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Regulatory exaptation of the catabolite repression protein (Crp)–cAMP system in *Pseudomonas putida*

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Summary

The genome of the soil bacterium *Pseudomonas putida* KT2440 encodes singular orthologues of genes *crp* (encoding the catabolite repression protein, Crp) and *cyaA* (adenylate cyclase) of *Escherichia coli.* The levels of cAMP formed by *P. putida* cells were below detection with a *Dictyostelium* biosensor *in vivo.* The $cyaA_{P.putida}$ gene was transcribed *in vivo* but failed to complement the lack of maltose consumption of a *cyaA* mutant of *E. coli*, thereby indicating that $cyaA_{P.putida}$ was poorly translated or rendered non-functional in the heterologous host. Yet, generation of cAMP by $CyaA_{P.putida}$ could be verified by expressing the $cyaA_{P.putida}$ gene in a hypersensitive *E. coli* strain. On the other hand, the $crp_{P.putida}$ gene restored the metabolic capacities of an equivalent *crp* mutant of *E. coli,* but not in a double *crp/cyaA* strain, suggesting that the ability to regulate such functions required cAMP. In order to clarify the breadth of the Crp/cAMP system in *P. putida*, *crp* and *cyaA* mutants were generated and passed through a battery of phenotypic tests for recognition of gross metabolic properties and stress-endurance abilities. These assays revealed that the loss of each gene led in most (but not all) cases to the same phenotypic behaviour, indicating a concerted functionality. Unexpectedly, none of the mutations affected the panel of carbon compounds that can be used by *P. putida* as growth substrates, the mutants being impaired only in the use of various dipeptides as N sources. Furthermore, the lack of *crp* or *cyaA* had little influence on the gross growth fingerprinting of the cells. The poor physiological profile of the Crp–cAMP system of *P. putida* when compared with *E. coli* exposes a case of regulatory exaptation, i.e. the process through which a property evolved for a particular function is co-opted for a new use.

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Introduction

The cAMP-responsive protein of *Escherichia coli* Crp (also called CAP) was the first transcriptional activator ever identified because of its positive role in expression of the *lac* operon (Ullmann and Monod, 1968; Zubay *et al.*, 1970). Early in the study of this protein, it became clear that Crp had the ability of occupying a target DNA sequence in the proximity of the RNAP binding site of *Plac* depending on the binding to cAMP as co-activator. Later work has shown that Crp is in fact an abundant protein which, depending on its relative location in respect to the transcription initiation of target promoters, both stimulates and inhibits a large number of genes in response to fluctuations in the levels of cAMP (Kolb *et al.*, 1993; Busby and Kolb, 1996). In fact, Crp controls so many genes in *E. coli* (Gosset *et al.*, 2004) that it can be considered more properly a nucleoid-associated protein than a specific transcriptional regulator (Grainger *et al.*, 2005).

The unique source of cAMP in *E. coli* is the conversion of ATP into cAMP through the activity of the enzyme adenylate cyclase (CyaA). Since *E. coli* bears several phosphodiesterases (Barth *et al.*, 2009), it is believed that occurrence of cAMP happens in pulses that reflect changes of the activity of the enzyme. This seems to be high when stimulated by the phosphorylated forms of the type EIIA proteins of the phosphoenolpyruvate: carbohydrate phosphotransferase system (PTS) in the absence of cognate sugars (Deutscher *et al.*, 2006). On this basis, Crp and its necessary partner cAMP has traditionally been associated to the phenomenon of catabolite repression, i.e. the mechanism by which bacteria employ preferentially one carbon source in respect to others when several of them are simultaneously available. The Crp/cAMP regulatory system has been explored in *E. coli* at length from such a metabolic control perspective (Bruckner and Titgemeyer, 2002; Nanchen *et al.*, 2008). More recent work has shown however (i) that the phenomenon of catabolite repression happens in other bacteria through mechanisms that are entirely alien to Crp (Bruckner and Titgemeyer, 2002; Rojo, 2010) and (ii) that *crp* and *cyaA*

orthologues found in many genomes of non-*E. coli* bacteria are not related to catabolite control. These two circumstances fully apply to the soil bacterium *Pseudomonas putida* KT2440. This microorganism displays sophisticated catabolite repression phenomena, none of which can be traced to Crp/cAMP (Phillips and Mulfinger, 1981; Rojo, 2010) to the point that pre-genomic studies on this genus even doubted the existence of cAMP in this bacterium (Vicente and Canovas, 1973; Botsford and Harman, 1992). Later work, however, showed that the genome of *P. putida* KT2440 does have recognizable *crp* and *cyaA* sequences, although their biological function is not apparent upfront (Weinel *et al*., 2002; Dos Santos *et al*., 2004). Its close relative *Pseudomonas aeruginosa* PAO1 bears also one *crp* homolog and two *cya* genes that encode enzymes of distinct protein families. In this case, the Crp variant has been named Vfr (*virulence factor regulator*) and its role in controlling pathogenesis-related traits is well established (West *et al*., 1994; Fuchs *et al*., 2010a,b).

Both $Crp_{E.coli}$ and Vfr belong to the Crp branch of the Crp–Fnr superfamiliy of dimeric transcriptional regulators (Korner *et al*., 2003) that includes the Clp protein of *Xantomonas campestris* (de Crecy-Lagard *et al*., 1990). They are characterized for possessing a typical helix–turn–helix (HTH) DNA binding motif in their carboxy-terminal domain, a distinct pocket for binding cyclic nucleotide monophosphate, and various protein surfaces for interacting with RNA polymerase (Kolb *et al*., 1993). In the instances where the issue has been examined, the cyclic nucleotide phosphate acts as an allosteric effector that modulates the affinity of the protein for DNA (Youn *et al*., 2008). The *E. coli* Crp–cAMP complex of *E. coli* seems to target sequences best containing 5′-TGTGA-6N-TCACA-3′ (extended: 5′-AAA**TGTGA**TCTAGA**TCACA** TTT-3′) although this optimal configuration hardly appears as such (see below). Also in *E. coli,* CRP controls > 100 promoters, many of them involved in metabolic functions (Grainger *et al*., 2005). The only possible origin of cAMP in *E. coli* (the ultimate physiological signal for Crp to work in this microorganism) is the soluble adenylate cyclase type I, encoded by *cyaA*. In contrast, the Vfr protein of *P. aeruginosa* is earmarked for controlling a distinct virulence programme, with little or no connection to metabolism (Kanack *et al*., 2006) and in concert or not with cAMP (Fuchs *et al*., 2010b). On top of this, *P. aeruginosa* can produce cAMP from either an orthologue of the $cyaA_{E.coli}$ gene (equally named CyaA) or a second membrane-bound type III cyclase (CyaB), which becomes active in response to environmental calcium (Smith *et al*., 2004). Just to complete the cycle, the levels of cAMP in *P. aeruginosa* are checked by a cognate phosphodiesterase named CpdA (Fuchs *et al*., 2010a). This information is largely derived from genetic data, because adenylate cyclases, in particular those of the cytoplasmic type I, have proven to be very difficult to purify and test *in vitro* (Yang and Epstein, 1983).

From a comparative genomics perspective, the Crp–cAMP system of *P. putida* holds an uncertain functional position between the *E. coli* and *P. aeruginosa* counterparts. On one hand, the $crp_{P.putida}$ and $cyaA_{P.putida}$ orthologues encode proteins with a very high similarity to those of *E. coli* (Weinel *et al*., 2002) but they have not been recognized thus far in any catabolite repression phenomena (Rojo, 2010). On the other hand, *P. putida* lacks the distinct type III, membrane-bound cyclase CyaB, which accounts for the virulence programme brought about by Vfr-mediated expression of several components of the quorum-sensing system (Albus *et al*., 1997; Lory *et al*., 2004). If $Crp_{P.putida}$ neither controls catabolite repression (as in *E. coli*) nor it regulates virulence (as in *P. aeruginosa*), then why is it conserved in *P. putida* and what is its role in this microorganism? This work aims at addressing this question, i.e. the function(s) of the *crp* and *cyaA* genes of *P. putida*, employing the sequenced strain KT2440 as the system of reference. Our approach has involved the combination of structural prediction tools with an analysis *in vivo* of the products encoded by *crp* and *cyaA* and the properties of *P. putida* variants lacking such genes. The results shown below suggest that the dissimilar roles of Crp in various bacteria constitute a case of regulatory *exaptation* (Gould and Vrba, 1982), i.e. a shift of function of the very same quality or behavioural trait of a biological object depending of its context and evolutionary history.

