Aromatic degradative pathways in *Acinetobacter baylyi* underlie carbon catabolite repression

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Carbon catabolite repression is an important mechanism allowing efficient carbon source utilization. In the soil bacterium *Acinetobacter baylyi*, this mechanism has been shown to apply to the aromatic degradative pathways for the substrates protocatechuate, *p*-hydroxybenzoate and vanillate. In this investigation, transcriptional fusions with the gene for luciferase in the gene clusters for the degradation of benzyl esters, anthranilate, benzoate, hydroxycinnamates and dicarboxylates (*are*, *ant*, *ben*, *hca* and *dca* genes) were constructed and established in the chromosome of *A. baylyi*. The respective strains revealed the presence of strong carbon catabolite repression at the transcriptional level. In all cases, succinate and acetate in combination had the strongest repressing effect, and pyruvate (or lactate in case of the *ben* and *hca* genes) allowed the highest expression when these carbon sources were supplied together with the respective inducer. The pattern of repression for the different cosubstrates was similar for all operons investigated and was also observed in the absence of the respective inducing compounds, indicating a mechanism that is independent of the respective specific regulators. Repression by acetate and succinate varied between 88 % for the *hca* genes and 99 % for the *pca* genes.

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INTRODUCTION

To cope with environmental and metabolic changes bacteria have complex regulatory networks. In their natural environments bacteria are usually exposed to a mixture of carbon sources. To allow the cells to use rapidly metabolizable carbon sources first, a global control mechanism represses the specific induction of other degradative pathways despite the presence of their respective substrates to ensure a hierarchical use of individual carbon sources. This higher level of gene regulation is termed carbon catabolite repression (Stülke & Hillen, 1999). There is a profound understanding of the molecular mechanism in both enterobacteria and Gram-positive bacteria. In both these groups of bacteria, sugars are preferred carbon sources and the phosphotransferase system has a central function in the mechanism. In contrast, in bacteria belonging to the genera *Pseudomonas* and *Acinetobacter*, organic acids play an important role as preferred carbon sources and the understanding of the molecular mechanism is far from complete (Gerischer, 2002; Gerischer *et al.*, 2008).

In *Acinetobacter baylyi* (Vanechoutte *et al.*, 2006), carbon catabolite repression has been described affecting the expression of the *pca-qui* operon, the *pob* operon and the *van* genes (Dal *et al.*, 2002). These genes code for enzymes

required for the utilization of protocatechuate (*pca*), *p*-hydroxybenzoate (*pob*), quinate, shikimate (*qui*) and vanillate (*van*). It could be shown that in the presence of the organic acids succinate and acetate in addition to the aromatic compounds the expression level of the *pca-qui* operon was repressed by at least 90 % during the growth phase. The *pca* genes are part of the β -ketoadipate pathway, which allows the organism to utilize a large variety of aromatic compounds (Williams & Kay, 2008). This pathway consists of two parallel branches, starting with protocatechuate (3,4-dihydroxybenzoate) or catechol (Cánovas & Stanier, 1967). The different aromatic substrates are converted to central metabolites via short funnelling pathways (Fig. 1) (Harwood & Parales, 1996). The expression of the genes required for catechol and protocatechuate degradation, respectively, and that of the genes for the funnelling pathways is regulated independently at the level of specific induction (Gerischer *et al.*, 2008). Most of the genes involved in aromatic catabolism are clustered in two of five islands of catabolic genes on the genome of *A. baylyi* (Barbe *et al.*, 2004).

Since a strong catabolite repression of the *pca* operon has been shown (Dal *et al.*, 2002; Siehler *et al.*, 2007) we address here the question if other operons linked with aromatic compound catabolism (*are*, *ant*, *ben*, *hca*, *dca*) also underlie this regulatory mechanism. To answer this question we produced transcriptional fusions between the catabolic genes and the *luc* gene, coding for luciferase.

Abbreviations: RLU, relative light units.

Fig. 1. Catabolic pathways for aromatic compound catabolism in *A. baylyi*. The degradation of dicarboxylic acids appears to proceed through classic β -oxidation and converges with the β -keto adipate pathway at the level of β -keto adipyl-CoA. The names of the respective genes are given next to the arrows.

