REVIEW ARTICLE

Carbon catabolite repression in *Pseudomonas*: optimizing metabolic versatility and interactions with the environment

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Abstract

Metabolically versatile free-living bacteria have global regulation systems that allow cells to selectively assimilate a preferred compound among a mixture of several potential carbon sources. This process is known as carbon catabolite repression (CCR). CCR optimizes metabolism, improving the ability of bacteria to compete in their natural habitats. This review summarizes the regulatory mechanisms responsible for CCR in the bacteria of the genus *Pseudomonas*, which can live in many different habitats. Although the information available is still limited, the molecular mechanisms responsible for CCR in *Pseudomonas* are clearly different from those of *Enterobacteriaceae* or *Firmicutes*. An understanding of the molecular mechanisms underlying CCR is important to know how metabolism is regulated and how bacteria degrade compounds in the environment. This is particularly relevant for compounds that are degraded slowly and accumulate, creating environmental problems. CCR has a major impact on the genes involved in the transport and metabolism of nonpreferred carbon sources, but also affects the expression of virulence factors in several bacterial species, genes that are frequently directed to allow the bacterium to gain access to new sources of nutrients. Finally, CCR has implications in the optimization of biotechnological processes such as biotransformations or bioremediation strategies.

Introduction

Free-living bacteria frequently have a versatile metabolism that allows the use of many different compounds as a source of carbon and energy. This facilitates survival in different habitats and adaptation to changing environmental conditions. Metabolic versatility is linked to a tight, although flexible regulation of the expression of metabolic pathways, regulation that is directed to optimize efficiency and ecological fitness. Several distinct global regulation networks participate in coordinating gene expression programmes under different situations (Cases & de Lorenzo, 2005). Some of them allow cells to respond to stress conditions such as starvation, oxygen (O_2) limitation, etc. Many bacteria also possess global regulation systems that coordinate metabolism under conditions of feast, i.e., when several possible carbon sources are available at concentrations that do not limit growth. Under these conditions, bacteria can either cometabolize the different carbon sources or assimilate preferentially one specific compound that provides the most efficient growth, inhibiting at the same time the uptake and/or the expression of the genes required for the catabolism of other nonpreferred compounds. The regulatory processes allowing for this selection of preferred carbon sources have been named carbon catabolite repression (CCR) or catabolite repression control (CRC). It was initially described in *Escherichia coli* for the hierarchical assimilation of sugars (Magasanik, 1970). CCR not only inhibits the expression of the pathways for nonpreferred compounds, but can also generate an important reorganization of the metabolism that requires the activation of several genes, a process named carbon catabolite activation (CCA). Both CCR and CCA can be included in the more general term carbon catabolite control (CCC). Catabolite repression is important for the competition of the different bacterial species in their natural habitats, because it plays a key role in determining growth speed and which carbon compound will be assimilated, thus allowing for a flexible and reversible specialization for a particular carbon source (Görke & Stülke, 2008; Moreno *et al*., 2009a). However, the identification of a growing list of

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genes controlled by CCC has shown that this global regulation process affects not only specific catabolic pathways, but many other genes that impact on important aspects of cell physiology and of the interaction of the bacterium with the environment. For example, in pathogenic bacteria, catabolite repression controls the expression of several virulence factors, which are frequently genes ultimately directed to facilitate the access to new carbon sources (Görke & Stülke, 2008).

CCR is a complex regulatory process that has been observed in most free-living heterotrophic bacteria, particularly in those having a versatile metabolism. In a given bacterial species, CCR can be mediated by diverse regulatory systems. Interestingly, the underlying molecular mechanisms can be quite different in distinct bacterial species. The factors and mechanisms involved have been studied mainly in *Enterobacteriaceae* (*E. coli* and *Salmonella*) and in *Firmicutes* (*Bacillus subtilis* and *Lactobacilli*) (reviewed in references Görke & Stülke, 2008; Deutscher, 2008). This review provides an overview on the regulatory mechanisms responsible for CCR in *Pseudomonas*. Although the information available is still fragmentary, it is clear that CCR mechanisms in *Pseudomonas* are very different from those operating in *Enterobacteriaceae* or in *Firmicutes* (Shingler, 2003; Rojo & Dinamarca, 2004; Cases & de Lorenzo, 2005; Görke & Stülke, 2008). This probably arises from differences in the general metabolism and in the ecological specialization of each bacterial group. To facilitate comparisons, the first section of this review provides a brief overview of the mechanisms of CCR in *E. coli* and *B. subtilis*. This is followed by a more extensive description of the CCR processes in *Pseudomonas*. Particular emphasis is on the differences observed between *Pseudomonas* and other model bacterial species, on the coexistence of several different CCR networks that respond to distinct metabolic signals and on the importance of CCR for the expression of genes relevant for biotechnological applications and for virulence.

CCR in the model bacteria *E. coli* and *B. subtilis*

In *E. coli* and *B. subtilis*, glucose is the preferred carbon source. It enters the cell through a phosphoenolpyruvate-carbohydrate phosphotransferase system (PTS) that couples glucose transport to its phosphorylation (see Fig. 1). The PTS system is formed by several enzymes that transfer the high-energy phosphate from phosphoenolpyruvate (PEP) to the sugar being internalized (Deutscher *et al.*, 2006). The first two enzymes, EI and HPr, are common for all sugars transported by this system (glucose, fructose, mannose, trehalose). The third enzyme, named EII, is sugar specific and phosphorylates and transports the sugar through the cytoplasmic membrane. The EII enzymes are usually composed of three domains included in different polypeptides. In *E. coli*, the glucose-specific EII enzyme has two polypeptides: the cytoplasmic $EIIA^{Glc}$ and the membrane-bound glucose transporter $EIICB^{Glc}$. The phosphoryl group is sequentially transferred from PEP to glucose via EI, HPr, $EIIA^{Glc}$ and $EIICB^{Glc}$. The glucose-specific enzyme $EIIA^{Glc}$ is the central regulator of CCR in *E. coli*, while in *B. subtilis* and *Firmicutes*, HPr plays this role (for recent reviews, see Deutscher *et al.*, 2006; Görke & Stülke, 2008; Fujita, 2009).

In *E. coli*, when glucose is actively transported and transformed to glucose-6-phosphate, the levels of the phosphorylated form of $EIIA^{Glc}$ are low, while those of the nonphosphorylated form are high (the phosphoryl group is drained by glucose). Nonphosphorylated $EIIA^{Glc}$ interacts with the transporters for several sugars that do not use the PTS system (lactose, maltose or melobiose), inhibiting their activity (Fig. 1a). Impeding the entry of these sugars into the cell hinders the induction of the corresponding catabolic genes. This regulation system has been termed 'inducer exclusion'. When glucose is exhausted, the levels of the phosphorylated form of $EIIA^{Glc}$ increase, while those of the nonphosphorylated form decrease. Under these conditions, inducer exclusion is relieved and non-PTS sugars such as lactose can enter the cell. This allows the induction of the corresponding catabolic genes, although the expression levels are usually low due to the relative weakness of the promoters involved. For this reason, full induction of the genes for the assimilation of non-PTS sugars such as lactose, maltose, arabinose, rhamnose, galactose or melobiose requires transcription activation by the cAMP–CRP complex. When glucose is being assimilated, cAMP levels are low and CRP is present in a monomeric state that is unable to bind DNA and activate transcription. However, when glucose is exhausted, the phosphorylated form on $EIIA^{Glc}$, together with an unknown soluble factor, interacts with and stimulates the activity of the adenylate cyclase, which generates cAMP from ATP (Park *et al.*, 2006; Bettenbrock *et al.*, 2007). This leads to dimerization of CRP as a cAMP–CRP complex, which can bind DNA and regulate the transcription of the target genes (Parkinson *et al.*, 1996; Tagami & Aiba, 1998; Busby & Ebright, 1999). Therefore, the phosphorylation state of $EIIA^{Glc}$ can regulate the expression of genes involved in the metabolism of several carbohydrates in two ways: through inducer exclusion and by controlling cAMP levels.