Results

The crp *gene of* P. putida *and its cognate product*

Figure 1A shows the context of the sole *crp* orthologue that can be found in the genome of *P. putida* KT2440 and which was recorded in the TIGR (now JCVI) database as PP0424 (http://cmr.jcvi.org). It is noteworthy that despite 62% of identity and 80% of similarity of the encoded product with the Crp protein of *E. coli* (Fig. S1), PP0424 was not annotated as such in the primary analysis, but as the cyclic nucleotide-binding transcriptional regulator Vfr. Yet, it does appear assigned with a tentative Crp function in other databases (e.g. http://www.pseudomonas.com). It is also remarkable that $crp_{P.putida}$ comes into sight as a stand-alone gene, likely to be expressed as a single cistron and thus unrelated to the adjacent ORFs. To verify that the $Crp_{P.putida}$ protein was produced *in vivo,* a mutant was generated in which the central portion of the PP0424 was replaced by a promoterless *xylE* gene (Fig. 1A). The resulting strain turned yellow upon spraying with catechol, indicating that the

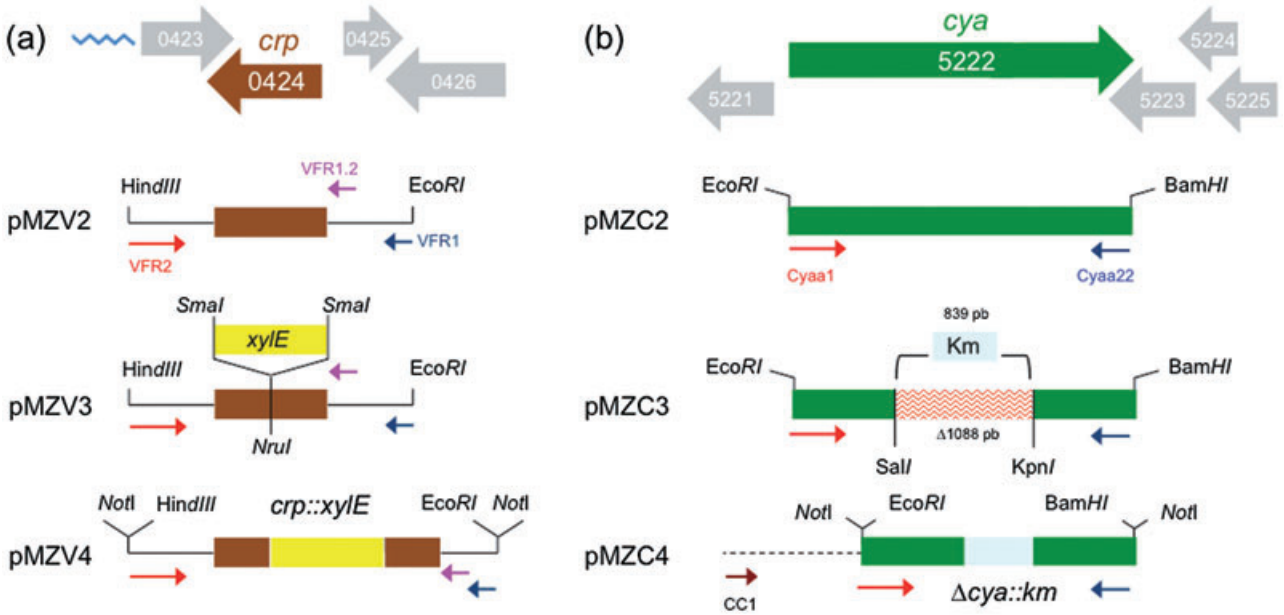

Fig. 1. Chromosomal context for *crp* and *cyaA* genes of *P. putida* and roadmap for generation of cognate mutants.
A. The site shown contains the sole *crp* orthologue (PP0424) found in the genome of *P. putida*. It is flanked by ORFs numbered PP0423, PP0425 and PP0426, all of them of unknown function. Note the orientation of adjacent genes, which make it unlikely co-transcription with *crp*. The various steps followed for cloning the gene, the name of the constructs and the designation of the oligonucleotide primers employed for amplification of the sequences of interest are indicated (see Table 1 and *Experimental procedures*).
B. Same, for the sole *cyaA* orthologue of *P. putida* (PP5222). Adjacent ORFs PP5221 and PP5224 have unknown functions, whereas PP5223 may encode a nucleoside diphosphate kinase regulator (*rnk*) and PP5225 seems to be a bacterial frataxin (*cyaY*), a protein involved in the biogenesis of iron–sulfur clusters. Note that *crp* and *cyaA* map in different locations of the *P. putida* chromosome.

transcription signals of *crp* were high enough for expression of the dioxygenase encoded by the reporter gene. To unequivocally assign the product of PP0424 to the Crp protein we run the experiment shown in Fig. 2, in which protein extracts from *E. coli* and *P. putida* and their *crp*-minus counterparts were probed in a Western blot with a rabbit anti-$Crp_{E.coli}$ serum (kindly provided by H. Aiba). The results clearly showed that the PP0424 was indeed expressed and its product recognized by the anti-Crp antibodies. Furthermore, since the antigenicity of the proteins of *P. putida* and *E. coli* are likely to be similar, the Western blot of Fig. 2 suggested that Crp could be less abundant in *Pseudomonas* cells.

As indicated in the alignment of Fig. S1, $Crp_{P.putida}$ bears a considerable conservation of the functional ingredients of the *E. coli* counterpart. These include the HTH domain for DNA binding as well as a suite of residues for cyclic nucleotide monophosphate binding. Moreover, the $Crp_{P.putida}$ sequence maintains the three regions identified in the *E. coli* protein as responsible of interacting with various sites of the RNAP: AR1, necessary for activation of class I and class II promoters through direct contacts with the α-CTD; AR2, required for class II promoters via interactions with the α-NTD; and the optional surface AR3, which seems to interact with region 4 of σ^{70} (West *et al.*, 1994; Niu *et al.*, 1996; Parkinson *et al.*, 1996;

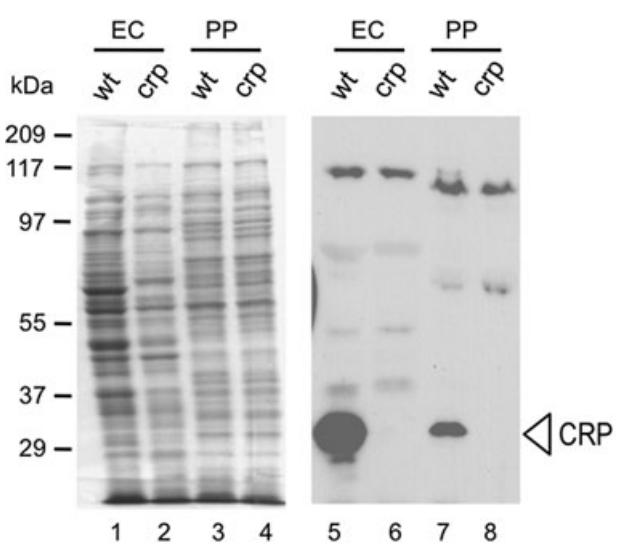

Fig. 2. Gross comparison of the proteomes of *crp* mutants of *E. coli* and *P. putida* and relative abundance of the Crp protein. Each of the *E. coli* (EC) and *P. putida* (PP) strains indicated was grown in LB medium up to an OD_{600} = 1.0 and an equivalent amount of whole-cell extracts run in a 12% denaturing PAGE system. Gels were then stained with Coomassie blue (left, lanes 1–4) or submitted to a Western blot with rabbit anti-$Crp_{E.coli}$ antiserum (right, lanes 5–8). Note the effect of *crp* in the *E. coli* proteome (compare lanes 1 versus 2) and the lack of effect in *P. putida* (lanes 3 versus 4). Compare also the relatively higher signal of the Crp protein in *E. coli* versus the corresponding band in *P. putida* (lanes 5 versus 7).

Busby and Ebright, 1999). Taken together, all these similarities would indicate that this protein is equivalent to the classical $Crp_{E.coli}$ transcription factor. However perusal of the gels of Fig. 2 suggests that this may not be the case, because the loss of Crp does not seem to bring about an alteration in the protein profile of *P. putida*. This is in contrast with the situation in *E. coli,* where the shortfall of Crp results in appreciable gross changes in the relative composition of the protein extract (e.g. Fig. 2). On this background, we examined in more detail the parts of the $Crp_{P.putida}$ protein that could account for a different functionality.

Figure S2 shows a blow-up of the amino acids that compose the DNA-binding, HTH domain of the *P. putida* protein and the sequence of the proteins and target sites for the *E. coli* and the *P. aeruginosa* counterparts. This analysis suggests that the DNA sequences bound by each the proteins are likely to be similar, but not necessarily identical. Relevant changes are also noticeable in the interaction pocket of the Crp proteins with cAMP (Fig. S3). In fact, simulations of the tridimensional structure of the cNMP crevice suggest that $Crp_{P.putida}$ could both bind cGMP and cAMP, this last with higher affinity (Fig. S3). The three questions derived from the analyses above were therefore whether $Crp_{P.putida}$ (i) binds the same target DNA sequences as $Crp_{E.coli}$, (ii) interacts with RNAP in the same fashion and (iii) responds to the same physiological signals, i.e. cAMP. The following sections report a series of experiments aimed at producing the corresponding answers.

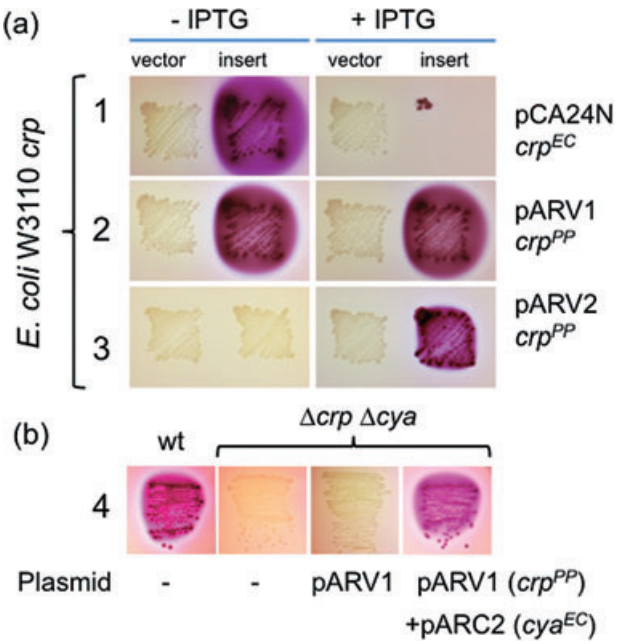

Fig. 3. Complementation analysis of *P. putida crp* and *cyaA* genes in *E. coli*.
A. Expression of *crp* genes in *E. coli* Δ*crp*. The host strain was transformed with plasmids bearing either the *crp* genes from *E. coli* (EC) or *P. putida* (PP) or their corresponding insert-less vectors as indicated. Each transformant was then patched on McConkey-maltose agar plates with or without IPTG. Note toxicity of $crp_{E.coli}$ when overexpressed from pCA24N*crp*.
B. Expression of $crp_{P.putida}$ in a double *crp cyaA E. coli* mutant. High-copy-number $crp_{P.putida}$-encoding plasmid pARV1 was transformed by itself in the host indicated or co-transformed with $cyaA_{E.coli}^{+}$ plasmid pARC2 in the same strain. Rescue of the Mal^{+} phenotype is observed only when the Δ*cyaA* Δ*crp* strain contains the two compatible plasmids (pARV1 + pARC2).