Using the detected luciferase activity we monitored the gene expression during growth on different carbon sources. We could demonstrate that all five operons underlie strong carbon catabolite repression.

METHODS

Bacterial strains and growth conditions. Strains of *Acinetobacter baylyi* (Table 1) were grown on mineral medium at 30 °C as described earlier (Trautwein & Gerischer, 2001). The carbon sources were used at the following final concentrations unless indicated otherwise: succinate, 10 mM and 30 mM (indicated in the text); pyruvate, 20 mM; lactate, 20 mM; acetate, 15 mM; succinate plus acetate, 15 mM each. For induction the following concentrations were used: *p*-hydroxybenzoate, 1 mM; benzoate, 1 mM; anthranilate, 1 mM; benzyl alcohol, 2 mM; *p*-coumarate, 1 μ M; adipate, 1 mM. Benzyl alcohol, *p*-coumarate and adipate were dissolved in DMSO.

Antibiotics for *A. baylyi* strains were used in the following concentrations: spectinomycin, 100 μ g ml⁻¹; ampicillin 150 μ g ml⁻¹. *Escherichia coli* strains were grown in LB medium at 37 °C supplied with antibiotics when appropriate (spectinomycin, 100 μ g

ml⁻¹, or ampicillin, 100 μ g ml⁻¹). For growth experiments the *A. baylyi* strains with the transcriptional gene fusions were precultured on mineral medium with same carbon source as later used in the experiment except for the aromatic component.

Plasmid and strain construction. Standard methods were used for plasmid isolation, DNA purification, restriction endonuclease cleavage, ligation and transformation (Sambrook & Russell, 2001). To obtain the DNA region of interest from the *A. baylyi* genome a PCR using *Pfu*-DNA polymerase was performed using the primers listed in Table 2 (benA1/benA2, hcaA1/hcaA2, dcaA1/dcaA2, areA1/areA2, antA1/antA2 for the respective genes). For the construction of pAC116 the PCR fragment containing the *benA, B* region was cleaved with *Hind*III and *Kpn*I and inserted into the corresponding sites of pUC18. To create plasmid pAC117 the 3254 bp *hca* region PCR fragment was prepared using *Nhe*I and *Nsi*I. To insert the fragment into pUC18, the vector was cleaved by *Pst*I and *Xba*I and ligated with the PCR fragment using the compatibility between the *Pst*I/*Nsi*I and *Xba*I/*Nhe*I ends. The *dca* PCR fragment was treated with *Psh*AI and *Kpn*I and cloned into pUC18 using *Hinc*II and *Kpn*I, yielding pAC118. The plasmid pAC119 was made by cloning a fragment of the *are* region digested by *Bst*BI and *Sac*I into the pUC18 *Acc*I and *Sac*I sites. To construct plasmid pAC123 the vector pBluescript II SK(+) was