This model leads us to speculate that, when glucose is not present, the levels of phosphorylated $EIIA^{Glc}$ and of cAMP are high. In fact, the levels of cAMP parallel those of the phosphorylated form of $EIIA^{Glc}$ under a wide range of conditions (Bettenbrock *et al.*, 2007). However, cAMP levels are very similar in cells growing on glucose or on lactose and significantly lower than when cells grow on poor carbon sources such as lactate or succinate (Inada *et al.*, 1996a; Hogema *et al.*, 1997). When cells grow on a mixture of

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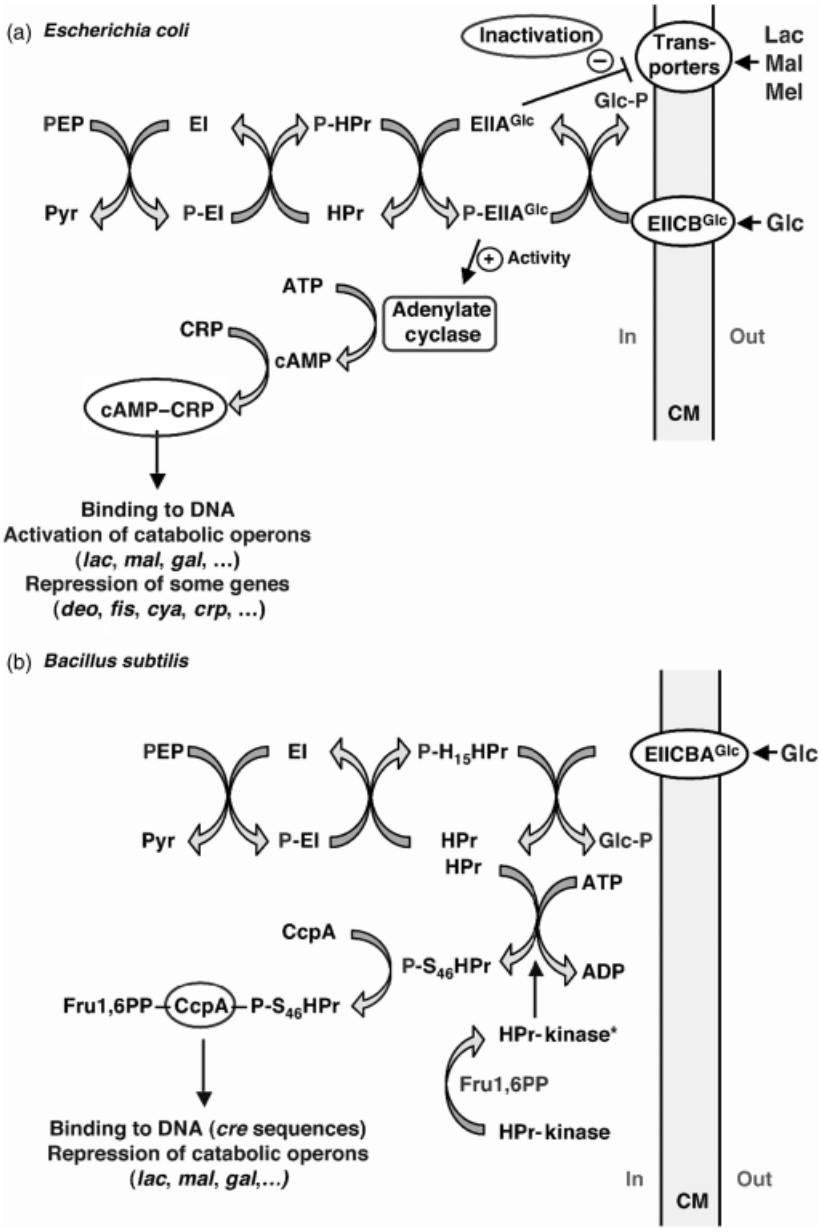

Fig. 1. Sugar transport and CCR in *Escherichia coli* and *Bacillus subtilis*. In both *E. coli* (a) and *B. subtilis* (b), the transport of glucose (Glc) through the cytoplasmic membrane (CM) is coupled to its phosphorylation by a multiprotein phosphorelay system named the PTS. The phosphorylation state of some components of the PTS triggers a CCR effect. In *E. coli*, CCR is mainly determined by the phosphorylation state of the $EIIA^{Glc}$ enzyme. Nonphosphorylated $EIIA^{Glc}$, which accumulates when glucose is actively transported into the cell, interacts with the transporters for nonpreferred sugars such as lactose (Lac), maltose (Mal) or melobiose (Mel), impeding their uptake and the subsequent activation of the catabolic operons for these sugars. When glucose is exhausted, $EIIA^{Glc}$ accumulates in its phosphorylated form. Nonpreferred sugars can then enter the cell and induce the expression of their catabolic pathways. Expression of these genes is further stimulated by the cAMP–CRP transcriptional regulator. This regulator binds DNA as a dimer, and dimers form only in the presence of cAMP, whose levels vary according to the phosphorylation state of $EIIA^{Glc}$. In *B. subtilis*, CCR is determined by the phosphorylation state of the HPr enzyme. This enzyme can be phosphorylated either at His-15 or at Ser-46. $HPr\text{-}His_{15}$ serves as a phosphate donor for the incoming glucose. $HPr\text{-}His_{46}$, which accumulates when glucose is being actively consumed, interacts with the the CcpA protein, forming a complex that binds to DNA at the *cre* sequences, inhibiting the expression of catabolic operons for nonpreferred sugars. The $CcpA\text{-}HPr\text{-}His_{46}$ complex is stabilized by fructose-1,6-biphosphate (Fru1,6PP), an intermediate of the glycolytic pathway. See text for a complete description.

glucose and lactose, there is a transient increase in the cAMP levels when glucose is exhausted, but these levels decrease again when cells resume exponential growth at the expense of lactose (Bettenbrock *et al.*, 2006). In addition, other sugars such as glucose-6-phosphate, glycerol-3-phosphate, xylose or gluconate, which are not transported by the PTS system and therefore are not expected to drain the pools of phosphorylated $EIIA^{Glc}$, also reduce the levels of both cAMP and CRP. Interestingly, these non-PTS sugars also exert a CCR effect on some catabolic pathways (Hogema *et al.*, 1997) and can trigger the inducer exclusion response. For example, glucose-6-phosphate inhibits the transport of lactose (Hogema *et al.*, 1998a) and even lactose partially inhibits the activity of the lactose permease to avoid excessive lactose uptake, which is detrimental for the cell (Hogema *et al.*, 1999). This effect has been traced to the phosphorylation state of $EIIA^{Glc}$, because several non-PTS sugars can cause partial dephosphorylation of this enzyme, thereby favouring inhibition of sugar permeases by the nonphosphorylated form of $EIIA^{Glc}$ (Hogema *et al.*, 1998b). It has been proposed that the metabolism of these non-PTS sugars decreases the PEP/pyruvate ratio, which leads to partial dephosphorylation of the PTS proteins (Hogema *et al.*, 1998b; Bettenbrock *et al.*, 2007). Because

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the phosphorylation state of $EIIA^{Glc}$ determines cAMP levels, the partial dephosphorylation exerted by the uptake and metabolism of lactose and other non-PTS sugars is paralleled by a decrease in cAMP levels, which, however, maintains concentrations that are high enough to allow cAMP–CRP regulation of several genes.