In vivo *activities of* $Crp_{P.putida}$

The experiments shown in Fig. 3 were made to examine the ability of $crp_{P.putida}$, expressed at various levels to restore maltose metabolism in *E. coli* strains with relevant genotypes. The reference condition is that of row 1, in which a *crp E. coli* strain was transformed with a high-copy-number plasmid (pCA24N*crp*, Table 1) which expressed $crp_{E.coli}$ under the control of a heterologous IPTG-inducible promoter. This strain was plated along with the corresponding insert-less vector on McConkey-maltose medium. As shown in Fig. 3 (row 1), $crp_{E.coli}$ restored the Mal^{+} phenotype even when expressed through the escape of the vector promoter in the absence of induction. Figure 3 (row 1) shows also that IPTG addition was toxic to the host Δ*crp E. coli* strain. The situation was similar, but not identical, when $crp_{E.coli}$ was replaced by $crp_{P.putida}$ in the same test system (Fig. 3, row 2). When cloned in a multicopy plasmid (pARV1), the gene from *P. putida* complemented the lack of *crp* in the host *E. coli* strain. However, when expressed from a lower-copy-inducible vector (pARV2), IPTG addition was required to attain the same effect (Fig. 3, row 3). These simple experiments revealed two important pieces of information. First, that $Crp_{P.putida}$ maintains the same RNAP-activating abilities of the *E. coli* protein; second, that the *P. putida* Crp recognizes the same target DNA sequence. Yet, the affinity for the target could be lower (or expression of $Crp_{P.putida}$ lesser in the hererologous host), since overproduction of $Crp_{P.putida}$ was required to fully complement the phenotype under examination (compare rows 1 and 3 of Fig. 3). In any case, cAMP was necessary to bring about complementation, because $crp_{P.putida}$ failed to restore maltose metabolism in a double Δ*crp* Δ*cyaA* strain of *E. coli*, even when overexpressed (Fig. 3, row 4; see below). This is an important detail, because other members of the same Fnr/Crp family of proteins have been claimed to work independently of cAMP (e.g. Clp from *X. campestris* complements an *E. coli crp cyaA* double mutant; de Crecy-Lagard *et al.*, 1990). Furthermore, even Vfr of *P. aeruginosa* can operate without cAMP (Fuchs *et al.*, 2010b).

Table 1. Strains and plasmids.

Strain/plasmid	Description/relevant characteristics	Reference
Escherichia coli strains		
CC118λ*pir*	Δ(*ara-leu*), *araD*, Δ*lac*X174, *galE*, *galK, phoA, thi1, rpsE, rpoB, argE (Am), recA1,* lysogenic λ*pir*	de Lorenzo and Timmis (1994)
DH5α	*supE44,* Δ*lacU169, (ϕ80 lacZDM15), hsdR17, (rk–mk+), recA1, endA1, thi1, gyrA, relA*	Hanahan and Meselson (1983)
HB101	Sm^r, *hsdR*$^-$*M*$^+$, *pro, leu, thi, recA*	Sambrook *et al.* (1989)
W3110	Prototrophic, F$^-$, λ$^-$, IN (*rrnD-rrnE*)1, *rph-1*	Hayashi *et al.* (2006)
W3110 *crp*	*crp* deletion derivative of W3110 strain	This work
W3110 *cyaA*	*cyaA* deletion derivative of W3110 strain	This work
W3110 *crp cyaA*	Double *crp cyaA* deletion derivative of W3110	This work
TP610	*cyaA*-less derivative of C600 bearing cAMP-hypersensitivity allele *cyaA-610*	Hedegaard and Danchin (1985)
Pseudomonas putida strains		
KT2440	Prototrophic, wild-type strain	Nelson *et al.* (2002)
KT2442	KT2440, spontaneous rifampycin resistant	Regenhardt *et al.* (2002)
MAD1	KT2442, Tel^R, chromosomal insertion of mini-Tn*5* [*xylR*, *Pu–lacZ*]	Fernandez *et al.* (1995)
crp	MAD1 derivative, *crp::xylE*	This work
cyaA	MAD1 derivative, Δ*cyaA::km*	This work
crp cyaA	MAD1 derivative, Δ*cyaA::km, crp::xylE*	This work
Plasmids		
pUC18Not	Ap^R, *oriV* ColE1, cloning vector, polylinker flanked by NotI sites	Herrero *et al.* (1990)
pKNG101	Sm^R, *sacB*$^+$, *oriV* R6K, *oriT*, suicide delivery vector	Kaniga *et al.* (1991)
pUCKm	Ap^R, Km^R, *oriV* ColE1, inserted with an excisable promoterless Km resistance gene	Cases *et al.* (1999)
pVLT31	Tc^R, *oriV* RSF1010, broad-host-range LacI^q/IPTG-inducible expression vector	de Lorenzo *et al.* (1993)
pGEMT	Ap^R, multicopy cloning vector	Promega
pVTR-A	Cm^R, *oriV* PSC101, LacI^q/IPTG-inducible expression cassette inserted as a NotI segment	Perez-Martin and de Lorenzo (1996)
pMZC2	Ap^R. pUC18Not inserted with the *P. putida cyaA* gene sequence amplified with oligos cyaAa1 and cyaAa2.2 (Fig. 1B)	This work
pMZC3	Ap^R, pMZC2 derivative inserted with a Δ*cyaA::km* segment Km^R (25 μg ml^{-1}; Fig. 1B)	This work
pMZC4	Sm^R, Km^R; pKNG101 derivative inserted with Δ*cyaA::km* segment of pMZC3	This work
pMZC5	Tc^R. pVLT31 inserted with the *P. putida cyaA* gene sequence amplified with oligos CC1 and CC2 (i.e. same insert as pMZC6)	This work
pMZC6	Ap^r. pUC18Not inserted with the *P. putida cyaA* gene sequence amplified with oligos CC1 and CC2 (*Experimental procedures*)	This work
pMZV1	Ap^R. pGEMT inserted with the *P. putida crp* gene sequence amplified with oligos VFR1 and VFR2 (Fig. 1A)	This work
pMZV2	Ap^R. pUC18Not inserted with the same $crp_{P,putida}$ sequence as pMZV1 (Fig. 1A)	This work
pMZV3	Ap^R. pMZV2 derivative inserted with a promoterless *xylE* gene in the NruI site of the $crp_{P,putida}$ sequence (Fig. 1A)	This work
pMZV4	Sm^R, C2,3O$^+$; pKNG101 derivative inserted with the *crp::xylE* segment of pMZV3	This work
pCA24N	Cm^R; *oriV* ColE1 cloning and IPTG-inducible expression vector	Mori *et al.* (2000)
pCA24N *crp*	Cm^R; pCA24N inserted with *crp* gene of *E. coli*	Mori *et al.* (2000)
pARV1	Ap^R. pUC18Not inserted with the *P. putida crp* gene sequence amplified with oligos vfrMyc1 and vfrHis-C (*Experimental procedures*). The encoded Crp sequence is added with a terminal 6xHis fusion	This work
pARV2	Tc^R. pVLT31 bearing with the *P. putida crp* gene sequence assembled in pARV1	This work
pARC2	Cm^R. pVTR-A with its NotI insert altogether replaced by a segment bearing the *cyaA* gene of *E. coli* amplified with oligos aaECcyaA-F and aaECcyaA-R (*Experimental procedures*). $cyaA_{E.coli}$ expressed through its native transcription and translation signals	This work
pARC3	Tc^R. pVLT31 inserted with the *B. pertussis* gene amplified from plasmid pDIA5240 with oligos aaBPcyaA-F and aaBPcyaA-R	This work
pDIA5240	Ap^R, *oriV* ColE1, expression plasmid for the catalytic subunit of the adenylate cyclase gene of *Bordetella pertussis*	Ladant *et al.* (1992)
pRK600	Cm^R; *oriV* ColE1, *tra*$^+$*mob*$^+$ of RK2, helper plasmid for mobilization in tripartite conjugations	Kessler *et al.* (1992)
pXYLE10	Km^R. Multicopy plasmid inserted with a promoterless *xylE* gene encoding catechol 2,3 dioxygenase (C2,3O)	Stein (1992)