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
<i>A. baylyi</i>		
ADP1	Wild-type (strain BD413, ATCC 33305)	Vaneechoutte <i>et al.</i> (2006)
ADPU47	<i>pcaI-luc</i> transcriptional fusion inserted into <i>pcaI</i>	Siehler <i>et al.</i> (2007)
ADPU92	<i>benA-luc</i> transcriptional fusion inserted into <i>benA</i>	This study
ADPU93	<i>hcaA-luc</i> transcriptional fusion inserted into <i>hcaA</i>	This study
ADPU94	<i>dcaA-luc</i> transcriptional fusion inserted into <i>dcaA</i>	This study
ADPU95	<i>areA-luc</i> transcriptional fusion inserted into <i>areA</i>	This study
ADPU96	<i>antA-luc</i> transcriptional fusion inserted into <i>antA</i>	This study
<i>E. coli</i>		
DH5 α	General cloning strain	Hanahan (1983)
Plasmids		
pAC116	2444 bp fragment containing <i>benA,B</i> region in pUC18	This study
pAC117	3254 bp fragment containing the <i>hcaA,K</i> region in pUC18	This study
pAC118	2964 bp fragment containing the <i>dcaA</i> region in pUC18	This study
pAC119	2993 bp fragment containing the <i>areA</i> region in pUC18	This study
pAC120	<i>benA-luc</i> transcriptional fusion inserted into pAC116	This study
pAC121	<i>hcaA-luc</i> transcriptional fusion inserted into pAC117	This study
pAC122	<i>dcaA-luc</i> transcriptional fusion inserted into pAC118	This study
pAC123	4070 bp fragment containing the <i>antA,B</i> region in pBluescript II SK(+)	This study
pAC124	<i>areA-luc</i> transcriptional fusion inserted into pAC119	This study
pAC125	<i>antA-luc</i> transcriptional fusion inserted into pAC123	This study
pFW11_luc	Contains the <i>Photinus pyralis</i> luciferase gene	Podbielski <i>et al.</i> (1999)
pUC18_luc	<i>luc-aad9</i> cassette from pFW11_luc cloned into pUC18, with <i>Acd</i> and <i>SmaI</i>	Siehler <i>et al.</i> (2007)

was cleaved by *Bam*HI and *Eco*RV and ligated with the *Eco*RV/*Bgl*II-treated PCR fragment containing the *ant* region of *A. baylyi*.

To construct transcriptional fusions between the catabolic genes and the gene for luciferase the *luc-aad9* cassette was prepared from pFW11_luc using *Xho*I and *Sma*I. The cassette was inserted into pAC117 using the *Xho*I and *Sna*BI sites, generating plasmid pAC121, and into pAC118 using the *Xho*I and *Stu*I sites to create plasmid pAC122. In pAC117 the transcriptional fusion was placed 500 bp from the start of the *hcaA* gene sequence, creating a 507 bp deletion in this gene. The *luc-aad9* cassette in pAC122 was introduced 73 bp from the *dcaA* gene start, replacing a 69 bp *dcaA* fragment. For the

construction of pAC120 and pAC124, the plasmid pUC18_luc was used. From this plasmid the *luc-aad9* cassette could be obtained as a *Pst*I fragment and cloned into pAC116 and pAC119 linearized with *Nsi*I. Plasmid pAC119 has two *Nsi*I recognition sites in close proximity. The cleavage by *Nsi*I created a deletion of 787 bp in the *areA/salD* gene region and was used to insert the *luc-aad9* cassette. The *areA-luc* fusion is located 445 bp downstream of the transcriptional start of *areA*. In plasmid pAC116 the *Nsi*I insertion site placed the *luc-aad9* cassette 1103 bp downstream from the *benA* gene start. To create plasmid pAC125, the *luc-aad9* cassette was removed from plasmid pFW11_luc using the *Xmn*I and *Sma*I sites and cloned into pAC123 cut with *Nru*I. The cassette was introduced 88 bp after the *antA* gene start. In all cases, the orientation of the cloned fragments was verified by restriction analysis.

For the construction of *A. baylyi* strain ADPU92 the *A. baylyi* DNA containing the *benA-luc* transcriptional fusion was separated from the vector backbone of pAC120 using the *Dra*III and *Eco*RI sites. The linear fragment was used for transformation of *A. baylyi* strain ADP1. A selection for spectinomycin resistance encoded by *aad9* of the *luc-aad9* cassette was applied to ensure the homologous recombination of *A. baylyi* DNA. The other *A. baylyi* strains containing the transcriptional fusion were made as for strain ADPU92 except for the restriction sites used to remove the insert from the corresponding plasmids. To generate strain ADPU93, plasmid pAC121 was cleaved by *Bam*HI and *Bgl*II; to obtain strain ADPU94 the *dcaA-luc* fusion was cut out from pAC122 by *Eco*47III and *Sac*I. The plasmid pAC124 was linearized by *Pml*I and *Pst*I and used to create strain ADPU95. ADPU96 was generated by transformation of *A. baylyi* with the *Pml*I–*Mlu*I fragment from pAC125.

