 1998; Cowles et al., 2000). The conversion of phenol into catechol may be under positive transcriptional control since the non-syntenic coding regions *cg2966* and *ce2219* both are preceded by a convergently transcribed gene that encodes an AraC-type regulator (Figure 4B; Table 1).

Recently, it was shown that the *catA* gene, encoding catechol 1,2-dioxygenase, is under direct transcriptional control by the RipA repressor, the expression of which is negatively regulated by the DtxR protein (Wennerhold et al., 2005). Transcriptional regulation by RipA is realized by the interaction of the protein with a 14-bp operator sequence that is located 337 bp upstream of the translational start codon of the *catA* gene but only 68 bp upstream of the *benA* translational start (Figure 4C). Binding of the RipA protein to this regulatory region could therefore also interfere with the expression of at least the *benABCD* genes (Figure 4B). However, this direct regulatory interaction of RipA with the *catA-benA* intergenic region apparently links catechol metabolism with the availability of iron since both proteins, RipA and DtxR, constitute a hierarchical regulatory network to control iron homeostasis in *C. glutamicum* at the transcriptional level (Wennerhold et al., 2005; Brune et al., 2006). The physiological link between catechol and iron metabolism is reasonable when considering that catechol 1,2-dioxygenases are iron-containing enzymes (Yamahara et al., 2002). In the presence of excess iron, the iron-sensing regulator DtxR represses transcription of the *ripA* gene, thereby indirectly provoking derepression of the *catA* gene, whereas under iron limitation, repression of *ripA* transcription by DtxR is relieved and *catA* gene expression is repressed by the synthesized RipA protein to reduce cellular iron consumption (Figure 4D). The transcriptional response of *C. glutamicum* to the presence of benzoate or catechol is therefore superimposed by the response of a superior regulatory network that senses the availability of iron within the cell.

TRANSCRIPTIONAL REGULATION OF THE PUTATIVE HYDROXYQUINOL PATHWAYS OF *CORYNEBACTERIUM GLUTAMICUM*

Further experiments concerning the utilization of aromatic compounds as the sole carbon and energy source showed that *C. glutamicum* is able to use resorcinol, 2,4-dihydroxybenzoate and 3,5-dihydroxytoluene for growth (Figure 5A; Shen et al., 2005b). Cultivation of *C. glutamicum* with these carbon sources was associated with hydroxyquinol 1,2-dioxygenase activity. Genome data mining revealed the presence of two gene clusters (Figure 5B, D) encoding putative hydroxyquinol 1,2-dioxygenases and maleylacetate reductases (Kalinowski et al., 2003; Shen et al., 2005b). The putative hydroxyquinol 1,2-dioxygenase genes (*cg1311* and *cg3385*) were individually expressed in *E. coli* and the purified proteins showed hydroxyquinol 1,2-dioxygenase activity in enzyme assays. Whether these proteins are indeed required for the assimilation of the aforementioned aromatic compounds remains to be elucidated.

Transcriptional regulation of expression of the first gene cluster comprising *cg1309* to *cg1311* is apparently exerted by the TetR-type repressor Cg1308 (Table 1; Brune et al., 2005) which is encoded on the opposite DNA strand upstream of *cg1309* (Figure 5B). Deletion of the *cg1308* coding region in the *C. glutamicum* genome resulted in elevated expression of *cg1309*, *cg1310* and *cg1311*, demonstrating that transcription of these genes was derepressed in the mutant strain and is thus under negative transcriptional control. A 20-bp palindromic sequence is