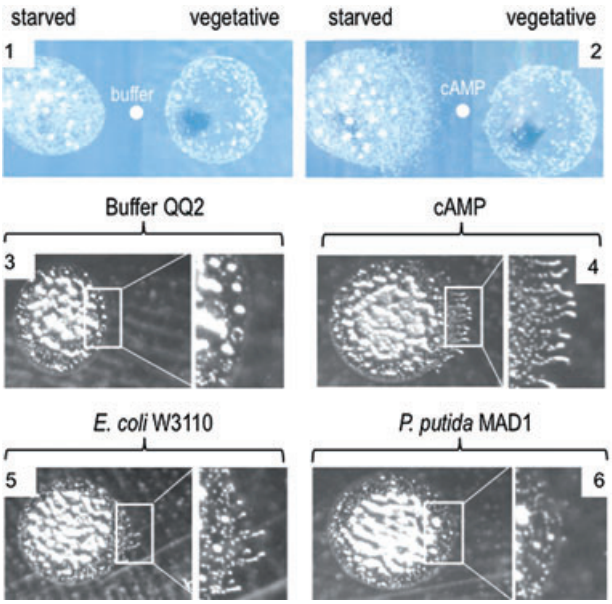

Fig. 4. *Dictyostelium discoideum* cAMP chemotaxis test *in vivo*. Colonies of the sensor slime mold were grown on the surface of either nutrient-rich (allowing vegetative state) or starvation media as indicated, the last conditions endowing *D. discoideum* with a chemotactic motion towards cAMP (panels 1–4). For the experiments, the same starved colonies were laid in the proximity of concentrated supernatants of either *E. coli* or *P. putida* cultures (panels 5 and 6). Note dendritic extensions towards the *E. coli* sample in contrast with the maintenance of the *D. discoideum* colony rim in the case of *P. putida*.

cAMP in P. putida

On the basis of the results above we set out to investigate the occurrence of cAMP in *P. putida*. To this end we set up an *in vivo* biosensor based on the chemotaxis of *Dictyostelium discoideum* towards cAMP, which is a differentiation signal for starved cells of this slime mold at concentrations in the nM–μM range (Saran *et al*., 2002). The corresponding experiment is shown in Fig. 4. As a control, the upper panels 1 and 2 of Fig. 4 display how the rims of starved and vegetative colonies of *D. discoideum* were affected differently by cAMP: starved cells presented a dendritic growth towards the compound that was not apparent in vegetative cells. A closer view of such colonies (Fig. 4, panels 3 and 4) allowed differentiating the presence or absence of cAMP in the neighbourhood. These conditions were used for testing the release of compounds able to elicit the same chemotactic response in *E. coli* and *P. putida* cells. Panels 5 and 6 of Fig. 4 indicated that this was the case for *E. coli* but not for *P. putida*, thereby confirming that the production of cAMP – if any – was negligible under these conditions. Taken together, these set of data raised an intriguing paradox. In one hand, cAMP is necessary for $Crp_{P.putida}$ activity in *E. coli,* but in the other, cAMP production was not detected in any significant amount in the native host. Yet, the genome of *P. putida* does bear a *bona fide cyaA* gene encoding adenylate cyclase (Fig. 1A), the mRNA of which can be detected perfectly well in a microarray experiment (Yuste *et al*., 2006). The standing questions were therefore whether the *cyaA* gene product is functional for cAMP synthesis and whether the compound is the actual signal for Crp to operate in *P. putida* (see Fig. S3 on the possibility that cGMP binds $Crp_{P.putida}$). The experiments presented below were made to clarify these uncertainties.

The cyaA *gene of* P. putida *and its cognate product*

As mentioned above, there is only one recognizable *cyaA* gene in *P. putida.* It corresponds to the ORF PP5222 (Fig. 1B; Fig. S4), which encodes a putative protein of 951 amino acids belonging to the class I of soluble adenylate cyclases (http://www.uniprot.org/uniprot/Q88CF9; Danchin, 1993). As was the case with $crp_{P.putida}$, the $cya_{P.putida}$ gene stands alone in the chromosome, apparently encoding a mono-cistronic RNA. Transcription of $cyaA_{P.putida}$ was not only detected in a genome-wide analysis of the *P. putida* transcriptome (Yuste *et al*., 2006) but also inferred from the resistance to > 25 μg ml^{-1} kanamycin that results from inserting a promoterless Km^R gene in the midst of the $cyaA_{P.putida}$ coding sequence (Fig. 1B). To test production of cAMP borne by the cognate product, we placed the $cyaA_{P.putida}$ gene in an expression vector and had it induced in a Δ*cyaA* strain of *E. coli*. As controls we employed $cyaA_{E.coli}$ as well as the gene of the adenylate cyclase of *Bordetella pertussis*, which belongs to a different protein family (Ladant *et al*., 1992). The results of these experiments are shown in Fig. 5, rows 1–3. While the controls $cyaA_{E.coli}$ and $cya_{B.pertussis}$ were able to complement the lack of *cyaA* in a maltose metabolism test in *E. coli*, $cyaA_{P.putida}$ failed to do so. This indicated that cAMP was not produced in *E. coli,* at least to the levels needed to bring about complementation. On the other hand, $crp_{P.putida}$ had no effect on the lack of maltose consumption of a Δ*cyaA* Δ*crp* strain of *E. coli,* even when overproduced with IPTG (Fig. 3, row 4; see above). These results established that $Crp_{P.putida}$ does not function separately from cAMP, at least in this type of essays.

Taken together, these data led to a paradox. On one hand, $Crp_{P.putida}$ needs cAMP to work *in vivo*. On the other hand, we were unable to find any production of cAMP either in *P. putida* cells as such (see Fig. 4) or in a heterologous complementation test of $cyaA_{P.putida}$ in *E. coli* (Fig. 5). One possibility is that a small molecule different from cAMP is the one that actually elicits the regulatory ability of the protein. As mentioned above, cGMP could be a candidate to this, but no recognizable guanidyl cyclases can be made out in the genome of *P. putida,* and its residual production from side reactions is probably insignificant (Linder, 2010). A simpler explana-

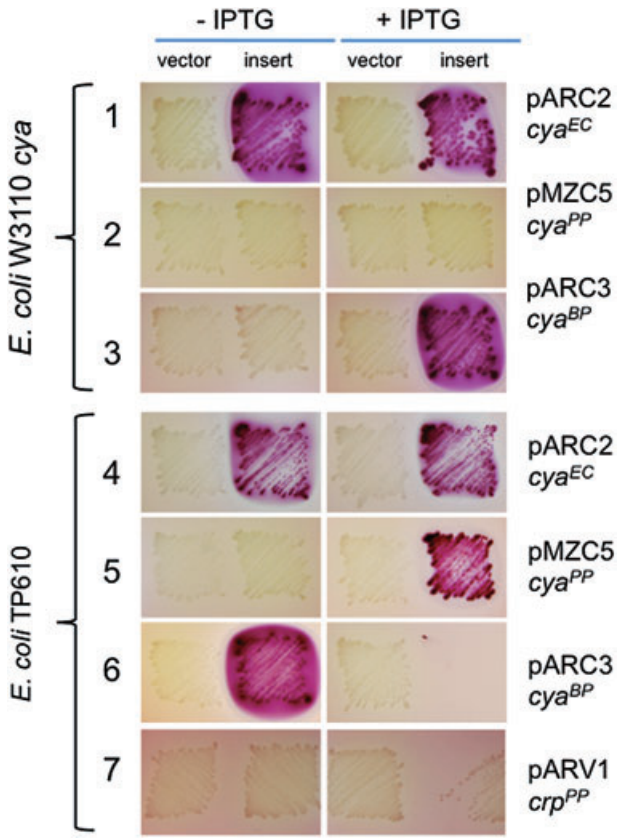

Fig. 5. Detection of cAMP production borne by the CyaA product of *P. putida*. The *ΔcyaA* strain indicated and the cAMP-hypersensitive host *E. coli* TP610 were transformed with plasmids bearing the *cyaA* genes from *E. coli* (EC), *P. putida* (PP) and *B. pertussis* (BP) along with their corresponding insert-less vectors as specified. Transformants were patched on McConkey-maltose plates as in Fig. 3. Note that plasmid pMZC5-expressing $cyaA_{P.putida}$ failed to restore the Mal$^+$ phenotype in the conventional Δ*cyaA* host (row 2) but it did in the specialized strain TP610 (row 5). In the same conditions, the $cya_{B.pertussis}$ variant is toxic for the same hypersensitive host (row 6). Observe also that overproduction of $crp_{P.putida}$ cannot complement strain *E. coli* TP610, even if overproduced (row 7), indicating that in no case $Crp_{P.putida}$ can work independently of cAMP for activation of the maltose operon.

tion could be that $Crp_{P.putida}$ has a higher affinity for cAMP in a way that allows the active Crp–cAMP complex to form with intracellular concentrations of the effector below the detection limit of the *in vivo* tests described above. To examine this possibility, we resorted to the use of a specialized strain of *E. coli* (named TP601; Hedegaard and Danchin, 1985), which is hypersensitive to cAMP. This means that a lower intracellular concentration of cAMP could be sufficient to restore the phenotypes derived from the loss of *cyaA*. As before, this strain was separately transformed with expression plasmids bearing $cyaA_{E.coli}$ and $cya_{B.pertusis}$ as controls, and $cyaA_{P.putida}$ as test case (Fig. 5, rows 4–6). Using this experimental set-up we were finally able to observe that overproduction of $cyaA_{P.putida}$ in this *E. coli* strain restored maltose metabolism, suggesting that cAMP was indeed produced – even if in very small amounts. On the other hand, overproduction of $crp_{P.putida}$ in the same strain had no effect (Fig. 5, row 7), thereby confirming the absolute requirement of cAMP for the protein to exert its regulatory role. An interesting sidelight of the results shown in Fig. 5 is what appears to be a strong threshold effect of intracellular cAMP concentrations. For instance, comparison of rows 3 and 6 (cells transformed with a $cya_{B.pertusis}$ expression plasmid) revealed that residual production of the protein in the non-induced cells complemented the lack of *cyaA* in cAMP-hypersensitive strain *E. coli* TP610 but not in the ordinary counterpart *E. coli* W3110Δ*cyaA*. In no case we found intermediate red tones in the colonies of *E. coli*, thereby pointing out that complementation of cAMP deficit was an all-on or all-off occurrence. This could explain that a lesser level of expression of $cyaA_{P.putida}$ in a standard Δ*cyaA E. coli* strain fails entirely to complement the mutation (Fig. 5, row 2), whereas the same construct fully reinstates the phenotype in a cAMP-hypersensitive strain (Fig. 5, row 5). In summary, this set of experiments indicated that $Crp_{P.putida}$ and cAMP act in concert, the logic prediction being that phenotypes resulting from the loss of either of the corresponding genes in *P. putida* should be equivalent. This prediction was thus addressed next.