The cAMP–CRP complex controls the activity of close to 200 promoters (Kolb *et al.*, 1993; Zheng *et al.*, 2004), including those for the adenylate cyclase (Inada *et al.*, 1996b), for the major glucose transporter $EIICB^{Glc}$ (Kimata *et al.*, 1997), and for many genes involved in carbohydrate metabolism. This fact, together with the variations in cAMP levels and with the different affinities of cAMP–CRP for each of its target sites, makes it difficult to generate a unifying picture for all the genes involved.

The relative importance of inducer exclusion and of the cAMP–CRP complex in *E. coli* CCR differs depending on the genes considered. For example, inducer exclusion is the main factor determining CCR of the *E. coli lac* operon, while cAMP–CRP is more important for CCR in the case of the glycerol utilization genes (Inada *et al.*, 1996a; Holtman *et al.*, 2001; Bettenbrock *et al.*, 2006). Finally, it should be noted that the regulation of carbohydrate metabolism in *E. coli* includes several other global regulators such as Mlc (Plumbridge, 2002) or Cra (Saier & Ramseier, 1996), which also control the expression of the PTS and sugar assimilation genes.

In *B. subtilis*, CCR is exerted mainly by directly regulating the transcription of genes relevant for the transport and metabolism of carbon sources (for recent reviews, see Deutscher *et al.*, 2006; Deutscher, 2008; Görke & Stülke, 2008; Fujita, 2009). The major transcriptional regulator of CCR in *B. subtilis* (Henkin *et al.*, 1991), and in many other related Gram-positive bacteria (Warner & Lolkema, 2003), is the catabolite control protein A (CcpA), which belongs to the LacI/GalR family of regulators (Fig. 1b). CcpA binds to a *cis*-acting palindromic sequence named the catabolite-responsive element (*cre*) (Weickert & Chambliss, 1990). Depending on the location of the *cre* sites at the target promoters, binding of CcpA to DNA can activate or repress transcription. Approximately 10% of the *B. subtilis* genome is under the influence of CcpA (Miwa *et al.*, 2000; Moreno *et al.*, 2001). For example, CcpA regulates the expression of several genes coding for transporters of tricarboxylic acid (TCA) cycle intermediates, of many genes involved in the metabolism of carbon, nitrogen and phosphate and some respiration genes (see Fujita, 2009 for a review). A key aspect of CcpA function is that efficient binding to the *cre* sites requires the interaction of CcpA with the allosteric cofactor $P\text{-}Ser_{46}$-HPr, a component of the PTS (Deutscher *et al.*, 1995; Schumacher *et al.*, 2004). The interaction between CcpA and $P\text{-}Ser_{46}$-HPr, well as the ability of the complex to bind to the *cre* sites, is enhanced by the presence of the glycolytic intermediates fructose-1,6-biphosphate or glucose-6-phosphate, whose levels are high when glucose is actively consumed (Deutscher *et al.*, 1995; Schumacher *et al.*, 2007). Therefore, in Gram-positive bacteria, the PTS system for sugar transport also plays a key role in CCR, but in this case, the regulatory signal is transduced by the PTS component HPr, rather than by $EIIA^{Glc}$, as it occurs in *E. coli*.

Bacillus subtilis HPr can be phosphorylated at two different residues. The phosphoryl group derived from PEP is transferred by the EI protein to HPr residue His-15, from which it is imparted to $EIICBA^{Glc}$ and finally to an incoming glucose molecule, yielding glucose-6-phosphate. When cells are actively metabolizing glucose and the levels of the glycolytic intermediate fructose-1,6-biphosphate are high, the bifunctional protein HPr-kinase/phosphorylase (HPrK/P) phosphorylates HPr at residue Ser-46 (Reizer *et al.*, 1998, and references therein). In this case, the phosphoryl group derives from ATP. When glucose is exhausted and glycolysis activity declines, the levels of fructose-1,6-biphosphate decrease, conditions under which the HPrK/P dephosphorylates $P\text{-}Ser_{46}$-HPr. In this way, the glycolytic intermediate fructose-1,6-biphosphate behaves as a key signalling molecule in the regulation of carbon metabolism in *B. subtilis*. The ATP/Pi ratio, which is high in cells assimilating glucose and low when a poor carbon source is being used, also modulates the HPrK/P activity (reviewed in Deutscher *et al.*, 2006). Sugars other than glucose can also trigger CCR. There is a correlation between the extent to which each sugar causes repression and the amount of HPr phosphorylated at Ser-46, suggesting that the hierarchy of CCR is determined by the activity of HPrK/P (Singh *et al.*, 2008).

Bacillus subtilis contains an HPr-like protein named Crh (Galinier *et al.*, 1997). *Bacillus subtilis* HPr and Crh exhibit 45% sequence similarity and are structurally similar (Favier *et al.*, 2002), but HPr His-15 is replaced with a Gln at Crh. Therefore, Crh can be phosphorylated at residue Ser-46, but not at position 15, meaning that Crh cannot participate in glucose transport. However, $P\text{-}Ser_{46}$-Crh can interact with CcpA, forming a complex that can partly substitute for $P\text{-}Ser_{46}$-HPr in CCR. However, Crh and HPr have different impacts on CCR, in part because CcpA has a much lower affinity for $P\text{-}Ser_{46}$-Crh than for $P\text{-}Ser_{46}$-HPr, because Crh expression is much weaker than that of HPr and because the formation of the CcpA-$P\text{-}Ser_{46}$-Crh complex is not enhanced by fructose-1,6-biphosphate (Görke *et al.*, 2004; Schumacher *et al.*, 2006).

Bacillus subtilis has other catabolite control proteins in addition to CcpA. For example, CcpB, CcpC, CcpN and CggR control the expression of specific groups of genes (for reviews, see Deutscher, 2008; Fujita, 2009). Some additional transcriptional regulators that mediate CCR contain PTS

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regulation domains. These regulators control the expression of genes encoding sugar-specific PTS components and their activity is regulated by phosphorylation through proteins of the PTS system (reviewed in Deutscher *et al.*, 2006; Deutscher, 2008).

CCR in *Pseudomonas*: preferred substrates

Pseudomonas are ubiquitous bacteria that can live under a wide range of environmental conditions, for example in soil or water ecosystems, or associated with plants, animal or human tissues (Palleroni & Moore, 2004). Some species, as for example *Pseudomonas aeruginosa*, can behave as severe opportunistic pathogens. Other species, such as *Pseudomonas fluorescens* or *Pseudomonas putida*, can be beneficial for plants and thrive in the plant rhizosphere (Lugtenberg & Dekkers, 1999; Molina *et al.*, 2000; Martins dos Santos *et al.*, 2004). A common characteristic of *Pseudomonas* is their considerable metabolic versatility, being able to assimilate a wide range of compounds. However, glucose does not play the same central role in *Pseudomonas* as it does in *E. coli*, *B. subtilis* or lactic acid bacteria. In fact, the preferred carbon sources for *Pseudomonas* are some organic acids or amino acids, rather than glucose. For example, in the presence of succinate and glucose, the expression of enzymes of the *P. aeruginosa* central pathway for glucose catabolism such as glucose-6-phosphate dehydrogenase or 2-keto-3-deoxy-6-phosphogluconate aldolase is repressed until succinate is consumed (reviewed in reference Collier *et al.*, 1996). The expression of genes for the assimilation of other sugars such as gluconate, glycerol, fructose and mannitol is also inhibited by succinate or acetate. Glucose, however, has a repressing effect on the expression of several genes, for example on the *P. aeruginosa* regulons for mannitol or histidine utilization, on the *P. aeruginosa* amidase genes (Collier *et al.*, 1996 and references therein), on the *P. putida* pWW0 plasmid genes for the degradation of toluene (Holtel *et al.*, 1994; Cases *et al.*, 1999; del Castillo & Ramos, 2007), on the *P. putida* genes involved in the assimilation of methylphenol (Müller *et al.*, 1996) and phenylacetic acid (Schleissner *et al.*, 1994) or on the genes required to degrade styrene in *P. fluorescens* ST (Santos *et al.*, 2000; Rampioni *et al.*, 2008). Induction of these genes is also inhibited when cells are grown in complete media, where amino acids are the carbon source used, or in a synthetic medium containing casamino acids (an acid hydrolysate of casein). Therefore, there is a sequential hierarchy where some organic acids and amino acids stand as the most preferred compounds, and hydrocarbons as the less preferred compounds, glucose lying in between. Even among amino acids, there is also a hierarchy of preference: when grown in a complete medium containing all 20 amino acids, *P. putida* preferentially uses Pro, Ala, Glu, Gln, His, Arg, Lys, Asp and Asn, while it represses genes required for the assimilation of Val, Ile, Leu, Thr, Phe, Tyr, Gly and Ser (Hester *et al.*, 2000b; Morales *et al.*, 2004; Moreno *et al.*, 2009a).

