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The β-ketoadipate pathway of *Acinetobacter baylyi* undergoes carbon catabolite repression, cross-regulation and vertical regulation, and is affected by Crc

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The degradation of many structurally diverse aromatic compounds in *Acinetobacter baylyi* is accomplished by the β-ketoadipate pathway. In addition to specific induction of expression by certain aromatic compounds, this pathway is regulated by complex mechanisms at multiple levels, which are the topic of this study. Multiple operons feeding into the β-ketoadipate pathway are controlled by carbon catabolite repression (CCR) caused by succinate plus acetate. The pathways under study enable the catabolism of benzoate (*ben*), catechol (*catA*), *cis*,*cis*-muconate (*catB*,*C*,*I*,*J*,*F*,*D*), vanillate (*van*), hydroxycinnamates (*hca*), dicarboxylates (*dca*), salicylate (*sal*), anthranilate (*ant*) and benzyl esters (*are*). For analysis of CCR at the transcriptional level a luciferase reporter gene cassette was introduced into the operons. The Crc (catabolite repression control) protein is involved in repression of all operons (except for *catA*), as demonstrated by the analysis of respective *crc* strains. In addition, cross-regulation was demonstrated for the *vanA*,*B*, *hca* and *dca* operons. The presence of protocatechuate caused transcriptional repression of the *vanA*,*B*- and *hca*-encoded funnelling pathways (vertical regulation). Thus the results presented extend the understanding both of CCR and of the effects of Crc for all aromatic degradative pathways of *A. baylyi* and increase the number of operons known to be controlled by two additional mechanisms, cross-regulation and vertical regulation.

INTRODUCTION

The bacterium *Acinetobacter baylyi* is a soil organism known to be able to use aromatic substances through the β-ketoadipate pathway (Harwood & Parales, 1996). Numerous more complex aromatic compounds can be converted into the two central starting compounds of the β-ketoadipate pathway, protocatechuate (PCA) and catechol, by additional short metabolic pathways (funnelling pathways). The expression of all the respective operons is thoroughly controlled by specific inducers. Furthermore, to cope with an array of environmental changes, the β-ketoadipate pathway and its funnelling pathways are controlled by a regulatory network the complexity of which is only beginning to be elucidated (Vaneechoutte *et al.* 2006; Gerischer, 2008; Williams & Kay, 2008). One part of this network is carbon catabolite repression (CCR) (Cánovas & Stanier, 1967; Tresguerres *et al.*, 1970; Dal *et al.*, 2005; Fischer *et al.*, 2008). The molecular mechanisms of CCR are well understood in *Escherichia coli* and Gram-positive bacteria such as *Bacillus subtilis*, but not in bacteria belonging to the genera *Pseudomonas* and *Acinetobacter*. In these bacteria, organic acids such as succinate and acetate, as well as the protein Crc (catabolite repression control), play an important role in CCR (Wolff *et al.*, 1991; Zimmermann *et al.*, 2009). *A. baylyi* Crc has been shown to be involved in the degradation of the *pca-qui* transcript, which encodes enzymes dealing with quinate and PCA degradation (Zimmermann *et al.*, 2009). In *Pseudomonas putida*, Crc affects the expression of genes involved in aromatic compound degradation (*ben*, *cat*, *pca* and *pobA*) (Morales *et al.*, 2004). Direct binding of Crc to the RNA region directing translation of the regulators BenR and AlkS was demonstrated, indicating translational repression (Moreno *et al.*, 2007; Moreno & Rojo, 2008).

In addition to CCR, cross-regulation becomes effective when mixtures of substrates feeding into both branches of the pathway are presented to an organism. In *A. baylyi*, cross-regulation results in a dominance of the catechol branch over the PCA branch (Brzostowicz *et al.*, 2003; Siehler *et al.*, 2007). There is evidence that transcriptional

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Abbreviations: CCR, carbon catabolite repression; PCA, protocatechuate; POB, *p*-hydroxybenzoate.

regulators BenM and CatM, which can bind regulatory regions upstream of *pcaU* (Gerischer *et al.*, 1998), are involved in this cross-regulation. Finally, vertical regulation has also been observed in the presence of PCA. For example, the degradation of *p*-hydroxybenzoate (POB) is repressed by its own reaction product, PCA (Brzostowicz *et al.*, 2003; Siehler *et al.*, 2007).

In this investigation we analysed more operons with respect to CCR, cross-regulation and vertical regulation. Given the indicated global nature of the mechanism it is relevant to know whether multiple operons are affected, and in the future to examine whether the same mechanism is the cause. We used chromosomal luciferase reporter gene fusions in operons encoding enzymes for the degradation of benzoate, benzyl esters, anthranilate, hydroxycinnamates, dicarboxylic acids, vanillate, salicylate and catechol to gain a more comprehensive understanding of gene expression within the aromatic degradative pathways of *A. baylyi*. We addressed the involvement of Crc by investigating *crc* strains in parallel. We showed that all the operons analysed are affected by CCR; the withdrawal of Crc derepresses gene expression in most cases. Cross-regulation and/or vertical regulation was observed in all operons investigated (cross-regulation in *hca*, *van* and *dca*; vertical regulation in *hca* and *van*).