Phenotypic characterization of cyaA *and* crp *mutants of* P. putida

In order to confirm the functional partnership of *crp* and *cyaA* in *P. putida* we exploited the wealth of information that can be derived from passing each of the strains through a phenotypic microarray (PM™) platform (see *Experimental procedures*), which reveals growth characteristics of bacteria exposed to large set of nutritional situations and environmental stresses. To this end, the isogenic wild-type, *cyaA* and *crp* strains described above were subject to a considerable number of growth and stress endurance conditions (Table S1). To our surprise, only a few of the growth settings exposed differences between the wild-type strain and any of the mutants. Yet, when such differences materialized, most of them were shared by both the *crp* and the *cyaA* strains (Fig. 6). In one case, both mutants acquired a higher tolerance to pyrophosphate and protamine as well as a noticeable resistance to colistin (Haagensen *et al.*, 2007). At the same time, the mutants lost concurrently the ability to use a large number of dipeptides as the only N source and became more sensitive to some antibiotics and antimetabolites. These results established the mutual dependence of $Crp_{P.putida}$ and $CyaA_{P.putida}$ to regulate these functions, as predicted by the complementation assays in

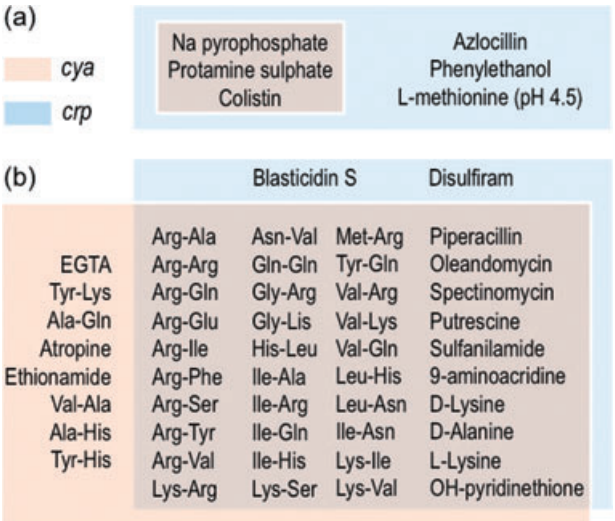

Fig. 6. Convergence of *crp* and *cyaA* phenotypes revealed through phenotypic microarray tests. The figure summarizes the most relevant results of the growth and stress-endurance conditions made on *cyaA* and *crp* mutants of *P. putida* (see Table S1 for full list).
A. Gain of function of each of the mutants in respect to the wild-type.
B. Loss of function of each of the mutants in respect to the wild-type. Note both overlapping and separate traits brought about by each of the mutations.

E. coli discussed before. Yet, some phenotypes were not shared by both mutants. For instance, the *crp* strain seemed to be more tolerant to the potent β-lactam antibiotic azlocillin and to the biocide phenylethanol, whereas the *cyaA* strain became more sensitive to EGTA and ethionamide. It could well happen that in some instances both cAMP and Crp could interact with other molecular partners, an issue that deserves further work. Yet, it is to be expected that the cAMP-Crp association is the one that makes most regulatory effects to happen.

Metabolic properties of P. putida *lacking Crp and CyaA*

Whether together or by separate, the phenotypic effects of lacking *cyaA* or *crp* were not trivial to explain mechanistically. Yet, we noticed that most phenotypes – shared or not by the *crp* and the *cyaA* mutants – can be traced to envelope-associated functions (peptide permeases, membrane-disrupting agents). In contrast, none of the gross abilities for metabolism of C and N sources appeared to be significantly affected. This makes a dramatic difference with the loss of the Crp–cAMP system of *E. coli,* which prevents cells from growing on a number of sugars and N compounds (Busby and Kolb, 1996).

In order to uncover metabolic effects of Crp–cAMP in *P. putida* which could have been missed in the PM™ tests above, we examined the growth fingerprinting (Isalan *et al.*, 2008) of each of the strains in four different liquid media. This extraordinarily sensitive method allows detection of minute changes in the metabolic profile of otherwise isogenic bacteria by generating the differential A_{600} value along the growth curve. In this way, minor variations in the C-consuming and N-consuming regime of the cells – which are often imperceptible in a standard growth curve – become translated into a defined diagnostic pattern which merges global gene expression levels, timing and metabolic effects (Isalan *et al.*, 2008).

Figure 7 summarizes such experiments made in three different media with ammonia as only N source along with succinate, glucose or fructose as sole C and energy substrates. While succinate forces an altogether gluconeogenic metabolism, glucose is consumed by *P. putida*

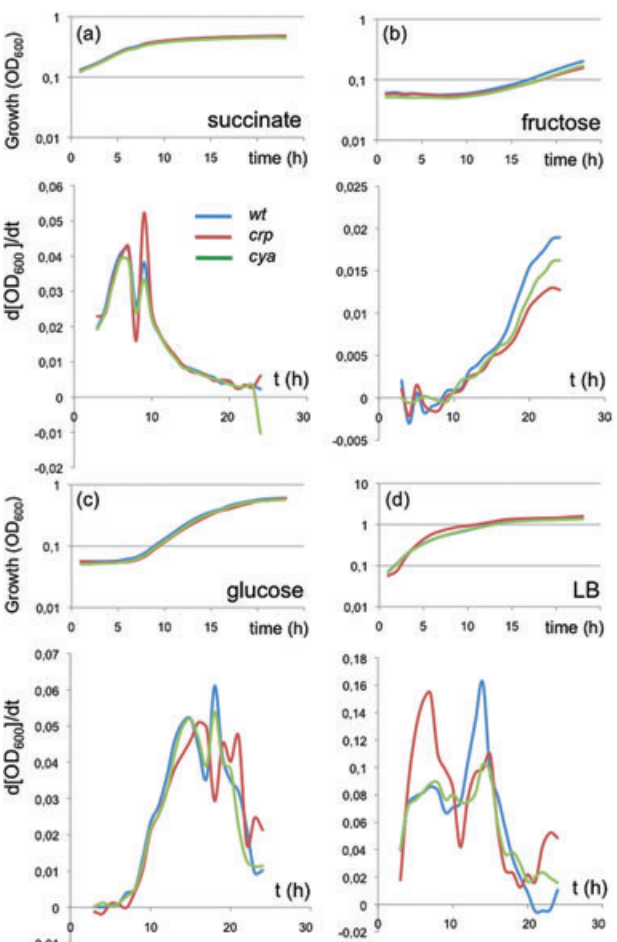

Fig. 7. Growth fingerprinting of *P. putida crp* and *cyaA* mutants. Triplicate cultures of each of the strains indicated were grown at 30°C in the media specified and their OD_{600} recorded at short intervals (see *Experimental procedures*) as displayed in the upper panels. Average OD_{600} increments with time were then derivated [$d(OD_{600})/dt$] and plotted along the entire period, originating distinct patterns (lower panels) which were diagnostic of metabolic and regulatory status (Isalan *et al.*, 2008). Notice the similarity of wild-type, *crp* and *cyaA* strains in succinate (A) and fructose (B), and the manifestation of growth outliers in glucose (C) which become more pronounced in rich LB medium (D).

through the Entner–Doudoroff (ED) pathway (del Castillo *et al*., 2007). Fructose, on the other hand, is converted into fructose-1,6-bisphosphate which can then be channelled both into the glycolytic route or the ED pathway (Velazquez *et al*., 2004). Growth on these substrates thus covered a considerable range of metabolic conditions. The first remarkable feature of these analyses (Fig. 7A) is the virtually identical growth fingerprints of the wild-type strain and the mutants in succinate medium. This indicated that the Crp–cAMP system has little, if any, effect on the general anabolism of carbon in this bacterium. A similar situation became evident in cultures with fructose as carbon source (Fig. 7B). In this case, both mutants displayed a small, but still noticeable delay in their growth rates as compared with the wild-type strain. However, the gross growth patterns were virtually the same in the three cases, suggesting a minor influence of the mutations on the fructose glycolysis/ED metabolic domain – certainly not a big effect. In contrast the growth fingerprints of the tested strains bifurcated when they were grown in glucose, particularly during the exponential-stationary-phase transition. Whereas the patterns of the wild-type strain and the *cyaA* mutant were almost interchangeable, the Crp-less strain diverged in at least five positions of the growth curve. Such deviations reflect metabolic differences in glucose brought about by Crp in the absence of cAMP. In any case, it must be noted that these differences never translated into a major modification of growth properties (see non-derived growth curves of Fig. 7C).