There are some apparent exceptions to these preferences, however, because *P. putida* strain CSV86 has been reported to use naphthalene (an aromatic hydrocarbon) in preference to glucose (Basu *et al.*, 2006, 2007a). Organic acids such as succinate do not inhibit naphthalene degradation in this strain, but impair glucose assimilation by impeding glucose transport and the expression of glucose-assimilating enzymes (Basu *et al.*, 2007b). The ability to degrade naphthalene could be transferred to *Stenotrophomonas maltophilia* by conjugation, suggesting that catabolic genes are located on a conjugative element (Basu & Phale, 2008). Transconjugants also showed preferential utilization of naphthalene over glucose, suggesting that the conjugative element possesses determinants that confer this trait. The authors proposed that the repression elicited by naphthalene on glucose utilization could be due to the accumulation of organic acid intermediates during assimilation of the aromatic compound (Basu *et al.*, 2007a).

Glucose not only has a different influence in CCR in *Pseudomonas* as compared with *E. coli* or *B. subtilis*, but also shows striking differences the way in which it is transported and metabolized. As explained in previous sections, in *Enterobacteria* and *Firmicutes*, glucose is transported through the cytoplasmic membrane and phosphorylated by a PTS system, yielding glucose-6-phosphate. Some components of this PTS system directly participate in the CCR response (see Fig. 1). Unlike in *E. coli* and *B. subtilis*, the uptake of glucose in *Pseudomonas* is not carried out by a PTS transport system. Glucose crosses the outer membrane into the periplasmic space through the OprB-1 porin (Fig. 2). Thereafter, glucose can be directly transported into the cell, or converted to gluconate or to 2-ketogluconate in the periplasmic space, compounds that are internalized using specific transporters (Lessie & Phibbs, 1984; Schleissner *et al.*, 1997; del Castillo *et al.*, 2007). At least in *P. putida*, the direct import of gluconate into the cell normally accounts for only 10% of glucose metabolism, the remaining 90% occurring by direct uptake of glucose and of the 2-ketogluconate generated in the periplasmic space from glucose (del Castillo *et al.*, 2007). Once inside the cell, glucose, gluconate and 2-ketogluconate are processed to yield 6-phosphogluconate, which is further oxidized through the Entner–Doudoroff pathway (Fig. 2; Lessie & Phibbs, 1984; del Castillo *et al.*, 2007).

Metabolic flux ratio analyses, together with previous genetic and biochemical data, indicate that in *E. coli* and *B. subtilis*, glucose is mainly assimilated through the glycolytic (Emden–Meyerhof–Parnas) pathway, the Entner–Doudoroff pathway playing a minor role (Fuhrer *et al.*,

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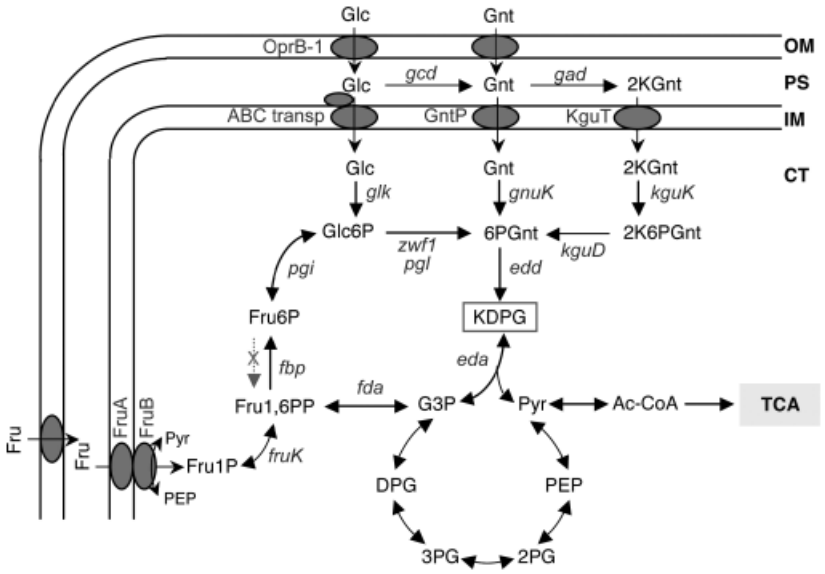

Fig. 2. Sugar transport and metabolism in *Pseudomonas putida* and *Pseudomonas aeruginosa*. Fructose is the only carbohydrate known to enter the cell through a PTS system in *Pseudomonas*. All other sugars use non-PTS transport systems. The genes coding for the enzymes that undertake some relevant steps, and the name of some transporters, are indicated. The lack of phophofructokinase in most *Pseudomonas* species, which transforms Fru6P into Fru1,6PP, is highlighted with a crossed arrow. OM, outer membrane; PS, periplasmic space; IM, inner membrane; Fru, fructose; Glc, glucose; Gnt, gluconate; 2KGnt, 2-ketogluconate; Glc6P, glucose-6-phosphate; 6PGnt, 6-phosphogluconate; 2K6PGnt, 2-keto-6-phosphogluconate; Fru1P, fructose-1-phosphate; Fru6P, fructose-6-phosphate; Fru1,6PP, fructose-1,6-biphosphate; KDPG, 2-keto-3-deoxy-6-phosphogluconate; G3P, glyceraldehyde-3-phosphate; DPG, 1,3-diphosphoglycerate; 3PG, 3-phosphoglycerate; 2PG, 2-phosphoglycerate; PEP, phosphoenolpyruvate; Pyr, pyruvate; TCA, tricarboxylic acid cycle.

2005, and references therein). Most *Pseudomonas* species (*P. aeruginosa*, *P. putida*, *P. fluorescens*, *Pseudomonas syringae*, *Pseudomonas mendocina* and *Pseudomonas entomophyla*, but not *Pseudomonas stutzeri*) lack phosphofructokinase, a key enzyme of the glycolytic pathway that transforms fructose-6-phosphate into fructose-1,6-biphosphate (see Fig. 2). The lack of phosphofructokinase impedes the assimilation of glucose through the glycolytic pathway. Accordingly, metabolic flux ratio analyses performed in *P. putida* and *P. fluorescens* showed that glucose is metabolized through the Entner–Doudoroff pathway, via 6-phosphogluconate (Fuhrer *et al.*, 2005; del Castillo *et al.*, 2007).