PCR analysis was employed to verify the correct integration of the constructs into the corresponding location on the chromosome of *A. baylyi* strain ADP1. For this PCR the primer pair comprised one primer hybridizing in the *luc-aad9* cassette (*luc* primer) and a second primer (*ben3*, *hca3*, *dca3*, *are3*, *antA1*) binding in the immediate

Table 2. Oligonucleotides

Primer	Sequence (5'–3')
hcaA1	CGCTTTGAGCAAGCGTAAAC
hcaA2	CAGATTGAGCGCGAACTG
hca3	GCCTCTTGTCGAATCAGTGATG
dcaA1	CTTCTTCTGCTGGAACCATGAC
dcaA2	TGGCAGGAGTATTCAGGTACAC
dca 3	GATCGACTGGTTGGCTAATGC
benA1	GCGGCTTTGGTGAAGCTTTGCTCC
benA2	AAAGCACGGCAGGTACCACACG
ben3	GTACGTTTAATGGCGGGATCAC
luc	AAACCGGGAGGTAGATGAGATG
areA1	ATCGGCTGCTAGAGTAAG
areA2	ACTCAGTGGCAATGGATG
are3	CCCTGCAATAGGTGAGATATCG
antA1	ACATCCAGCACCACTTTACG
antA2	CCTGATTTGCCCTTGCCATTG

vicinity of the fusion on the chromosome but outside the DNA used for transformation. In all strains the expected PCR fragment could be amplified, thus confirming the desired construction.

Transformation of *A. baylyi* strain ADP1. For transformation of *A. baylyi*, 200 µl cell suspension of an overnight culture was transferred in fresh mineral medium supplied with 10 mM succinate and incubated for 2 h at 30 °C to allow the cells to start growing again. To 500 µl of these cells 0.1–1 µg linear DNA was added and incubated at 37 °C for 3 h or overnight. Then 100 µl volumes of the cells were spread on selective plates and incubated at 30 °C. Grown *A. baylyi* mutants were transferred on fresh selective plates several times to ensure a pure culture.

PCR. Cells from a bacterial colony diluted in water were used as chromosomal templates. The conditions using *Pfu* DNA polymerase and *Taq* DNA polymerase were 95 °C for 3 min, followed by 25–30 cycles of denaturation at 95 °C for 1 min, annealing at 53–68 °C for 1 min (depending on the primers), and extension at 72 °C for an appropriate length of time. The primers used are listed in Table 2. For working with *Pfu* DNA polymerase the extension time was doubled.

Determination of luciferase enzyme activity. D-Luciferin was added to a sample of *A. baylyi* cells taken at different times during growth. The luciferase enzyme activity was detected as described earlier (Siehler *et al.*, 2007). The resulting light emission was measured and expressed in relative light units (RLU). The results were normalized by dividing the RLU by the respective OD₆₀₀ value. The data presented for each culture are taken from mid-exponential-phase cultures and are means based on samples from at least three independent cultures. Error bars indicate standard deviation.

RESULTS

Construction of *A. baylyi* strains with transcriptional fusions between *benA*, *hcaA*, *dcaA*, *areA* or *antA* and the luciferase gene

To detect the effect of catabolite repression we produced transcriptional fusions between the relevant structural gene promoter and the gene for *Photinus pyralis* luciferase. For the construction the region of interest was amplified by PCR and introduced into a cloning vector. The *luc* cassette, containing the luciferase (*luc*) gene and a spectinomycin-resistance gene, was inserted into the genes *benA*, *hcaA*, *dcaA*, *areA* and *antA*. The plasmid backbone was separated from the gene construct and the latter was introduced into the chromosome of *A. baylyi* strain ADP1 via homologous recombination, using the ability for natural transformation of this organism. The newly generated strains contained one copy of the respective transcriptional fusion per cell and thus allowed the determination of gene expression. To examine the expression patterns of the respective promoters we used different carbon sources (lactate, pyruvate, succinate, acetate and a combination of succinate and acetate). The gene expression was induced by adding the specific aromatic compound to the medium. Due to the insertion of the *luc* cassette the formation of the respective enzyme in each pathway was prevented and further degradation of the respective metabolites was blocked. Therefore the aromatic compounds were added in low

concentrations as an inducer. Their concentration was assumed to remain unchanged due to the interrupted catabolism, thus ensuring permanent induction.