An interesting result was produced when the same strain set was grown in rich LB medium, because outlier growth signatures became clearly evident. The precise composition of this classical formulation varies from supplier to supplier, but it typically consists of a pancreatic digest of casein (containing approximately 7.7% carbohydrates) and yeast extract (17.5% carbohydrates), which provides a complex source of nitrogen and carbon sources (Baev *et al*., 2006). Furthermore, the medium contains amino acids and other organic acids as well as a diversity of precursors of macromolecules. Cells growing in this medium pass through a large number of metabolic regimes as available precursors are consumed sequentially and have thus to be synthesized rather than just taken from the external milieu. The growth fingerprints of wild-type, *crp* and *cyaA* strains of *P. putida* are shown in Fig. 7D. A close examination of the corresponding patterns revealed at least two gross physiological states in which the mutants behaved different from the wild type. In the first (approximately time 2–10 h) the *crp* mutant displayed a peak of rapid growth which was not shared by either the wild-type or the *cyaA* strain. As before, this effect was not traceable to cAMP, but to Crp alone. In the second part of the growth fingerprint (after 10 h), the two mutants behaved identically, but unlike the wild-type bacteria. It is possible that in this late metabolic state, both cAMP and Crp acted in concert to bring about the observed behaviour. Since the nature of the C sources seems to influence little the impact of *crp* and *cyaA* on the gross physiological status of *P. putida,* it is plausible that the changes observed in LB reflect variations in N metabolism and/or transport of precursors rather than connections to catabolism of C compounds.

Discussion

The question addressed in this work was whether the presence of *bona fide* orthologues of the *crp* and *cyaA* genes of *E. coli* in the genome of *P. putida* implies also the conservation of their biological function. In a first approach, the complementation experiments above indicated that the intrinsic mechanistic utility of Crp (i.e. binding and bending DNA in response to cAMP) was fully preserved. The protein surfaces of Crp that interact with various sites of the *E. coli*'s RNAP (AR1, AR2 and AR3; Fig. S1) seem to be maintained as well, although whether this feature is instrumental in *P. putida* remains to be demonstrated. The data above suggest also that $cyaA_{P.putida}$ may give rise to only small amounts of cAMP because of the low expression or activity of the corresponding cyclase (and perhaps further decreased by endogenous phosphodiesterases; Fuchs *et al*., 2010b). If so, Crp^{PP} could have evolved a higher affinity for this effector to counter this fact. Finally, $Crp_{P.putida}$ appears to work mostly in conjunction with endogenous cAMP but can also exert some of its functions by itself or together with other thus far unknown (small or big) molecular partners. Taken together, it seems that the structural efficacy of the Crp–cAMP duo for activating and repressing transcription is thus kept the same in both *E. coli* and *P. putida*. However, the maintenance of a given regulatory device does not mean keeping the biological function(s) regulated by it. In *E. coli*, Crp stays at the summit of the regulatory hierarchy together with other six global factors (Martinez-Antonio and Collado-Vides, 2003) while in *P. putida*, global phenotypic effects of *crp* and *cyaA* are hardly detectable, at least with the gross tests employed in this work.

Also in *E. coli*, the only known mechanism to change CyaA performance (and thus intracellular cAMP levels) involves direct protein–protein contacts of the enzyme with the phosphorylated forms of EIIA-type enzymes of the phosphoenolpyruvate-dependent carbohydrate transport system (Park *et al*., 2006). Whether this mechanism is conserved in *P. putida* is unknown, but certainly the only sugar that is transported through PTS in this microorganism is fructose (Velazquez *et al*., 2004). It would be surprising that the Crp–cAMP system were linked only to this sugar. Instead, it is likely that $CyaA_{P.putida}$ expression

and/or activity is controlled by other, thus far unknown physiological or environmental signals. On the other hand, the Crp protein of *E. coli* targets a well-defined consensus sequence (Fig. S2), which often appears in the genome of this bacterium in the proximity of metabolism-related operons (Grainger *et al.*, 2005). As shown above, overexpressed $Crp_{P.putida}$ complements the *E. coli* counterpart in a maltose metabolism assay, indicating that the *P. putida* protein recognizes a similar, but perhaps non-identical target sequence. The utilization of the $Crp_{E.coli}$ DNA-binding consensus sequence to scan the *P. putida* chromosome with various windows of permissiveness (Table S2) produces a considerable list of ORFs, which have upstream Crp sites located at various distances of the coding sequences. Out of those ORFs that are annotated, very few of them have a metabolic role and none of them is functionally related to their *E. coli* equivalents. Although such a list is merely indicative (the target consensus sequence for $Crp_{P.putida}$ might be significantly different; Fig. S2), the noticeable presence of envelope-related functions is worth of note. The corollary of these observations is that – despite a virtually identical mechanistic performance – the Crp–cAMP regulatory device of *P. putida* seems to be mostly alien to the biological (metabolic) functions that shape its predominant duty in *E. coli.* It is perfectly possible that some metabolic promoters of *P. putida* are still under a degree of control by Crp, but either there is a physiological back-up to them, or the effect is not enough to be detected through the growth phenotypes described above.

The conclusion of these analyses is that the very same Crp–cAMP component (along with one or more phosphodiesterases; Fuchs *et al.*, 2010a) has been recruited in different bacteria to control dissimilar sets of biological functions. It is difficult to trace the evolutionary history of such a device, but cAMP seems to be produced in virtually all biological systems (Botsford and Harman, 1992) and thus appears to be a versatile signal-carrier molecule between regulatory modules. Whether the metabolic control functions of the system appeared earlier or later, we argue that the Crp–cAMP arrangement constitutes an obvious occurrence of evolutionary exaptation at a molecular level. This concept, which originated in Darwinian Zoology (Gould and Vrba, 1982), refers to the process by which a fixed feature that was originally selected to perform a given function may later execute another quite unlike role, i.e. was co-opted for its current function. The archetypical example of exaptation is the evolutionary history of feathers, which were probably selected first for thermal insulation, and only later were they co-opted for flight. The key angle here is that functional re-assignment happens with little or no concurrent structural modification. In this way, one specific trait that has evolved under one set of conditions can be co-opted to serve a different function under a second set of circumstances. In our case, it is most likely that an ancestral form of the Crp–cAMP system was selected for controlling expression of one or more distinct functions, but was then co-opted in subsequent hosts to regulate entirely different functional ensembles. Even different strains of the same species can select the same transcriptional factor for controlling dissimilar sets of functions. For instance, a *crp* mutant of the solvent-tolerant *P. putida* strain DOT-T1E was unable to employ ammonium salts as sole N source (Daniels *et al.*, 2010) in contrast with the soil and rhizosphere-thriving *P. putida* KT2440 counterpart described in this work. That habitat-dependent exaptation happens in transcriptional factors argues in favour that regulatory control systems can evolve in a fashion that is independent of the functions that they regulate (de Lorenzo and Perez-Martin, 1996). Perhaps this is not true for all types of proteins, but the basic function Crp–cAMP system seems to enjoy a considerable level of orthogonality (i.e. context-independence; Silva-Rocha and de Lorenzo, 2008) that accounts for its regulatory flexibility in diverse bacteria.

The emergence of regulatory systems in prokaryotes is still a controversial matter. Bacteria with small genomes often bear few or no transcriptional factors, the number of which increases exponentially with genome size (Kunin *et al.*, 2003; Konstantinidis and Tiedje, 2004). Yet, whether this is because earlier bacteria had a very limited repertoire of TFs or because such TFs were lost along a genome-reduction process remains unclear (Tamames *et al.*, 2007). This makes it difficult to retrospect the earliest possible regulatory role of the Crp–cAMP system to only metabolic functions or other tasks. The work presented in this article exposes how regulatory innovations that may have been selected in a first instance to fulfil a given function can end up controlling a class of genes altogether different from the ones they were initially associated with. Such a regulatory exaptation thus provides an evolutionary mechanism through which regulons governed by the same transcription factor can dramatically diversify in different hosts. Our results also leave the question open on whether the emergence of a regulatory niche creates a selective pressure for the shaping of a new regulatory device or it is the appearance of a fresh transcriptional control mechanism that expands the connectivity and the dynamic range of a pre-existing network.

Experimental procedures

Strains, plasmids and growth conditions

The bacterial strains and the plasmids used in this work are listed in Table 1. All *Pseudomonas* strains were derived from the reference strain KT2440. *P. putida* MAD1 is a rifampicin-

resistant KT2440 variant which has been inserted in its chromosome with a *Pu–lacZ* transcriptional fusion along with the *xylR* regulator of the pWW0 plasmid assembled in a tellurite-resistance mini-Tn*5* vector (Fernandez *et al*., 1995). The *E. coli* strain of reference was W3110, on which various mutations were engineered as described below. Unless indicated otherwise, cells were grown at 30°C (*P. putida*) or 37°C (*E. coli*) either in rich LB medium or in synthetic mineral M9 medium (Sambrook *et al*., 1989) supplemented with 0.2% of the C sources indicated in each case and amended, where necessary, with suitable antibiotic to retain plasmids at the following concentrations: ampicillin (Ap) 100 μg ml^{-1}, chloramphenicol (Cm) 40 μg ml^{-1}, kanamycin (Km) 50 μg ml^{-1}, potassium telurite (Tel) 80 μg ml^{-1}. For complementation experiments, McConkey agar base (Miller, 1972) was prepared with 1.0% maltose. Where required, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the media to a final concentration of 1 mM. Phenotypic Microarray tests (PM™; Bochner *et al.,* 2001; Bochner, 2003) were run on the Biolog platform (http://www.biolog.com/).

Recombinant DNA techniques

General methods for DNA manipulation were performed as described previously (Sambrook *et al*., 1989). Genomic DNA was extracted from *P. putida* and specific chromosomal segments were amplified by means of polymerase chain reactions (PCR) using 100 ng of genomic DNA or 10 ng of plasmid as templates in a buffer containing 1.5 mM $MgCl_2$, 0.2 mM of deoxynucleoside triphosphates (dNTP) and 0.2 μM of the oligonucleotides indicated in each case. Reactions were run by first setting an initial denaturalization 4 min at 94°C followed by 35 cycles of denaturalization (1 min, 94°C), annealing (1 min, 55°C–65°C), extension (2 min at 72°C) and final extension (7 min, 72°C). In some cases, the same PCR reaction was directly run on a small amount of bacterial biomass picked from isolated colonies grown on agar plates. Where necessary, amplified DNA segments were purified with a PCR cleaning kit from Roche (Cat 11 732 676 001).