The metabolic differences between *Pseudomonas* and *Enterobacteria* or *Firmicutes* may facilitate a different life-style of each bacteria or different strategies to compete in the habitats they colonize. When living in soils, *Pseudomonas* probably use compounds derived from the decomposition of plant or animal tissues, for example amino acids, sugars, aromatic compounds or diverse organic acids. When living associated with plant roots as part of the rhizosphere, *Pseudomonas* can feed on the organic compounds excreted by the roots (the root exudates). The composition of the exudates varies depending on the plant species considered, type of soil, etc., but can include several organic acids (citric, succinic, malic, lactic, etc.), sugars (glucose, fructose, xylose), amino acids, fatty acids, nitrogenated bases, etc. (Lugtenberg *et al.*, 2001). The concentration of organic acids usually exceeds that of sugars (Kamilova *et al.*, 2006). As mentioned above, *Pseudomonas* recognize many organic acids such as succinate, pyruvate or acetate as preferred compounds. However, not all organic acids can induce a CCR effect and, even more intriguingly, some of them have a clear effect in some cases, but not in others. For example, citrate allows a good expression of the *P. putida* GPo1 OCT plasmid alkane degradation pathway (Yuste *et al.*, 1998; Staijen *et al.*, 1999), but has a repressive effect on the *P. putida* H phenol degradation genes (Müller *et al.*, 1996) and on the *P. putida* CA-3 pathway for styrene assimilation (O'Leary *et al.*, 2001). This may be due to specific differences in the bacterial strains or in the genes analysed or the experimental conditions used.

The PTS–sugar transport system and the cAMP–CRP complex are not key players in CCR in *Pseudomonas*

In *E. coli* and *B. subtilis*, components of the PTS–sugar transport system play a key role in CCR. In *Pseudomonas*, however, only fructose is transported into the cell through a PTS system (Velázquez *et al.*, 2007), and fructose does not play an important role in CCR (Cases *et al.*, 1999; Velázquez *et al.*, 2004; Kim *et al.*, 2009). All other sugars metabolized

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by pseudomonads are transported through PTS-independent systems. In fact, an *in silico* analysis of the distribution of PTS proteins in 206 genomes covering the major bacterial groups has suggested that the use of PTS proteins for sugar transport is a characteristic found in *Enterobacteriaceae*, *Vibrionales* and *Firmicutes*, but it is not the rule for all bacteria (Cases *et al*., 2007). In many cases, PTS proteins are probably used as carbon-sensing devices with regulatory roles to control metabolism, rather than to transport sugars.

Glucose abundance in the growth medium affects cAMP levels in *E. coli* significantly, which in turns triggers a global regulatory response through the cAMP–CRP global transcription factor. This regulatory process does not operate in *Pseudomonas*. On the one hand, cAMP levels in *P. aeruginosa* grown in repressing carbon sources are very similar to those in cells grown in a nonrepressing carbon source, and addition of cAMP to the medium does not alter the succinate-triggered CCR response (reviewed in Collier *et al*., 1996). A protein showing high similarity to *E. coli* CRP is present in several *Pseudomonas* (close to 90% similarity, depending on the *Pseudomonas* species considered). In *P. aeruginosa*, this protein has been named Vfr. Several reports have shown that Vfr is a global regulator that participates in the control of other regulators such as the stationary-phase sigma factor RpoS and the flagellar regulator FleQ, and of genes involved in type-III secretion, in the quorum-sensing response and in the production of several virulence factors (West *et al*., 1994; Albus *et al*., 1997; Beatson *et al*., 2002; Dasgupta *et al*., 2006; Ferrell *et al*., 2008; Fox *et al*., 2008; Davinic *et al*., 2009). However, Vfr has no effect on the CCR induced by succinate on the expression of several genes tested (Suh *et al*., 2002). A recent report has shown, however, that *P. putida* CRP is required for the assimilation of some amino acids and organic acids as a source of carbon, and of some amino acids, urea and ammonium as a source of nitrogen (Daniels *et al*., 2010). Although direct evidence for a role of *P. putida* CRP in CCR is lacking, it is at least a global regulator involved in carbon and nitrogen metabolism.

Regulatory systems responsible for CCR in *Pseudomonas*

Currently, the regulatory factors or systems known to be involved in CCR in *Pseudomonas* are the Crc protein (together with CbrA, CbrB and CrcZ, which modulate Crc availability), the Cyo terminal oxidase and the PTS^{Ntr} system. The following sections summarize what is currently known about these regulatory systems.

The Crc global regulator

The Crc regulator was initially described in *P. aeruginosa* as a protein involved in the succinate-induced repression of several genes involved in the transport and metabolism of glucose and mannitol, as well as in the repression of the amidase (MacGregor *et al*., 1991, 1996; Wolff *et al*., 1991). Crc was later shown to also participate in the repression induced by succinate, or by components of a complete medium, of several catabolic genes, including the *bkd* genes required for the assimilation of branched-chain amino acids in *P. aeruginosa* and *P. putida* (Hester *et al*., 2000a, b), the *P. putida* chromosomal genes required for the assimilation of benzoate, 4-hydroxybenzoate and 4-hydroxyphenylpyruvate (Morales *et al*., 2004), the alkane degradation genes encoded in the *P. putida* GPo1 OCT plasmid (Yuste & Rojo, 2001), the toluene degradation genes encoded in the *P. putida* pWW0 plasmid (Aranda-Olmedo *et al*., 2005; del Castillo & Ramos, 2007) and the phenol degradation genes from *Pseudomonas* species EST1001 (Putrins *et al*., 2007). A proteomic and transcriptomic analysis of the role of Crc in *P. putida* grown in a complete medium, where amino acids are the main carbon source, showed that that inactivation of the *crc* gene modifies the expression of at least 134 genes (Moreno *et al*., 2009a). Most of them are involved in the transport and assimilation of amino acids or sugars (Fig. 3). Crc modifies the expression of several porins and transporters for the uptake of amino acids or dipeptides and inhibits the genes needed to assimilate valine, isoleucine, leucine, tyrosine, phenylalanine, threonine, glycine and serine. Crc does not inhibit the pathways for proline, alanine, glutamate, glutamine and histidine, amino acids that are good carbon sources for *P. putida*. In the case of arginine, lysine, aspartate and asparagine, which can be assimilated through several pathways, Crc favours one particular route, inhibiting other alternatives. Therefore, not all amino acids are equally preferred. Crc organizes the hierarchical assimilation of the preferred amino acids over the nonpreferred ones, also favouring the preferential use of a particular assimilation route when there are several options available (summarized in Fig. 4a).

In cells growing in a complete medium, *P. putida* Crc inhibits the expression of the OprB1 porin, responsible for glucose uptake, and of the inner-membrane transporters for glucose and fructose, as well as several genes required for the assimilation of these sugars (Moreno *et al*., 2009a). The absence of Crc leads to an increased expression of the genes required for the uptake of gluconate and its transformation into 6-phosphogluconate. This could be an indirect effect of the Crc inhibition of glucose uptake, which would facilitate the accumulation of glucose into the periplasm and its subsequent transformation into gluconate. It should be noted, however, that Crc inhibits further assimilation of the 6-phosphogluconate generated from gluconate (Fig. 3). Although amino acids exert a clear Crc-dependent CCR control over the assimilation of sugars, glucose can also induce a CCR effect on the assimilation of other