Carbon catabolite repression is observed in the *ben* and *ant* operons

Benzoate and anthranilate are independently converted to catechol, allowing further degradation to proceed via the β -ketoadipate pathway. The *antABC* genes coding for anthranilate dioxygenase (Bundy *et al.*, 1998; Eby *et al.*, 2001) and the *benABC* genes coding for benzoate dioxygenase (Neidle *et al.*, 1991) are homologous. The *ben* operon is regulated by BenM, a LysR family regulator able to activate transcription synergistically, responding to two effectors (benzoate and muconate) (Bundy *et al.*, 2002; Collier *et al.*, 1998; Ezezika *et al.*, 2007a, b). In studies using a chromosomal *antA-lacZ* transcriptional fusion it could be shown that the *antABC* expression is induced by anthranilate, but a regulatory protein has not been identified (Bundy *et al.*, 1998). According to these data the *ben-luc* and *ant-luc* constructs were induced by adding benzoate or anthranilate, respectively, to the medium.

The expression of the *antA* gene in strain ADPU96 was investigated during growth on different non-inducing carbon sources with or without the inducer anthranilate (Fig. 2). In all cases the inducing effect of anthranilate (16–58-fold) was obvious. Despite the presence of the same concentration of the inducer in all cultures, there were big differences in expression depending on the nature of the non-inducing carbon source. Growth on a combination of succinate plus acetate and inducer resulted in a repression

Fig. 2. (a) *ant-luc* transcriptional fusion in *A. baylyi* strain ADPU96. (b) Luciferase activity of strain ADPU96 measured after growth with the indicated carbon sources in the presence (white bars) or absence (black bars) of 1 mM anthranilate.

by 95 % as compared to pyruvate. Growth on succinate and inducer had the same repressing effect whereas lactate and acetate each had an intermediate repressing effect. Pyruvate was the carbon source allowing the highest induction.

The strain containing the *benA-luc* fusion was induced by adding 1 mM benzoate. Using succinate and acetate as a carbon source led to a strong decrease of *ben* promoter activity in comparison with the least repressing substrate (54-fold, Fig. 3). Succinate and acetate alone caused a light repression (about 2-fold). The highest induction of the *ben-luc* fusion was observed on lactate. Thus, the *benA* promoter showed a similar repression pattern to the *antA* promoter, with the slight difference that lactate and not pyruvate was the least repressing carbon source.

Repression of the *are* operon by additional carbon sources

The *areA*, *-B*, *-C* and *-R* genes, encoding enzymes which convert benzyl esters such as benzyl acetate through benzyl alcohol and benzaldehyde to benzoate, are located at one end of the *ben-cat* cluster (Jones *et al.*, 1999). The *are* operon is regulated by *areR*, a XylR-like transcriptional regulator (Jones & Williams, 2001). For the *areA-luc* construct the *luc* cassette was introduced into the *areA* gene and the transcription was induced by benzyl alcohol. In this strain the utilization of benzyl esters, catalysed by *AreA*, is blocked but the conversion of benzyl alcohol through benzaldehyde to benzoate followed by conversion to catechol is enabled by *AreCB*, because the genes are transcribed in the order *areCBA*. In this case the added

inducer can be used as a carbon source and was added to the medium at a higher concentration (2 mM).

Using pyruvate in mineral medium supplied with benzyl alcohol led to the highest expression levels (Fig. 4). The strongest reduction of activity was observed after simultaneous addition of succinate and acetate, or succinate alone (reduction by 96 % or 98 %, respectively). In summary, all three operons which funnel their substrates into the catechol branch show a transcriptional regulation which strongly depends on the nature of the carbon source(s) present in addition to the substrate inducer. In all cases succinate and acetate were the strongest repressing cosubstrates.