METHODS

Bacterial strains and growth conditions. Strains of *A. baylyi* were grown on minimal medium at 30 °C as described earlier (Trautwein & Gerischer, 2001). Carbon sources were used at the following concentrations unless indicated otherwise: pyruvate, 20 mM; lactate, 20 mM; gluconate, 20 mM; acetate, 15 mM; succinate, 30 mM; succinate and acetate, 15 mM each. The following concentrations were used for induction: benzoate, 0.5 mM; *p*-coumarate, 1 μM; adipate, 1 mM; vanillate, 0.5 mM; salicylate, 0.5 mM; anthranilate, 1 mM; benzyl alcohol, 2 mM. Benzyl alcohol, *p*-coumarate, vanillate and adipate were dissolved in DMSO. Antibiotics for *A. baylyi* strains were used at the following concentrations: 100 μg spectinomycin ml^{-1}; 20 μg streptomycin ml^{-1}.

Strains of *E. coli* were grown in LB medium at 37 °C. Antibiotics were used in the following concentrations: 100 μg ampicillin ml^{-1}; 100 μg spectinomycin ml^{-1}; 20 μg streptomycin ml^{-1}.

For growth experiments, *A. baylyi* strains (Table 1) with luciferase transcriptional gene fusions were precultured on minimal medium complemented with the carbon source that would later be used in the experiment (except for the aromatic compound).

Plasmid and strain construction. To integrate the luciferase reporter gene into specific genes, PCRs were performed with primers listed in Table 2, using chromosomal DNA from *A. baylyi* as a template. The fragments were cleaved with restriction enzymes and cloned (Table 1). After integration of the *luc* cassette, the fusion constructs were cleaved with the indicated enzymes and used for transformation of *A. baylyi*. The *aad9*-mediated spectinomycin resistance was used for selection. The restriction sites used for the plasmid and strain construction were native sites in all cases. Standard methods were used for plasmid isolation, DNA purification, restriction endonuclease cleavage, ligation and transformation. Transformation of *A. baylyi* was done as described by Fischer *et al.* (2008).

To verify that luciferase fusions were integrated into the genome of *A. baylyi* strain ADP1 at the correct position, PCR analysis was employed, using a gene-specific primer (catA5, catB1, vanB2, vanK1 and salA1) and the luc primer (Table 2), specific for the *luc-aad9* cassette. The gene-specific primer targeted a sequence outside the DNA that was used for transformation.

Plasmid pAC57 was used to disrupt the *crc* gene in all strains containing the chromosomal luciferase reporter gene fusion (Zimmermann *et al.*, 2009). pAC57 carries a *crc* gene that was rendered non-functional by the insertion of an Ω cassette, which carries a spectinomycin and streptomycin resistance gene. This construct was cleaved from the vector backbone by the restriction endonucleases *Xba*I and *Pst*I and used for transformation of strains containing a luciferase reporter gene fusion to create the respective *crc* strain (Table 1). Growth in the presence of spectinomycin and streptomycin was used to identify candidates with the desired modification. PCR analysis with primers crc3 and crc4 (Table 2) was employed to confirm the correct integration of the construct into the corresponding region on the chromosome. Again, primers targeted loci outside the DNA that had been used for transformation.

PCR. Cells of an overnight culture were suspended in water, boiled for 10 min, cooled on ice and centrifuged. The supernatant contained the chromosomal DNA and was used as template. The conditions using *Taq* DNA polymerase were 95 °C for 3 min, followed by 30 cycles of denaturation for 1 min at 95 °C, annealing for 1 min at 53 to 68 °C (depending on the primers) and elongation at 72 °C for a variable period (depending on the length of the amplified fragment).

Determination of luciferase activity. At different time points during growth, samples of *A. baylyi* cells were taken and mixed with D-luciferin. The luciferase activity was detected as described earlier (Siehler *et al.*, 2007). The measured relative light units (RLU) were divided by the respective OD_{600} to normalize the results. A value from the mid-exponential growth phase was read as characteristic for the strain and condition. Each growth experiment was carried out at least three times independently. The luciferase activity on different carbon sources was normalized to the corresponding activity on pyruvate (set to 100 %), or with the corresponding activity of the parental strain (crc^+) on succinate and/or acetate (set to 100 %) for the *crc* strains. Error bars indicate standard deviation.

RESULTS

CCR of operons encoding funnelling pathways for aromatic compound degradation in *A. baylyi*

To investigate CCR, growth experiments were performed with the strains carrying *catA-luc*, *catB,C,I-luc*, *vanA,B-luc*, *salA-luc* and *vanK-luc* transcriptional fusions (Fig. 1). Prior to this study, nothing was known about CCR of these operons by succinate and/or acetate. In all approaches, strains were grown on succinate and/or acetate with the specific aromatic inducer added (*catA-luc*, *catB,C,I-luc*, 0.5 mM benzoate; *vanA,B*, *vanK-luc*, 0.5 mM vanillate; *salA-luc*, 0.5 mM salicylate). We evaluated the effect of the carbon sources alone or in combination since it is known for the *pca-qui* operon that they have a much stronger effect in combination (Dal *et al.*, 2002). The resulting luciferase activities were compared with the activity after growth on the non-repressing carbon source pyruvate with the specific aromatic inducer (Table 3). The presence of