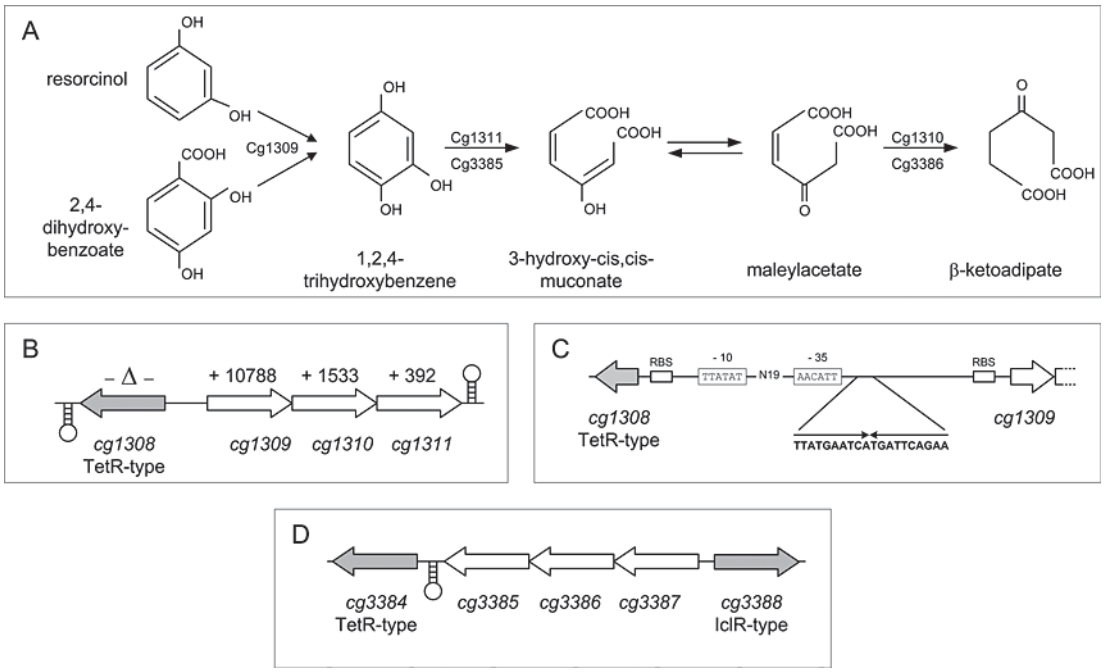

Figure 5. The putative hydroxyquinol pathway of *Corynebacterium glutamicum*. **A.** Predicted metabolic pathway for the conversion of resorcinol and 2,4-dihydroxybenzoate into β-ketoadipate. The corresponding reactions may be catalyzed by hydroxyquinol 1,2-dioxygenase (Cg1311/Cg3385), maleylacetate reductase (Cg1310/Cg3386) and a putative aromatic ring hydroxylase (Cg1309). **B.** Genetic organization of the *cg1308-cg1311* gene region of *C. glutamicum*. Predicted transcriptional terminators are shown by stem-loop structures. Deletion of the *cg1308* gene in *C. glutamicum* is indicated (-Δ-). Relative expression of the divergently transcribed genes *cg1309*, *cg1310* and *cg1311* in the mutant strain compared to that of the *C. glutamicum* wild-type is shown above the arrows. Values were measured by real-time reverse transcription-PCR assays. **C.** Detailed view of the *cg1308-cg1309* intergenic region. The predicted -10 and -35 regions of a *cg1309* promoter are shown along with a putative palindromic operator sequence of Cg1308. The 20-bp inverted repeat was identified by means of the REPuter program (Kurtz and Schleiermacher, 1999). RBS = ribosome-binding site. **D.** Genetic organization of the *cg3384-cg3388* gene region of *C. glutamicum*.

located in the short intergenic region of *cg1308* and *cg1309* and may represent an appropriate operator sequence for the TetR-type repressor Cg1308 (Figure 5C). Transcriptional regulation of the second gene cluster has not been investigated so far. Two potential regulatory genes representing candidates that may be involved in controlling expression of the *cg3387*, *cg3386* and *cg3385* genes flank this gene locus (Figure 5C). The *cg3384* gene encodes a repressor protein of the TetR family, whereas the *cg3388* gene codes for a transcriptional regulator of the IclR family (Table 1; Brune et al., 2005). Deletion of the *cg3384* coding region in the *C. glutamicum* genome did not affect expression of *cg3385*, *cg3386* and *cg3387*, suggesting that the TetR-type repressor Cg3384 is not involved in negative regulation of the expression of the hydroxyquinol pathway genes (Brinkrolf K, Brune I and Tauch A, unpublished data). Moreover, the *cg3388* gene provides the more attractive candidate for hydroxyquinol pathway regulation since genes encoding regulatory proteins of the IclR family are mostly located upstream of the target genes and are transcribed in the opposite direction (Figure 5D). The IclR-type regulator Cg3388 is somehow unusual since analysis of its protein architecture identified two winged helix DNA-binding domains and two GAF-like effector-binding domains (Gough et al., 2001; Martinez

et al., 2002). Compounds acting as effectors of the Cg1308 and Cg3388 regulators await experimental identification, but could be related to resorcinol, 2,4-dihydroxybenzoate, 3,5-dihydroxytoluene, or their degradation products (Shen et al., 2005b).