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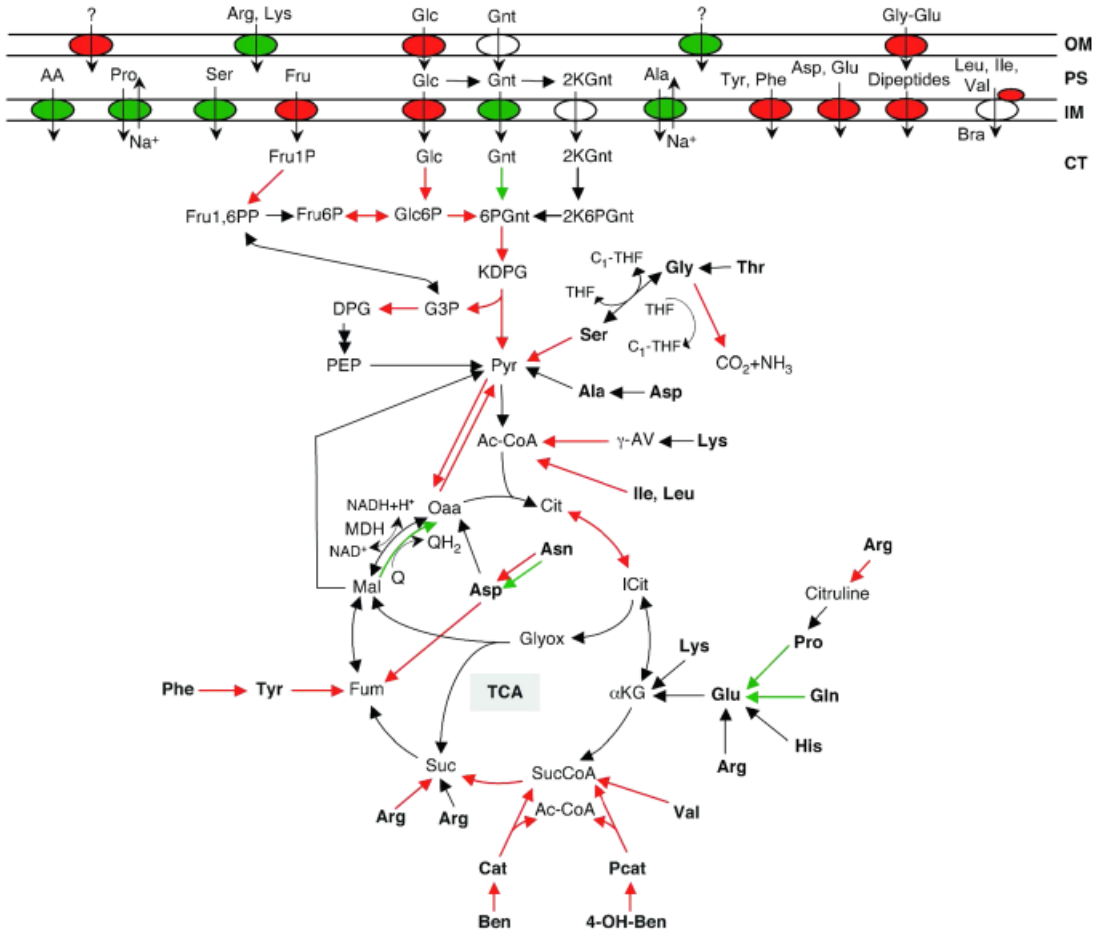

Fig. 3. Effect of Crc on the transport and metabolism of amino acids, sugars, benzoate and 4-OH-benzoate in *Pseudomonas putida*. The scheme only shows relevant routes; some steps are simplified and may be accomplished by several enzymes acting sequentially. Genes that are expressed at higher levels upon inactivation of Crc are indicated in red, while those with a lower expression in the *crc*-deficient strain are indicated in green. The same color code is used for membrane transporters, represented by ovals at the outer and inner membranes. Arrows in black (or transporters in white) correspond to steps (or transporters) that are not affected (or are not known to be affected) by Crc. AA, amino acids; OM, outer membrane; PS, periplasmic space; IM, inner membrane; and CT, cytoplasm. Abbreviations for sugars and their metabolites are as in Fig. 2. Common abbreviations are used for amino acids. Cit, citrate; ICit, isocitrate; αKG, α-ketoglutarate; SucCoA, succinyl-CoA; Suc, succinate; Fum, fumarate; Mal, malate; Oaa, oxalacetate; MDH, malate dehydrogenase; Glyox, glyoxylate; THF, tetrahydrofolate; Ben, benzoate; Cat, catechol; 4-OH-Ben, 4-OH-benzoate; Pcat, protocatechuate. Scheme modified from Moreno *et al*. (2009a).

compounds, although in the cases reported, the effect does not depend on Crc, but on the PtsN protein.

The proteomic and transcriptomic analyses described above, combined with earlier observations, show that Crc plays a key role in coordinating metabolism. Inactivation of *crc* in *P. putida* KT2442 reduces the growth rate by 8% when cells grow in a complete medium (Moreno *et al*., 2009a), suggesting that Crc optimizes metabolism to achieve a more efficient use of the resources present in the growth medium. Competition experiments performed in cells growing in a complete medium showed that wild-type *P. putida* KT2442 cells could easily outcompete an otherwise isogenic *crc* mutant derivative of this strain, which reduced its relative abundance by one half every 10 generations (Moreno *et al*., 2009a).

The consequences of Crc activity are not limited to optimizing the growth rate. Crc exerts, perhaps indirectly, an important influence in several aspects of cell biology. For example, in *P. aeruginosa*, a bacterium that can behave as an important opportunistic pathogen, inactivation of the *crc* gene impairs the formation of fully functional type IV pili, which in turn impairs twitching motility and biofilm formation (O'Toole *et al*., 2000). The capacity of *P. aeruginosa* to form biofilms is an important requirement for chronic colonization of human tissues (Costerton *et al*., 1999; Singh *et al*., 2000). The transition from planktonic (free swimming) growth to the development of a biofilm depends on environmental and physiological cues (O'Toole & Kolter, 1998).

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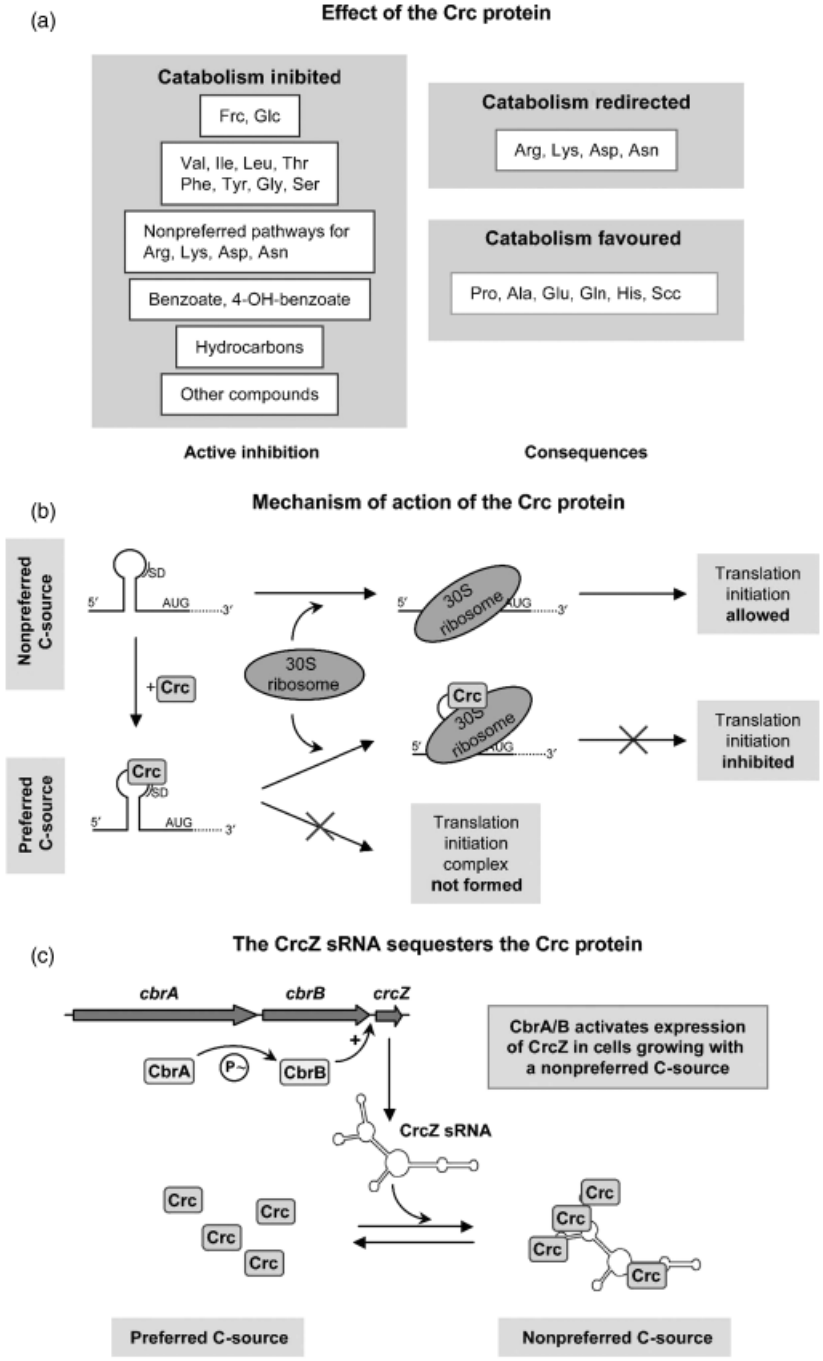