Construction of directed P. putida crp *and* cyaA *mutants*

The steps followed for generating a non-polar *crp*-null strain of *P. putida* are sketched in Fig. 1A. First, the *crp* gene coding sequence (PP0424) of *P. putida* KT2440 was amplified from chromosomal DNA by PCR using the oligonucleotides VFR1 (5′-GAATTCAGGCCCAGCAACAGCATTTCCATC-3′) and VFR2 (5′-AAGCTTTGCGATGGCCGCTACAACGTC-3′), which add terminal EcoRI and HindIII sites to the extremes of the resulting fragment respectively. The amplified product (1279 pb) was captured in the pGEMT vector (originating plasmid pMZV1) and the corresponding EcoRI–HindIII segment re-cloned in pUC18Not, which resulted in plasmid pMZV2. This construct was digested with NruI, which cleaves the *crp* sequence in its codon 116 leaving a blunt-end. This was exploited to insert a promoterless *xylE* gene (encoding catechol 2,3 dioxygenase, C2,3O) which was obtained by digestion of pXYLE10 plasmid (Table 1) with SmaI. The ensuing plasmid was named pMZV3. The NotI insert spanning the interrupted *crp* gene was cloned into the *sacB*$^+$ suicide delivery vector pKNG101 (Kaniga *et al*., 1991) originating pMZV4. Note that such NotI fragment containing the *crp::xylE* sequence in pMZV4 is 43 nucleotides smaller than this fragment pMZV3, as there is a indigenous NotI sequence 45 bp downstream from EcoRI site born by the VFR1 oligonucleotide. The pMZV4 plasmid was mobilized from its specialized host *E. coli* CC118λ*pir* into *P. putida* MAD1 by means of a triparental mating (de Lorenzo and Timmis, 1994) using *E coli* HB101 (RK600) as the helper strain. Co-integration of plasmid into the target chromosome was selected by plating the mating mixture on streptomycin plates. Resolution of the co-integrate was made happen by first growing individual colonies in non-selective LB medium devoid of antibiotics and then plating on agar medium with 5% sucrose. Clones growing in such a medium were sprayed with 1% catechol (to reveal the *xylE* gene) and screened for sensitivity to streptomycin, thereby verifying the predicted chromosomal recombination which leaves behind the *crp::xylE* insertion. A similar procedure was employed for generation of a *cyaA* mutant (Fig. 1B). In this case, the sequence of PP5222 (Fig. 1B) was amplified from genomic *P. putida* KT2440 DNA with oligonucleotides cyaAa1 (5′-GAATTCGCTTCCTGCATCTG AACCAGGG-3′) and cyaAa22 5′-GGGAAGCTTCCCCAGC ACATACA CGTC-3′), which add terminal EcoRI and HindIII sites to the corresponding segment. The 2240 pb PCR was cloned in pUC18Not as a EcoRI–HindIII insert, yielding pMZC2. This plasmid was then digested with KpnI/SalI to remove 362 codons internal codons of the *cyaA* gene, and the gap was replaced by promoterless kanamycin resistance cassette from plasmid pUCKm (Cases *et al*., 1999). The resulting plasmid was named pMZC3, the insert of which is 249 bp smaller than its counterpart in pMZC2 (Fig. 1B). The NotI insert of pMZC3 with the Δ*cyaA::km* sequence was excised and recloned in pKNG101, originating pMZC4. Delivery of the mutated gene to the chromosome of *P. putida* MAD1 was achieved as in the case of *crp::xylE* explained above, the final *cyaA*-minus strain being resistant to > 25 μg ml^{-1} kanamycin.

crp *and* cyaA *complementation assays*

For construction of a reliable recipient of adenylate cyclases of various origins, the *cyaA* coding sequence of the of *E. coli* K12 strain W3110 was cleanly deleted from the genome with the λ*red* recombinase method (Datsenko and Wanner, 2000). By the same token, the *crp* counterpart was removed with the seamless genomic deletion system of (Posfai *et al*., 1999). This procedure was also applied to the Δ*cyaA* strain for generating a double-deletion host Δ*crp* Δ*cyaA* (Table 1). These strains were employed along with the cAMP-hypersensitive strain *E. coli* TP610 (Hedegaard and Danchin, 1985) for complementation of the cAMP-Crp-dependent maltose consumption phenotype of *E. coli* in McConkey agar plates as explained in the text. The plasmids used in these experiments were constructed as follows. In order to create a control *cyaA*$^+$ plasmid, the *cyaA* coding region of *E. coli* was amplified along with 500 bp upstream and 200 bp downstream with primers aaECcyaA-F (5′-AAGGAAAAAAGCGGCCGCCAGTTCAA CGACCAGGCCCG-3′) and aaECcyaA-R (5′-TAAGGAATT

TGCGGCCGCGAGCCGCTGCACCAGGTATG-3′) to ensure inclusion of its own native promoter and terminator sequences. The flanking NotI sites generated with the same primers were then employed to clone the corresponding DNA segment in NotI-digested pVTR-A vector (Table 1), thereby originating plasmid pARC2, which expresses $cyaA_{E.coli}$ through its native transcription and translation signals. A second $cyaA^+$ control plasmid was engineered to express the catalytic domain of the adenylate cyclase of *B. pertussis* (i.e. residues 1–399 of its Cya protein). The corresponding DNA segment was amplified from plasmid pDIA5240 (Ladant *et al*., 1992) using primers aaBPcyaA-F (5′-TTACGAATTCTCACACAGGAAACAGCTA TGACC-3′) and aaBPcyaA-R (5′-TGAAAAGCTTCTAG CGTTCCACTGCGCCCAG-3′), which add terminal EcoRI and HindIII restriction sites to the amplified sequence. The resultant fragment was then digested with these enzymes and cloned in the matching sites of IPTG-inducible expression vector pVLT31, giving rise to pARC3. Finally, a test plasmid expressing the adenylate cyclase of *P. putida* was designed by amplifying the promoterless $cyaA_{P.putida}$ sequence (PP5222, Fig. 1B) with primers CC1 (5′-CGAATTCCGGGCTAAACTCG CCACAGCTTCAATG-3′) and CC2 (5′-CCCAAGCTTGGGATTTGCAGCACAAGGC TGTTCCAC-3′), which add the DNA segment of interest with EcoRI and HindIII sites. These were used for first cloning the entire $cyaA_{P.putida}$ in vector pUC18Not (originating plasmid pMZC6) and the same sites employed to ultimately insert the gene in the same expression vector (pVLT31) as that utilized for $cya_{B.pertussis}$ before. These operations originated plasmid pMZC5 (Table 1). To complete the entire set of complementation experiments, a $crp_{P.putida}$-expression plasmid was constructed as well. In this case, primers vfrMyc1 (5′-CCGGAATTCCGGATTCAAGGAGATATATCCA TGGTTGCCTCCGCCCTACCCG CCA-3′) and vfrHis-C (5′-CCCAAGCTTGGGCTAGTGATGGTGATGATGATGGCGGG TACCGTGGAC-3′) were used to both amplify the promoterless $crp_{P.putida}$ coding region (PP0424, Fig. 1A) and adding a 6xHis sequence at its 3′ end, the whole segment flanked – as in the previous instances – by EcoRI–HindIII sites. The corresponding segment was ligated to pUC18Not as a EcoRI/HindIII insert (originating pARV1) and subsequently recloned in expression vector pVLT31, generating plasmid pARV2. Other relevant plasmids are listed in Table 1. Every insert of the $cyaA^+$ and crp^+ expression plasmid was verified by DNA sequencing.

Protein techniques

For analyses of whole-cell protein extracts, bacteria were grown in LB medium until $OD_{600} = 1.0$. Cells from 1 ml of the cultures were spun down and directly resuspended in 100 μl of a loading buffer containing 2% sodium dodecyl sulphate (SDS), 5% glycerol, 60 mM Tris-HCl pH 6.8, 1% β-mercaptohetanol and 0.005% bromophenol blue. Samples were boiled for 5 min and then clarified by centrifugation 5 min at 10 000 r.p.m. Proteins were then analysed in a SDS-PAGE system with a 12% acrylamide/bisacrylamide (29:1) gel cast in a Mini-PROTEAN 3 Cell (Bio-Rad), following standard protocols. For visualization of proteins, the gel was stained with a 0.05% solution of Coomassie R-250 blue in methanol 50% and acetic acid 10%, and then de-stained in 10% methanol with 7% acetic acid. For immunodetection of Crp, proteins samples were separated by electrophoresis and transferred to a polyvinylidene difluoride membrane (PVDF, inmobilon-P, Milipore) using a semi-dry electrophoresis transfer apparatus (Bio-Rad). Blotted membranes were blocked for 1 h at 22°C (or 16 h 4°C) in 3% (w/v) skimmed milk, 1% (w/v) BSA, in PBS buffer containing 1% (v/v) Tween-20. The blots were subsequently incubated at room temperature in the same buffer (without detergent) with anti-CRP rabbit serum (kindly provided by H. Aiba) diluted 1:1000–1:5000 for 1 h. Detection of the Crp bands the membrane was treated with an anti-rabbit antibody conjugated with peroxidase and then soaked in a buffer 100 mM Tris-HCl pH 8.0 with1.20 mM luminol (Sigma) and 42 μM luciferin (Roche). Following a rapid rinse in PBS, the membrane was finally dropped in 0.0075% H_2O_2 (v/v). After a 1 min in the dark, the blots were exposed to an X-ray film (X-OMAT, Kodak).