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
A. baylyi		
ADP1	Wild-type (strain BD413, ATCC 33305)	Juni & Janik (1969)
ADPU92	*benA-luc* fusion, inserted into *benA*	Fischer *et al.* (2008)
ADPU93	*hcaA-luc* fusion, inserted into *hcaA*	Fischer *et al.* (2008)
ADPU94	*dcaA-luc* fusion, inserted into *dcaA*	Fischer *et al.* (2008)
ADPU95	*areA-luc* fusion, inserted into *areA*	Fischer *et al.* (2008)
ADPU96	*antA-luc* fusion, inserted into *antA*	Fischer *et al.* (2008)
ADPU97	*benA-luc* fusion, inserted into *benA*, Δ*crc*Ω, 50 bp *Bsg*I fragment	This work
ADPU98	*hcaA-luc* fusion, inserted into *hcaA*, Δ*crc*Ω, 50 bp *Bsg*I fragment	This work
ADPU99	*dcaA-luc* fusion, inserted into *dcaA*, Δ*crc*Ω, 50 bp *Bsg*I fragment	This work
ADPU100	*catA-luc* fusion, inserted into *catA*	This work
ADPU101	*catB,C,I-luc* fusion, inserted into *catI*	This work
ADPU102	*vanA,B-luc* fusion, inserted into *vanB*	This work
ADPU104	*catA-luc* fusion, inserted into *catA*, Δ*crc*Ω, 50 bp *Bsg*I fragment	This work
ADPU105	*catB,C,I-luc* fusion, inserted into *catI*, Δ*crc*Ω, 50 bp *Bsg*I fragment	This work
ADPU106	*vanA,B-luc* fusion, inserted into *vanB*, Δ*crc*Ω, 50 bp *Bsg*I fragment	This work
ADPU110	*salA-luc* fusion, inserted into *salA*	This work
ADPU111	*salA-luc* fusion, inserted into *salA*, Δ*crc*Ω, 50 bp *Bsg*I fragment	This work
ADPU112	*vanK-luc* fusion, inserted into *vanK*	This work
ADPU113	*vanK-luc* fusion, inserted into *vanK*, Δ*crc*Ω, 50 bp *Bsg*I fragment	This work
ADPU120	*areA-luc* fusion, inserted into *areA*, Δ*crc*Ω, 50 bp *Bsg*I fragment	This work
ADPU121	*antA-luc* fusion, inserted into *antA*, Δ*crc*Ω, 50 bp *Bsg*I fragment	This work
E. coli		
DH5α	F^- *ϕ*80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *deoR recA1 endA1 hsdR17*($r_k^- m_k^+$) *phoA supE44* λ^- *thi-1 gyrA96 relA1*	Hanahan (1983)
Plasmids		
pBluescript II SK(+)	Ap^r, *lacZ*	Stratagene; Alting-Mees & Short (1989)
pUC18	Ap^r, *lacZ*	Yanisch-Perron *et al.* (1985)
pFW11_luc	Contains the *Photinus pyralis* luciferase gene, *luc-aad9*	Podbielski *et al.* (1999)
pAC57	Δ*crc*Ω insertion, 50 bp deletion between two *Bsg*I restriction sites and insertion of the $\Omega Sm^r Spc^r$ cassette	Zimmermann *et al.* (2009)
pAC126	pUC18 with a 3371 bp *Hin*cII–*Xba*I fragment, containing *catA*	This work
pAC127	pBSKII with a 3709 bp *Eco*RI–*Sac*II fragment, containing the *catB,C,I,J,F* region	This work
pAC128	pBSKII with a 2267 bp *Psh*AI–*Xba*I fragment, containing the *vanA,B* region	This work
pAC129	pBSKII with a 2810 bp *Eco*RV/*Xba*I fragment, containing *vanK*	This work
pAC130	*luc*Spc^r *Xmn*I–*Bss*HII fragment from pFW11_luc ligated into pAC126 *Swa*I/*Bss*HII (*catA-luc* fusion; cleaved from the vector backbone with *Nco*I and *Pml*I)	This work
pAC131	*luc*Spc^r *Xmn*I–*Sma*I fragment from pFW11_luc ligated into pAC127 *Eco*47III (*catB,C,I-luc* fusion; cleaved from the vector backbone with *Pml*I and *Kas*I)	This work
pAC132	*luc*Spc^r *Xmn*I–*Nco*I fragment from pFW11_luc ligated into pAC128 *Swa*I/*Nco*I (*vanA,B-luc* fusion; cleaved from the vector backbone with *Xba*I and *Bsg*I)	This work
pAC133	pBSKII with a 4107 bp *Bgl*II–*Eco*RV fragment, containing *salA*	This work
pAC135	*luc*Spc^r *Xmn*I–*Kas*I fragment from pFWII_luc ligated into pAC133 *Eco*72I (*salA-luc* fusion; cleaved from the vector backbone with *Pvu*II)	This work
pAC139	*luc*Spc^r *Xmn*I–*Sma*I fragment from pFWII_luc ligated into pAC129 *Msc*I (*vanK-luc* fusion; cleaved from the vector backbone with *Xba*I and *Eco*RV)	This work

Table 2. Primers used in this study

Primer	Sequence	Plasmid construction or application
catA5	CAAGGCTGAGGCAAACCAAG	Amplification of *catA* with catA6, to generate pAC126
catA6	GCAATACGCTACGCCAGAC	
catB1	GACGCTTGTGCAGTATGAGTC	Amplification of the *catB,C,I,J,F* region with catB2, to generate pAC127
catB2	TTTGCGCTGTGGAATCGTG	
vanB2	TCCCAATCTGGACAGGAGATAC	Amplification of the *vanA,B* region with vanB3, to generate pAC128
vanB3	AAGCCATGTTCGAGCAAGG	
vanK1	TCATGCGTTGTTCGTCAAG	Amplification of *vanK* with vanK2, to generate pAC129
vanK2	CGGGAATACGATATCCCAACTC	
salA1	CTGAGTGCAGGTGTTGTTTG	Amplification of *salA* with salA2, to generate pAC133
salA2	GCCAGAACGTTATGGAATCG	
luc	AAACCGGGAGGTAGATGAGATG	Confirmation of the insertion of the *luc-aad9* cassette in the chromosome at the right position; used with catA5, catB1, vanB2, vanK1 or salA1
crc3	ATGATACCAAAGGATAGC	Confirmation of the insertion of the Ω cassette in the chromosome at the right position
crc4	TTGTGTAAGAAATTGGCG	Used with crc3