Fig. 4. CCR exerted by the Crc protein. (a) Effect of Crc. When cells grow in a complete medium containing all 20 amino acids, Crc triggers a repression process that inhibits the expression of the genes involved in the transport and catabolism of the nonpreferred amino acids, while it indirectly favours the assimilation of the preferred amino acids. In the case of Arg, Lys, Asp and Asn, which can be assimilated through several alternative pathways, Crc favours one of them inhibiting other alternatives. If other nonpreferred compounds such as fructose, glucose, benzoate, 4-OH-benzoate or several hydrocarbons (toluene, alkanes) are added to the medium, Crc also inhibits their transport and/or metabolism. In this way, Crc is believed to optimize metabolism and to increase the growth rate. (b) Mechanism of the Crc protein. Crc binds to target RNAs at specific sites located close to or overlapping the AUG translation start site, and inhibits the formation of a productive translation initiation complex. Crc may either compete with the 30S ribosome for binding to RNA or allow the 30S subunit to bind RNA, but trapping it into a nonproductive complex. The precise mechanism may depend on the mRNA considered and on the exact location of the Crc-binding sequence relative to the AUG start site. 'SD' indicates the Shine–Dalgarno sequence. (c) The CrcZ sRNA modulates Crc availability. The CrcZ sRNA contains five Crc-binding sites and can sequester Crc under conditions that generate low or no CCR. CrcZ levels vary according to the C source being used. The expression of *crcZ* requires the CbrA/B two-component system and the RpoN (σ^{54}) sigma factor.

Those *Pseudomonas* species for which the genome sequence is available contain a protein with an amino acid sequence showing $>85\%$ identity to *P. putida* Crc, although in many cases, it is annotated as an exodeoxyribonuclease III. This occurs because the Crc protein shows a low, but significant similarity to some well-characterized nucleases (24% identity, 43% similarity, to the *E. coli* K12 Exonuclease III). This has probably led to incorrect annotation of Crc as an Exonuclease III enzyme in several genomes. A protein showing 38–41% amino acid sequence identity (58–63% similarity) to *P. putida* Crc is present in several *Acinetobacter* strains, and has been experimentally characterized to be involved in CCR in *Acinetobacter baylyi* (Zimmermann *et al.*, 2009). Therefore, global regulators similar to Crc are probably present in many bacterial species, although it seems clear that Crc is not present in *E. coli* or in *B. subtilis*.

In spite of its amino acid sequence similarity to *E. coli* Exo III, the *P. aeruginosa* and *P. putida* Crc proteins show no

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nuclease activity, either on DNA or on RNA (MacGregor *et al.*, 1996; Ruiz-Manzano *et al.*, 2005; Moreno *et al.*, 2007, 2009b). Amino acid sequence comparisons show that Crc is related to a family of endonucleases–exonucleases–phosphatases that have two highly conserved residues at their catalytic site (Dlakic, 2000; Ruiz-Manzano *et al.*, 2005). These two residues are also present in the Crc protein, but their modification by site-directed mutagenesis does not impair the Crc function, further suggesting that Crc is not a nuclease (Ruiz-Manzano *et al.*, 2005). Rather, Crc has been shown to be an RNA-binding protein (Moreno *et al.*, 2007, 2009b). Perhaps Crc derives from nucleases, but its structure has evolved towards binding RNA specifically without degrading it.

Crc regulates gene expression post-transcriptionally (Hester *et al.*, 2000b; Moreno *et al.*, 2007; Putrins *et al.*, 2007; Moreno & Rojo, 2008; Zimmermann *et al.*, 2009). The molecular mechanism has been studied in detail in the case of the *P. putida* OCT plasmid alkane degradation pathway. The regulation of this pathway is explained in detail later in this review. In brief, the AlkS transcriptional activator induces the expression of the alkane degradation genes when alkanes are present. Crc binds to the 5′-end of the mRNA that codes for the AlkS protein, inhibiting its translation (Moreno *et al.*, 2007). AlkS is a very unstable protein that is always present in limiting amounts, so that controlling its levels is a good strategy to modulate the expression of the pathway genes (Yuste & Rojo, 2001; Moreno *et al.*, 2007). It should be noted that, although the direct effect of Crc is on *alkS* mRNA translation, decreasing the AlkS levels leads to an indirect decrease of the mRNA levels of all AlkS-dependent genes including *alkS* itself, because AlkS activates the transcription of its own gene.

The sequence recognized by Crc at the 5′-region of the *alkS* mRNA was been identified by chemical and enzymatic footprinting techniques using purified Crc protein and by band-shift assays using variant *alkS* mRNAs (Moreno *et al.*, 2009b). These approaches showed that Crc binds specifically to a short unpaired A-rich sequence located adjacent to the *alkS* AUG start codon. Binding of Crc to the mRNA does not degrade or cleave the nucleic acid, but rather prevents the formation of the 30S-$tRNA_f^{Met}$–RNA ternary complex *in vitro*, showing that the purified protein is able to inhibit the assembly of a productive translational initiation complex (Moreno *et al.*, 2009b). This agrees with the proposed role *in vivo* (Moreno *et al.*, 2007). It is likely that Crc inhibits translation initiation by imposing a direct steric hindrance on ternary complex formation, competing with the ribosome for a common binding site. However, it cannot be ruled out at present that Crc allows the ribosome 30S subunit to bind the RNA, but traps it into an inactive complex that cannot proceed towards further steps of the initiation process (Fig. 4b).

In an independent study (Sonnleitner *et al.*, 2009), a mutational approach led to the identification of a binding site for Crc at the *P. aeruginosa amiE* mRNA, which codes for an aliphatic amidase that allows cells to assimilate acetamide, and whose expression is subject to Crc-dependent CRC. The binding site found contained an AACA ACAA motif overlapping the Shine–Dalgarno sequence. Replacing this motif by an unrelated sequence relieved repression by almost threefold. Purified Crc could bind to a short *amiE* run-off transcript containing this sequence, indicating that this is a Crc-binding site.