Dictyostelium discoideum *chemotaxis assay*

To examine whether *E. coli* or *P. putida* could secrete compounds able to elicit a chemotactic response in the slime mold *D. discoideum* (Saran *et al*., 2002) cultures of *E. coli* W3110 and *P. putida* MAD1 were prepared in M9 minimal medium with 0.2% fructose as carbon source and let grow overnight to ensure its complete consumption. Cells were then spun down, the supernatants filtered through a 0.45 μm pore membrane and the filtrates concentrated 5× in a Speed Vac. Starvation was induced in *D. discoideum* by first growing the mold in nutrient medium and resuspending the cells for 16 h in QQ2 phosphate buffer (KH_2PO_4 16.5 mM; K_2HPO_4 3.9 mM; pH 6.2) devoid of nutrients. The suspension of *D. discoideum* was then adjusted to a concentration of 2.5×10^8 cells ml^{-1}, and 1 μl of the culture was placed in a QQ2-Agar plate. To induce chemotaxis, 1 μl of the concentrated supernatants of *P. putida* and *E. coli* were placed in the QQ2-Agar plate at 4 mm distance from the *D. discoideum* spot. The plates were then incubated in a humid chamber for 3 h at 22°C, after which formation of dendritic shapes towards the attractant at the rim of the *D. discoideum* population was considered an indication of chemotaxis and recorded with a Leica MZFLIII stereoscope.

Growth fingerprinting

Gross metabolic signatures of each of the strains in various media were extracted from OD_{600} time-courses as explained in Isalan and colleagues (2008). Plotting OD_{600} time derivative along growth (estimated as linear regression slope for sequential OD_{600} readings) gives a characteristic pattern that distinguishes between different strains and nutritional conditions. To ensure comparable start points, each of the strains under examination was separately grown overnight in LB medium. Cells were then collected, washed three times with 10 mM $MgSO_4$ and adjusted to an $OD_{600} = 2.0$. Two microlitres of each of these suspensions were then employed to inoculate 200 μl of the growth medium indicated in each case in 96-microwell plates (i.e. diluted 100 × in fresh medium). The plates were incubated at 30°C for 24 h, with 2 min of

orbital shaking every 15 min. The growth rates shown and their derivatives were the mean values from three replicates.

Bioinformatic tools

Protein sequences homologous to the Crp and CyaA products of *E. coli* were first searched in the *P. putida* KT2440 genome with the BLAST (Basic Local Alignment Search Tool) algorithms. This tool finds regions of sequence similarity, which yield evolutionary clues about the structure and function of the target sequence (Altschul *et al*., 1997). In our case, position-specific iterative BLAST (PSI-BLAST) was exploited to construct profiles from the first set of alignments and detect distant evolutionary relationships. Those sequences found with more than 80% similarity were then aligned with counterparts of enteric bacteria and *P. aeruginosa* using CLUSTAL-W (Larkin *et al*., 2007). Further analysis based on experimental data of the conserved DNA-binding domain of Crp and the cAMP binding site of CyaA confirmed the proposed *in silico* alignments and their unequivocal belonging to known protein families. Search of potential Crp-binding sequences through the genome of *P. putida* was made with the methods of van Helden (2003) and Thomas-Chollier and colleagues (2008). Structural predictions of the *P. putida* Crp protein were cast on the basis of the 1ruo crystal structure of the homologous *E. coli* protein (Guex *et al*., 2000). The model was constructed using SWISS-MODEL software and further analysed on the alignment generated by CLUSTAL-W and further refined by using the WHAT-IF tools (Arnold *et al*., 2006; Kiefer *et al*., 2009).

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Alignment of the Crp products of *E. coli, P. aeruginosa* and *P. putida*. The image shows the corresponding amino acid sequences along with an indication of the key functional ingredients as follows. : residues involved in cAMP binding (West *et al*., 1994). : stabilization of cAMP binding (West *et al*., 1994). : residues involved in Crp–RNAP interactions: AR1, AR2 and AR3 (activating regions: Niu *et al*., 1996; Busby and Ebright, 1999). : helix–turn–helix, DNA-binding motif (West *et al*., 1994). : residues that make direct contact with the DNA base edges (West *et al*., 1994; Parkinson *et al*., 1996). : residues that make contact with the phosphates of DNA. Note that the numbering of amino acids in the translated sequence of the Crp protein of *E. coli* is offset in respect to the numbering of the same residues in the tridimensional coordinates because of the lack of the leading Met in the purified and crystallized product. AR1 consists of the residues 156–164 of Crp. The side-chain of Thr-158 seems to be the most important for its function (it is conserved in *P. putida* and

P. aeruginosa). AR1 is necessary for activation of class I and class II promoters through direct interaction with the α-CTD (Parkinson *et al*., 1996; Busby and Ebright, 1999). AR2 is a specific determinant consisting of residues His-19, His-21, Glu-96 and Lys-101, which are necessary for the activation of class II promoters. In both *P. putida* and *P. aeruginosa*, the His-21 is substituted for an Arg. Moreover, His-19 is substituted in *P. putida* by a Glu. AR2 is proposed to interact with the α-NTD after initial binding of the RNAP to promoter DNA (Niu *et al*., 1996; Parkinson *et al*., 1996; Busby and Ebright, 1999). AR3 is the product of a non-native CAP–RNAP interaction at class II promoter and it is created by substitution of Lys-52 by a neutral or negatively charged residue. Interestingly, both in *P. putida* and *P. aeruginosa* there is a Glu at position 52 that probably makes functional the AR3 determinant as well. Several lines of evidence indicate that AR3, when operative, interacts with σ^{70} region 4 (residues 590–600; Parkinson *et al*., 1996; Busby and Ebright, 1999).

Fig. S2. DNA binding features of *P. putida* Crp protein as compared with counterparts of *E. coli* and *P. aeruginosa.*

A. Alignment of the HTH motifs. The amino acids that form the α-helixes of the DNA-binding domain of the *E. coli* protein are shown on top as reference. Note overall conservation but small changes in both the stabilization helix and the recognition helix. Although the secondary structure is kept, some changes become apparent in both the stabilization helix and the recognition helix of the motif. These are most likely translated in both a different affinity of the factor for target sequences as well as a non-equal DNA-binding specificity.

B. Consensus DNA-binding sequence for $Crp_{E.coli}$.

C. Same for Vfr (Crp of *P. aeruginosa*). This consensus is still the subject of a considerable controversy, as cAMP-free Vfr seems to bind non-identical DNA sequences as the cAMP–Vfr complex (Fuchs *et al*., 2010). Notice divergent optimal targets, surely derived from small changes in the corresponding HTHs noted above (Beatson *et al*., 2002; Kanack *et al*., 2006) leaving the possibility open that the best target for $Crp_{P.putida}$ is yet different from both.

Fig. S3. The cAMP binding site of Crp of *E. coli* versus *Pseudomonas*. The cAMP-binding domain of Crp proteins belongs to the larger family of cNMP-binding modules (Prosite Accession No. PDOC00691) which include also cAMP- and cGMP-dependent protein kinases and vertebrate cyclic nucleotide-gated channels.

A. Alignment of core cyclic nucleotide binding sites of homologous Crp proteins. The crystal structure of $Crp_{E.coli}$ reveals that up to six amino acid residues participate in such a pouch, five of them from one monomer (G71–E72/R82–S83 and S128) and the other from the second subunit of the dimeric structure (T127). Both $Crp_{P.putida}$ and Vfr (Crp of *P. aeruginosa*) keep these residues but have a number of changes in the intervening region.

B. Blow-up of the cAMP binding site of $Crp_{E.coli}$. Note the spatial orientation of the core G71/E72 and R82/S83 amino acids and the contribution of residues T127 of one subunit and S128 of the other monomer. These residues participate in inter-subunit communication and determine nucleotide selectivity.

C. Prediction of the cyclic nucleotide binding site of $Crp_{P.putida}$. Note the overall spatial conservation of the interaction pouch. However, a number of potentially important changes become apparent in this region. First, the Ser residue in position 128 of $Crp_{E.coli}$ changes into a Thr in the equivalent 133 amino acid of $Crp_{P.putida}$. Interestingly, a S128T exchange in $Crp_{E.coli}$ is known to make the mutant responsive to both cAMP and cGMP (Lee *et al*., 1994; Youn *et al*., 2008) posing the possibility that $Crp_{P.putida}$ uses both cAMP and cGMP as effectors (with uncertain outcome, though: see Fuchs *et al*., 2010). On the other hand, the Ser-63 residue, which lies outside the direct cAMP interaction sphere in $Crp_{E.coli}$, is changed to Ala in the matching position 64 of $Crp_{P.putida}$ (Fig. S1). The change S63A increases the binding of $Crp_{E.coli}$ for cAMP, what could be translated in a stronger affinity of the *P. putida* protein for its effector. Surprisingly, the most noticeable difference in the predicted cAMP binding site of $Crp_{P.putida}$ when compared with $Crp_{E.coli}$, i.e. the insertion of three extra residues between the two pairs of amino acids (GE and RS) that shape most of the pocket, does not have any foreseeable structural consequence for the interaction with the effector. In summary, the structural prediction analyses suggest that $Crp_{P.putida}$ may have a high avidity for cAMP and that cGMP could also act as a productive effector.

Fig. S4. Alignment of the CyaA products of *P. putida, P. fluorescens, P. aeruginosa* and *E. coli.* The comparison shown was generated with CLUSTAL 2.0.12 multiple sequence alignment.

Table S1. Phenotypic microarray data.

Table S2. Prediction of binding sites for Crp through the genome of *P. putida* KT2440.

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