The *hca* genes are strongly repressed by acetate and succinate

The Hca enzymes catalyse the conversion of derivatives of 4-hydroxycinnamic acid (chlorogenate, ferulate, caffeate and *p*-coumarate) into protocatechuate (Smith *et al.*, 2003) (Fig. 1). The HcaC CoA ligase initiates metabolism of all these substrates by converting the compounds to the corresponding hydroxycinnamoyl-CoA thioesters. They can act as inducers and relieve the repression caused by the repressor HcaR (a MarR-like regulator). To induce expression of the *hca* genes, *p*-coumarate was used. It was added in a low concentration (1 μ M) because of the known toxic effect caused by the presence of accumulated *p*-coumaroyl-CoA (Parke & Ornston, 2004).

Fig. 3. (a) Map of the *ben* operon, with the position of the *luc* cassette in *A. baylyi* strain ADPU92 indicated. (b) *benA* promoter activity in strain ADPU92. Luciferase activity was measured after growth with the indicated carbon sources in the presence (white bars) or absence (black bars) of 1 mM benzoate.

Fig. 4. (a) Map of the *are* gene region of *A. baylyi* strain ADPU95, showing the insertion position of the *luc* cassette replacing the indicated 787 bp *salD-areA* fragment. (b) *are* operon expression in strain ADPU95. The cells were grown on mineral medium containing the indicated carbon sources in the absence (black bars) or presence (white bars) of 2 mM benzyl alcohol.

A combination of succinate and acetate in the presence of *p*-coumarate led to a 14-fold reduced luciferase activity in comparison with lactate (Fig. 5). As in strain ADPU92 (*benA-luc*), lactate as a carbon source resulted in the highest *hca* operon expression, followed by pyruvate. Thus the *hca* operon underlies a strong catabolite repression following the same pattern as observed for other operons connected to the protocatechuate branch of the pathway.

The *dca* genes underlie catabolite repression

The *dca* operon is part of the *dca-pca-qui-pob-van-hca* supraoperonic gene cluster involved in aromatic compound degradation (Smith *et al.*, 2003). However, the enzymes encoded by the *dca* genes are required for the utilization of saturated, straight-chain dicarboxylic acids (Parke *et al.*, 2001). The degradation appears to proceed through classic β -oxidation. The required enzymes are encoded by two divergent gene clusters, *dcaECHF* and *dcaAKIJP*. The dicarboxylic catabolic pathway and the β -ketoadipate pathway converge at the level of β -ketoadipyl-CoA but individual enzymes catalyse for each pathway (an observation that also applies to the β -ketoadipate pathway itself) (Parke *et al.*, 2001; Young *et al.*, 2005).

The regulator protein of the *dca* operon is DcaR, which belongs to the IclR family (Parke *et al.*, 2001). For the luciferase activity assays the *luc* cassette was introduced into the *dcaA* gene. The specific gene expression was

Fig. 5. (a) *hca-luc* transcriptional fusion in *A. baylyi* strain ADPU93. Insertion of the *luc* cassette led to a deletion of 507 bp from the *hcaA* gene. (b) Luciferase activity of strain ADPU93 measured after growth with the indicated carbon sources in the presence (white bars) or absence (black bars) of 1 μ M *p*-coumarate.

induced by adding 1 mM adipate to the medium, which was sufficient since the degradation of the inducer was prevented by the *luc* gene fusion. As shown in Fig. 6 the activity on succinate plus acetate with adipate decreased by 93% in comparison to the combination of pyruvate and adipate. Lactate led to a slight repression, and acetate or succinate alone to a stronger repression of *dcaA* expression. These observations strengthened the evidence that all operons of the *dca-pca-qui-pob-van-hca* cluster show in principle the same expression pattern in response to the presence of additional carbon sources.

The carbon-source-dependent expression pattern is independent of the induction status

Looking at the results from the reporter gene studies done with all five operons we find a strong common pattern. All the constructs show the strongest decrease of activity on succinate plus acetate followed by succinate and acetate alone. The highest expression was observed when lactate or pyruvate was used as a carbon source.