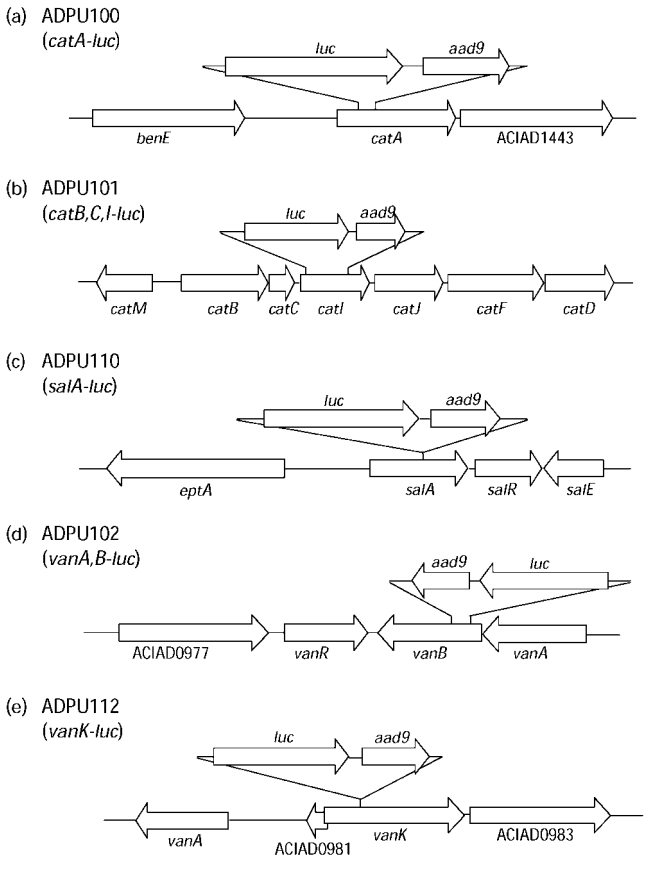

Fig. 1. Transcriptional fusions between *A. baylyi* genes and a luciferase reporter cassette (*luc-aad9*) made and used in this study. Arrangement and nomenclature of open reading frames is from the published and annotated genome sequence (http://www.genoscope.fr).

pyruvate in addition to the aromatic substrate is known to have no effect on the *pca-qui* expression level compared to growth solely on the aromatic substrate (Dal *et al.*, 2002). While the presence of succinate and acetate in combination caused strong repression of all operons analysed, succinate alone was able to repress the *catA*, *catB,C,I,J,F,D* and *sal* operons to a similar degree, while acetate failed to repress the *catB,C,I,J,F,D* and *sal* operons to that extent. However, the *vanA,B* and *vanK* operons showed only a slight increase on either succinate or acetate compared to both acids in combination. The strongest repression of promoter activity (up to 98 %) was observed for the *catA* operon. The activity under CCR conditions was comparable with the uninduced activity of this operon (data not shown). The *catB,C,I,J,F,D*, the *vanA,B* and the *vanK* operons showed slightly lower repression, while the *sal* operon showed only a moderate repression of promoter activity (up to 42 %).

Expression pattern in the presence of lactate and gluconate

Prior to this study, pyruvate (in addition to an inducing carbon source) was shown to be a non-repressing carbon source for the *ben*, *hca*, *dca*, *are* and *ant* operons of the β-ketoadipate pathway with regard to CCR (Fischer *et al.*, 2008). Lactate forms the precursor of pyruvate in the reaction catalysed by lactate/pyruvate dehydrogenase, suggesting that these two carbon sources might have related effects. Glucose is a repressing carbon source in *E. coli* and other bacteria and it was thus interesting to investigate its effect in *A. baylyi* CCR. While *A. baylyi* is able to use glucose, it first converts it to gluconate in the periplasm (Young *et al.*, 2005). Gluconate is a much better growth substrate than glucose and was thus chosen instead. Therefore, the effects of lactate and gluconate on the expression control of the operons named above were tested. Growth experiments were performed using three

Table 3. CCR of *A. baylyi* operons involved in aromatic compound degradation by succinate and acetate

Strain	Transcriptional fusion	Specific inducer (0.5 mM)	Luciferase activity after growth on specific inducer plus secondary carbon source(s)*		
			Succinate + acetate	Succinate	Acetate
ADPU100	*catA-luc*	Benzoate	1.68 ± 0.43	1.98 ± 0.58	1.71 ± 0.17
ADPU101	*catB,C,I-luc*	Benzoate	13.96 ± 1.0	14.16 ± 3.98	33.56 ± 6.87
ADPU102	*vanA,B-luc*	Vanillate	10.01 ± 1.25	18.73 ± 6.13	19.42 ± 2.60
ADPU111	*salA-luc*	Salicylate	57.60 ± 5.01	60.70 ± 1.93	75.30 ± 0.32
ADPU112	*vanK-luc*	Vanillate	11.68 ± 0.32	19.39 ± 1.93	15.06 ± 5.01

*Expressed as a percentage of the activity on pyruvate plus the aromatic inducer (set to 100 %). Values are means ± SD of at least three independent experiments.