CONCLUSIONS AND PROSPECTS

Microorganisms generally respond to changes in environmental conditions through the action of specific regulatory systems that not only detect the changes but also develop coordinated cellular responses to adapt to the new conditions. A specific transcriptional response is reasonable when, for instance, the bacterial cell is exposed to aromatic compounds that can be utilized as the sole carbon and energy source (Diaz and Prieto, 2000; Tropel and van der Meer, 2004). *C. glutamicum* possesses five ring-fission dioxygenases that are synthesized only upon exposure of the cells to a variety of aromatic compounds (Shen et al., 2005b). The corresponding dioxygenase genes and the entire enzymatic machinery necessary for the degradation of aromatic compounds are organized in gene clusters that are apparently under specific transcriptional control. As a result of genome data mining and microbiological studies (Brune et al., 2005; Shen et al., 2005b), we now have a profound understanding of the utilization of aromatic compounds as the sole carbon and energy source by *C. glutamicum*. This improvement also includes knowledge about the behavior of transcriptional regulatory proteins in the process of controlling gene expression of the respective catabolic pathways. Consequently, these data have been integrated into the CoryneRegNet database which allows the visualization of transcriptional regulatory networks by using qualitative regulatory interactions (Baumbach et al., 2006). With the exception of the *catA* (*cg2636*) gene, the regulatory interactions involved in the control of the expression of aromatic compound utilization are located separately in the transcriptional regulatory network of *C. glutamicum* and most likely represent single input motifs within the modular and hierarchical network structure (Shen-Orr et al., 2002; Baumbach et al., 2006).

In most cases a relatively clear picture has emerged for the physiological role of the regulatory proteins that control aromatic compound degradation by *C. glutamicum*. The respective transcriptional regulators have been classified into the AraC, IclR, LuxR, PadR, and TetR families of regulatory proteins (Brune et al., 2005). Whereas a number of AraC, IclR and PadR family proteins are already known to be involved in transcriptional control of catabolic pathways for aromatic compounds (Gerischer, 2002; Tropel and van der Meer, 2004; Molina-Henares et al., 2006), the participation of TetR and LuxR family proteins in the regulation of aromatic compound degradation has not been widely recognized so far (Tropel and van der Meer, 2004; Ramos et al., 2005). Moreover, it was possible to identify many of the molecular components that are involved in the regulatory processes as well as the environmental signals that trigger the expression of the catabolic pathways for aromatic compounds in *C. glutamicum*. Both, positive and negative control mechanisms apparently enable the *C. glutamicum* cell to express the respective pathways only in the presence of an inducing substrate. This observation is consistent with the standard model that a positive regulator is bound to its operator site to activate gene expression, whereas the interaction of a negative regulator with its cognate DNA-binding site is relieved by derepression, resulting in elevated transcription of the target genes. However, little is currently known about the exact mechanisms of transcriptional activation and (de)repression by the identified regulatory proteins and about the interaction between these

transcriptional regulators and their effector compounds. This lack of knowledge could be reduced by analyzing *in vitro* the DNA-binding properties of purified regulatory proteins in the presence and absence of potential effector compounds. Additionally, the elucidation of the molecular binding of experimentally identified effector compounds to purified regulatory proteins and determination of the affinity of the active regulator for its cognate operator sequence(s) may help to generate quantitative data that can be integrated into corresponding topological models of the *C. glutamicum* regulatory network. Accordingly, the aspired change-over from a static to a dynamic view of regulatory interactions will be an important challenge for future work in the field of genome-based systems biology of *C. glutamicum*.

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