This pattern was observed in the presence of the respective substrate inducer as well as in its absence (black bars in Figs 2–6). Thus the differential expression on the non-inducing carbon sources investigated here is independent of the induction status, an observation that had been made earlier for the *pca-qui* operon (Siehler *et al.*, 2007; Trautwein & Gerischer, 2001).

Fig. 6. (a) Map of the *dca* operon, with the position of the *luc* cassette in *A. baylyi* strain ADPU94 indicated. Insertion of the *luc* cassette led to the loss of 69 bp from the *dcaA* gene sequence. (b) Luciferase activity of strain ADPU94. The enzyme activities were measured after growth with the indicated carbon sources in the absence (black bars) or presence (white bars) of 1 mM adipate.

DISCUSSION

Strong repression of all operons investigated

All the operons we investigated showed a strong reduction of promoter activity when the carbon source combination succinate plus acetate in addition to the specific substrate was used (at least 90 %, Fig. 7). The respective genes are independent regulatory units and underlie specific regulation by different inducer metabolites and different regulatory proteins. Further, they are located in different regions of the chromosome of *A. baylyi*. These observations are thus in accordance with the phenotype of the global regulatory mechanism carbon catabolite repression.

Acetate is converted into acetyl-CoA (an energy-consuming step), which subsequently feeds into the glyoxylate cycle. Succinate is a metabolite of the citric acid cycle; acetyl-CoA becomes available by the action of malic enzyme forming pyruvate from malate. Both substrates are subsequently oxidized and used for the conservation of energy. Succinate and acetate (or the respective CoA esters) are also the products of the β -ketoadipate pathway, which in itself does not involve energy conservation. It thus makes sense that these compounds, when present at higher levels, lead to a repression of gene expression in the β -ketoadipate pathway. Pyruvate and lactate do not – these metabolites are just one or two steps away from acetyl-CoA (catalysed by pyruvate dehydrogenase and lactate dehydrogenase, respectively). It can be speculated that acetate and succinate are abundant substrates in the natural habitats of *A. baylyi*, whereas pyruvate and lactate are not, and therefore no regulatory response has been developed for the latter two. Of course, transport of the substrates into the cells is a process that may also be regulated and therefore also has to be considered. To our knowledge, the

Fig. 7. Extent of repression for the operons investigated. The luciferase activity in all constructed strains on succinate plus acetate (strongest reduction of activity; white bars, target gene indicated) was compared with the corresponding expression on pyruvate (set to 100 %, black bar), in the presence of the inducer in all cases. The data for the *pcal-luc* fusion are derived from measurements of strain ADPU47 growing with or without the inducer *p*-hydroxybenzoate (1 mM).

topic of expression/activity of transporters relevant for the substrates concerned in this study has not been investigated.

Operons affected by the repression in *A. baylyi*

Repression of catabolic enzymes forming the β -ketoadipate pathway was described about 40 years ago in *Moraxella calcoacetica* (Cánovas & Stanier, 1967), an organism later renamed *Acinetobacter calcoaceticus*. More recent reports have documented the repression by succinate of many different genes. An *A. baylyi* strain with additional copies of the *antABC* genes carried on a plasmid lost the ability to use anthranilate as a carbon source (Bundy *et al.*, 1998). The expression of the *antABC* genes *in trans* in *A. baylyi* was likely to cause the formation of a higher-than-normal level of catechol during anthranilate catabolism. Catechol probably contributed to the inhibition of growth. The strain grew, however, when succinate was provided together with anthranilate. It was proposed that succinate allows growth by reducing the expression of the *antABC* genes, supporting the findings presented here that succinate is a substrate causing carbon catabolite repression of the *antA,B,C* genes.

Further evidence for repression caused by succinate was reported by Jones & Williams (2001). Benzyl esterase activity on the aromatic compound alone was 33 % higher than in the presence of succinate plus benzyl alcohol. Benzyl alcohol dehydrogenase activity was reduced by 63 % when succinate was provided together with benzyl alcohol as compared to benzyl alcohol alone.