different carbon sources (pyruvate, lactate and gluconate) to characterize the expression of the *catA*, *catB,C,I,J,F,D*, *vanA,B*, *vanK* and *sal* operons (Table 4). Furthermore, expression levels of the *are* and *ant* operons were also analysed in the presence of gluconate as carbon source with addition of the aromatic inducer (*areA-luc*, 2.0 mM benzyl alcohol; *antA-luc*, 1.0 mM anthranilate). To summarize the observations: while the carbon source lactate turned out to be non-repressing for the expression of the *catB,C,I,J,F,D* and *sal* operons, a slight repressing effect on *vanA,B* and *vanK* expression was observed, as previously shown for the *ant* and *are* operons (Fischer *et al.*, 2008). Gluconate is non-repressing for the expression of the *sal* genes, but has a slight repressing effect on the *are* operon. Furthermore, gluconate has a moderate repressing effect on the expression of the *vanA,B*, *vanK*, *catB,C,I,J,F,D* and *ant* operons. The most remarkable result was obtained for the *catA* operon. Here, lactate is a repressing carbon source whereas the presence of gluconate causes an increased expression (more than twofold higher than in the presence of pyruvate). This is not an effect of Crc since the same expression pattern was seen in the *crc* strain (data not shown). It should be noted in particular that neither compound significantly repressed the *sal* operon.

Connection between CCR and Crc

A. baylyi Crc has been shown to strongly affect the stability of the *pca-qui* transcript but there is also a transcriptional effect (significantly increased expression under all conditions in the absence of Crc: Zimmermann *et al.*, 2009). To determine whether the Crc protein is involved in CCR of additional operons involved in aromatic compound degradation at the transcriptional level, *crc* was disrupted in strains carrying a luciferase reporter gene fusion (Table 1). Growth experiments were performed with *crc* strains containing a *benA-luc*, *hcaA-luc*, *dcaA-luc*, *catA-luc*, *catB,C,I-luc*, *salA-luc*, *vanA,B-luc*, *vanK-luc*, *areA-luc* or *antA-luc* transcriptional fusion and luciferase activity was determined (Table 5). Inducers were added as follows: benzoate, 0.5 mM (*benA-luc*, *catA-luc*, *catB,C,I-luc*); *p*-coumarate, 1 μM (*hcaA-luc*); adipate, 1.0 mM (*dcaA-luc*); salicylate, 0.5 mM (*salA-luc*); vanillate, 0.5 mM (*vanA,B-luc*, *vanK-luc*); benzyl alcohol, 2.0 mM (*areA-luc*); anthranilate,

Table 4. Effect of lactate or gluconate on the expression of the indicated operons

Strain	Transcriptional fusion	Specific inducer	Concn	Luciferase activity after growth on specific inducer plus secondary carbon source*	
				Lactate	Gluconate
ADPU95	*areA-luc*	Benzyl alcohol	2.0 mM	66 ± 12†	63 ± 6
ADPU96	*antA-luc*	Anthranilate	1.0 mM	45 ± 2†	16 ± 2
ADPU100	*catA-luc*	Benzoate	0.5 mM	10 ± 2	243 ± 16
ADPU101	*catB,C,I-luc*	Benzoate	0.5 mM	90 ± 12	35 ± 2
ADPU102	*vanA,B-luc*	Vanillate	0.5 mM	58 ± 5	48 ± 4
ADPU110	*salA-luc*	Salicylate	0.5 mM	90 ± 23	96 ± 11
ADPU112	*vanK-luc*	Vanillate	0.5 mM	46 ± 12	36 ± 4

*Expressed as a percentage of the activity on pyruvate plus the aromatic inducer (set to 100 %). Values are means ± SD of at least three independent experiments.
†Data from Fischer et al. (2008).

Table 5. Effect of *crc* deletion on the expression of the indicated operons under CCR conditions

Strain	Relevant characteristics	Specific inducer	Concn	Luciferase activity after growth on specific inducer plus secondary carbon source(s)*		
				Succinate + acetate	Succinate	Acetate
ADPU97	*benA-luc*, Δ*crc*	Benzoate	0.5 mM	2788 ± 917	13 ± 2	74 ± 45
ADPU98	*hcaA-luc*, Δ*crc*	*p*-Coumarate	1.0 μM	307 ± 146	139 ± 28	66 ± 21
ADPU99	*dcaA-luc*, Δ*crc*	Adipate	1.0 mM	1222 ± 195	757 ± 134	432 ± 38
ADPU104	*catA-luc*, Δ*crc*	Benzoate	0.5 mM	51 ± 10	109 ± 18	134 ± 27
ADPU105	*catB,C,I-luc*, Δ*crc*	Benzoate	0.5 mM	288 ± 26	121 ± 35	89 ± 18
ADPU106	*vanA,B-luc*, Δ*crc*	Vanillate	0.5 mM	822 ± 352	575 ± 198	720 ± 213
ADPU111	*salA-luc*, Δ*crc*	Salicylate	0.5 mM	185 ± 67	282 ± 106	160 ± 71
ADPU113	*vanK-luc*, Δ*crc*	Vanillate	0.5 mM	288 ± 31	432 ± 46	437 ± 141
ADPU120	*areA-luc*, Δ*crc*	Benzyl alcohol	2.0 mM	914 ± 18	721 ± 411	24 ± 6
ADPU121	*antA-luc*, Δ*crc*	Anthranilate	1.0 mM	143 ± 18	269 ± 51	92 ± 22

*Expressed as a percentage of the activity in the parental strain (crc^+) grown under the same conditions. Values are means ± SD of at least three independent experiments.

1.0 mM (*antA-luc*). Almost all the operons (with the exception of *catA*) responded with a derepression of promoter activity on succinate plus acetate to various degrees (from 3-fold for the *sal* operon, up to 28-fold for the *ben* operon) in comparison with the crc^+ strain. On succinate or acetate alone, the results were comparable to those on succinate and acetate except for a few cases, the most notable of which being the *ben* operon. Here, the absence of *crc* caused a repression on succinate but no significant effect on acetate. Obviously, Crc negatively affects transcription at most promoters tested. The observation of the derepression in the absence of Crc first made for the *pca-qui* operon can now be extended to almost all additional operons investigated here.

Cross-regulation

It was shown earlier that *pobA*, which encodes the enzyme for the degradation of POB to PCA, is strongly repressed when benzoate is present in addition to POB, although POB is the specific inducer for *pobA* expression (Brzostowicz *et al.*, 2003). This interaction between the two branches of the β-ketoadipate pathway was named cross-regulation. Here, the effect of different benzoate concentrations (0.1–5.0 mM) on the transcriptional activity of the *hca*, *vanA,B* and *dca* operons was determined (Fig. 2a). Probably due to the toxicity of benzoate, strains grown in the presence of higher amounts of benzoate (3.0 and 5.0 mM) showed a decrease in the growth rate, but reached the same final optical density as the culture grown without benzoate in the medium (data not shown). Since the effects were observed at much lower benzoate concentrations, the growth inhibition at higher benzoate concentration is not disturbing. For the *hca* and *vanA,B* operons, a strong decrease in transcriptional activity was observed with increasing benzoate concentrations. This repression is even stronger than CCR by succinate and acetate in combination (90 % for *vanA,B*; Table 3, and 93 % for *hca* (Fischer *et al.*, 2008)). For the *dca* operon, a slightly different behaviour was observed: benzoate concentrations lower than 1.0 mM led to increased promoter activity, while higher concentrations caused repression which was not as strong as in the case of *van* and *hca*.

Vertical regulation

It was shown earlier that PCA inhibits *pobA* expression in the presence of the specific inducer POB (Brzostowicz *et al.*, 2003). Here, we investigated the effect of PCA (0.1–5.0 mM) on promoter activity of the *hca* and *vanA,B* operons in the presence of the specific aromatic inducer. In contrast to cultures grown in the presence of higher concentrations of benzoate, no decrease in growth rate was observed for cultures grown in the presence of higher amounts of PCA. Both operons are affected by vertical regulation as well (shown in Fig. 2b). At higher concentrations of PCA, the expression of both operons decreased, as observed for the *pobA* gene. We refer to this type of regulation as vertical regulation, since a downstream metabolite (with respect to the catabolic pathway, PCA) affects the expression of an operon encoding a pathway for its generation from various precursors.

DISCUSSION

Strong repression of all investigated operons

Earlier studies analysing the *ben*, *dca*, *hca*, *are* and *ant* operons (Fischer *et al.*, 2008) and the *pca-qui* operon (Dal *et al.*, 2005; Siehler *et al.*, 2007) revealed a strong repression of promoter activity when the organic acids succinate and acetate were present in addition to the specific aromatic inducer of the respective pathway. Here, we extended these observations by analysing additional operons all connected to the degradation of aromatic compounds (*catA*,

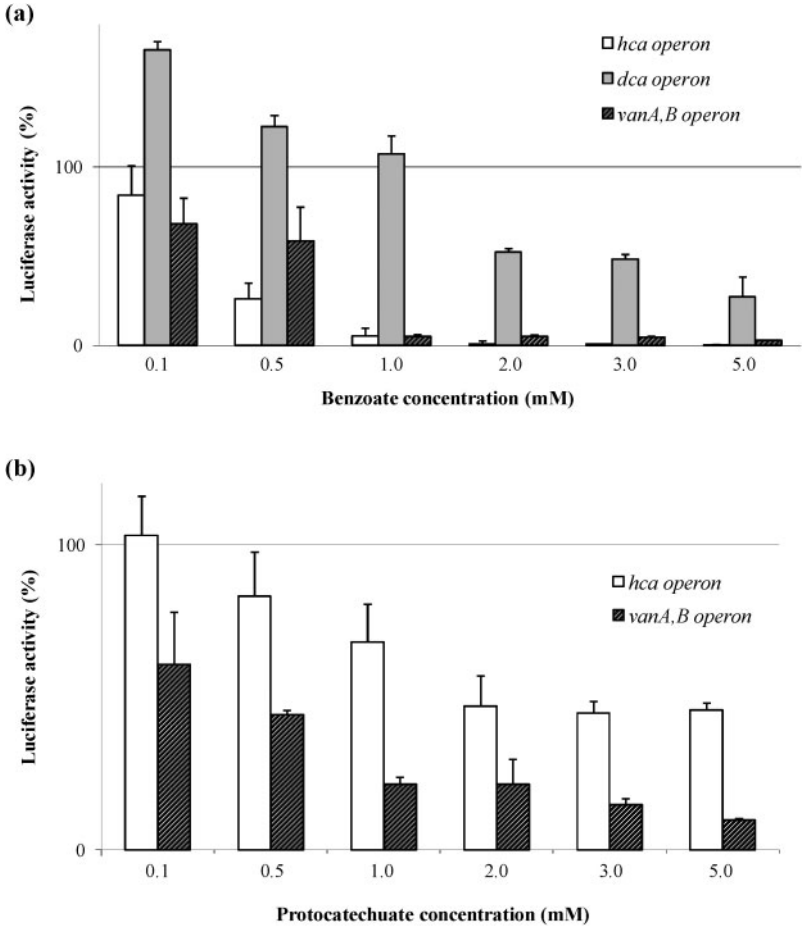

Fig. 2. Repression of operons in the presence of benzoate (cross-regulation) or PCA (vertical regulation). Luciferase activity of *A. baylyi* strains containing the indicated transcriptional *luc* fusions in the presence of the specific inducer and increasing concentrations of (a) benzoate or (b) PCA. The activity of each operon in the absence of benzoate or PCA was set to 100 %. Each growth experiment was carried out at least three times independently. Error bars indicate standard deviation.