In the *hca* operon, a transcriptional *hcaE-lacZ* fusion was used to detect the promoter activity (Parke & Ornston, 2004). The detected β -galactosidase activity at the expense of succinate and *p*-coumarate decreased by 53 % in comparison with the activity measured on *p*-coumarate alone. Using ferulate as substrate in the presence of succinate reduced the activity by 65 %.

All these data suggested that multiple operons are repressed in the presence of succinate and an aromatic compound. Our results provide more evidence for this. In terms of quantitative expression differences it has to be kept in mind which method was used. It has been documented that measurements based on luciferase or β -galactosidase do differ with respect to induction or repression factors, which is due to the lower stability of the luciferase protein (Siehler *et al.*, 2007).

All the operons investigated here are part of two large clusters that each encode pathways for catabolism of plant-derived carbon sources of mainly aromatic nature (*are-sal-ben-cat* and *dca-pca-qui-pob-hca*) (Young *et al.*, 2005).

Clues to the molecular mechanism

Several lines of evidence indicate an involvement of Cyo (cytochrome *o* ubiquinol oxidase) in catabolite repression (Dinamarca *et al.*, 2002; Morales *et al.*, 2006; Petruschka

et al., 2001). Furthermore, evidence for a role of protein IIA^{Ntr} (PtsN) in catabolite repression of *Pseudomonas putida* has been presented (Cases *et al.*, 2001). In some cases evidence has been adduced that the specific regulator protein is the target of catabolite repression. An example is (methyl)phenol degradation in *P. putida* H (Müller *et al.*, 1996) or catabolite repression of the *alk* genes in *P. putida* GPo1 via downregulation of the level of the AlkS protein (Yuste & Rojo, 2001). It should be noted that both alkane and phenol degradation pathways are encoded on a plasmid of *P. putida*. Furthermore, transcription requires σ^{54} (RpoN)-containing (*phl* operon) or σ^S -containing (*alk* operon) RNA polymerase. Neither of these two conditions applies to the operons under catabolite repression control in *A. baylyi* (except for the *are* operon, which depends on σ^{54}). Nevertheless an involvement of the regulators cannot be ruled out at this point. For PcaU, the activator/repressor protein of the *pca-qui* operon, it has been documented that the *pcaU* transcript level corresponds to changes in the amount of the *pca-qui* transcript levels under all conditions tested. Whether the *pcaU* transcript level simply is affected by the same mechanism as the *pca-qui* transcript or is involved in causing the repression is unknown (Siehler *et al.*, 2007).

Crc is the best-studied known player in catabolite repression

The first potential component of the mechanism described is the Crc (catabolite repression control) protein (MacGregor *et al.*, 1991). Inactivation of the *crc* gene relieved the repression of a number of genes implicated in the metabolism of carbohydrates (Wolff *et al.*, 1991) and nitrogenated compounds in *Pseudomonas aeruginosa* and *P. putida* (Collier *et al.*, 1996; Hester *et al.*, 2000a).

In the *P. putida* OCT plasmid alkane degradation pathway Crc participates in the repression observed when cells grow in rich medium, but it plays no role in the repression caused in mineral medium by organic acids (Yuste & Rojo, 2001). The number of genes that display an influence by Crc in their expression has been increased by proteome and RT-PCR analyses to include two of the three aromatic catabolic pathways (Morales *et al.*, 2004).

Crc seems to be involved in post-transcriptional regulation in *P. putida* (Hester *et al.*, 2000b). In recent reports it could be demonstrated that Crc can bind specifically to the translation initiation region of two mRNAs for transcriptional regulators (AlkS, BenR), suggesting that Crc modulates gene expression by hindering the access of ribosomes to the ribosome-binding site (Moreno & Rojo, 2007; Moreno *et al.*, 2007).

A. baylyi contains a Crc homologue. Its inactivation caused withdrawal of catabolite repression upon *pca-qui* operon expression. In this organism a very strong increase in transcript stability was observed upon *crc* deletion for the *pca-qui* operon (U. Gerischer and others, unpublished

data). This may be indicative of different activities of Crc in different organisms and/or at different genes. It will be exciting to explore Crc function with respect to the *A. baylyi* operons investigated here.

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