catB,C,I,J,F,D, *vanA,B*, *sal*, *vanK*, repression between 42 and 98 %; Tables 3 and 5). Furthermore, the investigation included the additional carbon sources lactate and gluconate. While pyruvate generally can be regarded as neutral for gene expression (Dal *et al.*, 2002), the presence of lactate or gluconate has different effects on the expression of the operons analysed, ranging from no effect to a slight repression in most cases (Table 4). A surprising result is the induction of *catA* by gluconate whereas all other tested carbon sources caused strong repression at this operon. Thus in this single case, gluconate has an even smaller negative effect on induction than pyruvate. In contrast, pyruvate has a small repressing effect, which is negligible when looking at the strong repression in all other cases. The repression of the operons encoding the funnelling reactions by elevated concentrations of succinate and acetate is meaningful in the context of energy preservation. The β-ketoadipate pathway and all the funnelling reactions do not lead to energy conservation. It thus seems advantageous to express these funnelling pathways only to the least necessary extent (an argument which also applies to the other regulatory phenomena, cross-regulation and vertical regulation, discussed below). Energy conservation occurs subsequently by oxidation of succinyl-CoA and acetyl-CoA. Lactate and pyruvate cause no or only a moderate repression of the operons (with the exception of lactate on *catA*). These two substrates may not be such abundant carbon sources for *A. baylyi* in its natural habitat and thus no regulatory mechanism may have evolved. The degradation of gluconate by *A. baylyi* is energy consuming, because gluconate has to be taken into the cell by active transport and then be converted to 6-phosphogluconate, which is also an energy-requiring step. Gluconate degradation then is accomplished by a modified Entner–Doudoroff pathway (Barbe *et al.*, 2004; Young *et al.*, 2005). Furthermore, the utilization of gluconate or glucose is unusual among species of *Acinetobacter*; thus it seems to fit that there is no strong repression of aromatic degradative pathways by gluconate as observed for succinate and acetate.

Crc is involved in the transcriptional expression control of numerous aromatic degradative pathways

The absence of Crc had a significant effect on the expression of the operons *are*, *sal*, *ben*, *catB,C,I,J,F,D*, *dca*, *hca*, *vanA,B*, *vanK* and *sal*, as shown here and earlier for *pca-qui* and *pobA* (Dal *et al.*, 2002; Zimmermann *et al.*, 2009). The inactivation of *crc* resulted in a significant transcriptional derepression of most operons analysed. Only *catA*, forming a separate regulatory unit in *A. baylyi*,

did not show a significant dependence on Crc. While the results presented here focus on the transcriptional level, *A. baylyi* Crc also acts post-transcriptionally by (directly or indirectly) dramatically changing the mRNA half-life of the *pca-qui* transcript (Zimmermann *et al.*, 2009). In *P. putida* it has been shown that Crc directly affects translation of the regulator AlkS (Yuste & Rojo, 2001). Similar observations have been made for *P. putida* BenR mRNA. In these bacteria, Crc blocks translation by binding the translation initiation site (Moreno & Rojo, 2008). In summary, Crc turns out to have complex effects at both the transcriptional and the post-transcriptional level. Furthermore, Crc affects all operons investigated so far, an observation supported by investigations of *P. putida* Crc, which is known to be a truly global regulator (Moreno *et al.*, 2009).

Cross-regulation

Cross-regulation takes place between the two branches of the β-ketoadipate pathway, indicating that these branches are not regulated independently (Fig. 3). Our results suggest that the consumption of benzoate (degraded via the catechol branch) is favoured over substrates of the PCA branch (e.g. vanillate or other hydroxycinnamates), as shown by the transcriptional repression of the respective operons in the presence of benzoate. Furthermore, the presence of increased benzoate concentrations led to a repression of the *dca* genes. The repression of the *vanA,B* and *hca* operons caused by benzoate is even stronger than the repression caused by the organic acids succinate and acetate in combination. It is important to note that all strains used for the analysis of cross-regulation are able to degrade benzoate to *cis,cis*-muconate via the catechol branch. Thus, *cis,cis*-muconate is available as an effector for BenM and CatM. These transcriptional regulators are in fact responsible for the regulated expression of the *ben* and *cat* genes in response to benzoate and *cis,cis*-muconate (Craven *et al.*, 2008). However, while no interaction of the two regulators with the *pobA* promoter could be detected, both BenM and CatM are able to bind to a fragment upstream of *pcaU*, an interaction that is promoted by *cis,cis*-muconate (Brzostowicz *et al.*, 2003). Subsequently, it was shown that *pca-qui* expression also undergoes cross-regulation by benzoate (Siehler *et al.*, 2007). It is still

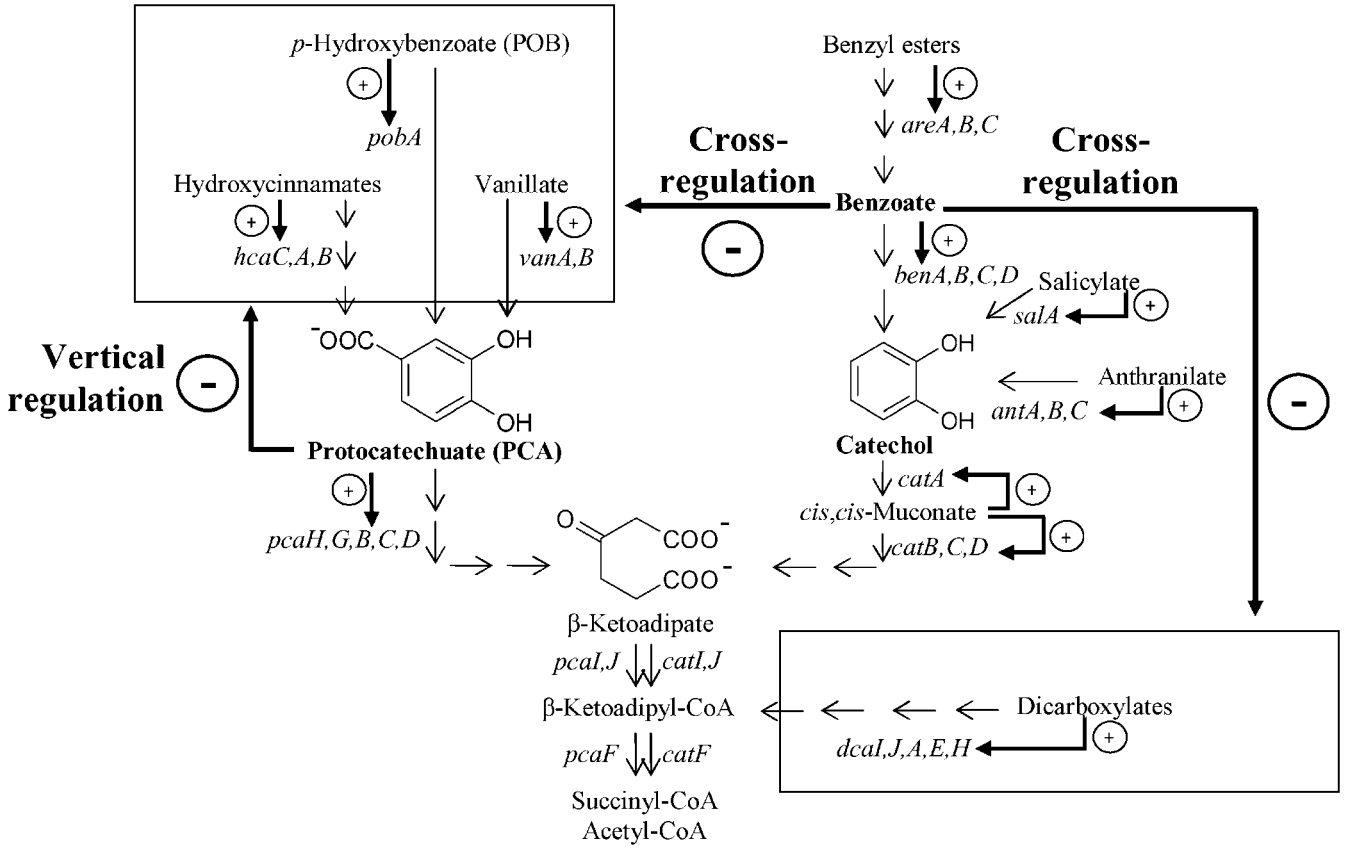

Fig. 3. Summary of the regulatory levels within the aromatic catabolic pathways of *A. baylyi*. All operons are affected negatively in expression by CCR triggered by succinate and acetate (not included in the scheme). Thin arrows indicate the direction of the metabolic pathway; thick arrows indicate transcriptional regulation effects. Repression (−) or activation (+) is symbolized by plus or minus signs next to the arrows.

unclear how these observations connect with the cross-regulation of *pobA*. It may be that POB transport is limited when the *pca-qui* operon (including *pcaK*) is repressed, but the involvement of the second POB transporter, VanK, needs to be characterized. Studies on VanK suggest an involvement in PCA uptake, since a double knockout of *vanK* and *pcaK* prevents growth on PCA, while a knockout of *pcaK* alone does not (D'Argenio *et al.*, 1999). However, it has to be determined whether there is involvement of VanK in POB uptake. For all other operons under study, the presence of binding sites for BenM or CatM needs to be evaluated to determine if these proteins are centrally involved in the mechanism of cross-regulation.

Vertical regulation

We showed here that the addition of PCA to the medium containing the specific inducers (vanillate, *p*-coumarate) triggers a mechanism that represses the *vanA*,*B* and *hca* operons encoding funnelling reactions to PCA. This regulatory mechanism was termed vertical regulation (Fig. 3). An analogous observation was made earlier for *pobA*. PobR had been excluded as a mediator of this effect (Siehler *et al.*, 2007). Furthermore, PcaU, the transcriptional regulator of the *pca* genes, is naturally able to bind PCA but is not able to bind the *pobA* promoter (Siehler *et al.*, 2007). The molecular basis of vertical regulation observed for the *pobA* promoter thus remains unknown, but the phenomenon seems to be common, as shown for the *vanA*,*B* and *hca* operons. The biological relevance appears to be that increased internal PCA concentrations prevent the expensive expression of any funnelling pathway leading to more PCA production. Only when the internal PCA level falls under a certain level will this mechanism cease to be active and more PCA can be produced from the respective precursor. Furthermore, external PCA in concentrations high enough to promote growth will also lead to the repression of funnelling pathways producing it, which also supports the organism in saving resources.

Conclusion

The first mechanisms of regulation found within the pathways for aromatic compound degradation in *A. baylyi* included induction brought about by specific regulators and their respective effectors. In the current study, additional levels of regulation were characterized more comprehensively, namely carbon catabolite repression, cross-regulation and vertical regulation. All of these consist of repressing effects. Besides efficient induction in response to the presence of the substrates, three different mechanisms decreasing expression under several conditions override specific induction. This indicates that the expression of genes for the degradation of aromatic compounds only occurs under well-defined conditions and in a well-defined order to best fit the needs of the bacterium.

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