Crc also binds to and inhibits the translation of the mRNA coding for the *P. putida* BenR protein, which is the transcriptional activator of the *benABCD* genes that code for the first enzymes of the chromosomal benzoate degradation pathway (Moreno & Rojo, 2008). Inhibiting *benR* translation indirectly reduces the transcription of the *benABCD* genes. A binding site for Crc at *benR* mRNA was localized upstream of the Shine–Dalgarno sequence (Moreno *et al.*, 2009b). The similarity between the *alkS* and the *benR* targets for Crc is restricted to the sequence AA($^{C}/_{U}$)AAUAA. This sequence is highly similar to the AACAACAA motif identified as a Crc-binding site at *amiE* mRNA (Sonnleitner *et al.*, 2009). In addition, sequences resembling this consensus are present close to the translation initiation sites of 20 genes detected as being regulated by Crc in a proteomic analysis (Moreno *et al.*, 2009a, b). Eight of these mRNAs contain the sequence AA($^{C}/_{U}$)AA($^{C}/_{U}$)AA, while the other 12 mRNAs show a less-conserved A-rich sequence. Although the real significance of these presumed Crc targets remains to be experimentally validated, the consensus derived will clearly be useful to identify Crc-binding sites, and to differentiate between the direct and the indirect effects of this global regulator.

In *A. baylyi*, the expression of genes implicated in the degradation of several aromatic compounds is subject to a strong CCR effect (Dal *et al.*, 2002; Fischer *et al.*, 2008). Although the regulation of some of these pathways appears to be rather complex (Brzostowicz *et al.*, 2003; Siehler *et al.*, 2007), the Crc regulator present in this bacterial species is involved in the CCR of the *pca-qui* genes, which are expressed as a long polycistronic transcript and encode enzymes required for the assimilation of protocatechuate and quinate. *Acinetobacter baylyi* Crc was found to reduce the stability of the *pca-qui* transcript up to 14-fold (Zimmermann *et al.*, 2009). It is at present unknown whether *A. bayly* Crc binds to this mRNA. If it does, there are several ways in which Crc could affect its stability. In many cases, impeding mRNA translation can lead to increased sensitivity to cellular nucleases and, therefore, to a reduced mRNA stability (Iost & Dreyfus, 1995; Kaberdin & Blasi, 2006). Therefore, *A. baylyi* Crc may bind the *pca-qui* transcript inhibiting translation initiation, indirectly facilitating its

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degradation. Alternatively, binding of Crc to the *pca-qui* transcript may stabilize an RNA structure that is attacked by RNAses, leading to a direct degradation of the transcript without the need to inhibit translation. A direct nuclease activity of *A. baylyi* Crc cannot be discarded at present, but has not yet been proven.

It is still unclear as to which are the ultimate signals that regulate Crc function. The repressing effect of Crc varies according to the carbon source being used. It is very strong in cells growing exponentially in a complete medium, or in a minimal salts medium containing all 20 amino acids, but is much milder in a minimal salts medium containing organic acids as the carbon source. In *P. putida*, transcription of the *crc* gene, and the amounts of Crc protein, are higher under conditions in which CCR is strong, particularly when cells grow exponentially in a complete medium (Ruiz-Manzano *et al.*, 2005; Aranda-Olmedo *et al.*, 2005; Yuste *et al.*, 2006). However, these changes are not high (of about fourfold) and may not fully explain the strong fluctuations in Crc activity that can be observed. In addition, expression of the *P. aeruginosa crc* gene was found to be similar in cells growing at the expense of preferred (succinate) or nonpreferred (glucose or mannitol) carbon sources (Sonnleitner *et al.*, 2009). This suggests the existence of factors that regulate Crc function. In *P. aeruginosa*, a 407 nt small RNA named CrcZ has been found that seems to sequester Crc when cell grow under conditions that do not generate catabolite repression (Sonnleitner *et al.*, 2009). The CrcZ sRNA contains five AANAANAA motifs in a predicted unpaired configuration, and binds Crc *in vitro* with a high affinity. In succinate grown cells, inactivation of *crcZ* led to the total repression of *amiE* expression, while overexpression of CrcZ relieved the repression effect. The levels of CrcZ in the cell vary according to the carbon source being used and correlate with the strength of the CCR effect. These levels are 2.5-fold higher in cells growing at the expense of a poor carbon source such as mannitol, which generates no CCR, than in cells using succinate, which induces a strong CCR. Interestingly, growth at the expense of glucose, which induces a moderate CCR effect, leads to intermediate levels of CrcZ. Thus, it seems that CrcZ modulates the levels of free Crc protein in the cell, thereby controlling the strength of the Crc-dependent CCR effect (Fig. 4c).

The *crcZ* gene maps downstream of the genes encoding the CbrA/CbrB two-component sensor-regulator system, an arrangement that is conserved in pseudomonads. Interestingly, the expression of *crcZ* is activated by CbrB and depends on the RpoN (σ^{54}) sigma factor (Sonnleitner *et al.*, 2009). The CbrA/CbrB system controls the utilization of several carbon and nitrogen sources, probably maintaining the C/N balance (Nishijyo *et al.*, 2001; Li & Lu, 2007; Zhang & Rainey, 2008). The signals triggering the activity of CbrA/CbrB are still unclear (Zhang & Rainey, 2008). However, modulation of the levels of free Crc protein by the CbrA/CbrB system imposes a further layer of regulation over the Crc-dependent CCR control, connecting CCR with the global coordination of the carbon and nitrogen metabolism.

The cytochrome *o* ubiquinol oxidase: a connection with the electron transport chain

Two different and independent random mutagenesis approaches aimed at identifying the genes involved in CCR in *P. putida* led to the isolation of multiple mutants that could partially relieve the repression exerted by succinate, or by amino acids, on the induction of the phenol degradation pathway encoded in the pPGH1 plasmid of *P. putida* H (Petruschka *et al.*, 2001) and of the alkane degradation pathway encoded in the OCT plasmid of *P. putida* GPo1 (Dinamarca *et al.*, 2002). Most of the mutants mapped within the *cyoABCDE* cluster that encodes the cytochrome *o* ubiquinol oxidase (Cyo), a terminal oxidase of the electron transport chain that is believed to play an important role in cells growing under an ample supply of O_2. Most bacteria contain several terminal oxidases, each of them usually having a different affinity for O_2, a different redox potential, and a different efficiency as a proton pump. Cells carefully coordinate the composition of the electron transport chain, and in particular, the levels of each terminal oxidase, according to the growth conditions and metabolic needs (Poole & Cook, 2000; Richardson, 2000). As it occurs in *P. aeruginosa* (Williams *et al.*, 2007), *P. putida* has a branched electron transport chain with at least five terminal oxidases (Fig. 5). Selective inactivation of each of the five *P. putida* terminal oxidases showed that only Cyo, but not the other four identified terminal oxidases, participates in CCR (Morales *et al.*, 2006). The finding that Cyo may participate in the global control of catabolic pathways was initially unexpected, but provides a way to sense the energy status of the cell by monitoring the flow of electrons through the transport chain. Transcriptomic assays showed that inactivation of the Cyo oxidase not only affects the induction of the alkane or phenol degradation pathways, but also modifies the mRNA levels of > 100 genes in cells growing exponentially in a rich medium (Morales *et al.*, 2006). This again suggests that Cyo participates in a global regulation process. Most of the genes affected by Cyo correspond to porins, to transporters of organic acids, aromatic compounds or other substrates, to diverse transcriptional regulators and to components of the electron transport chain, including terminal oxidases that substitute for Cyo when the latter is absent. The Crc and Cyo 'regulons' overlap very little, indicating that they are separate global regulation systems. Inactivation of the *P. putida* Cyo oxidase impairs the use of some amino acids, benzoate, fructose and diverse organic acids as the carbon source, as well as the use of

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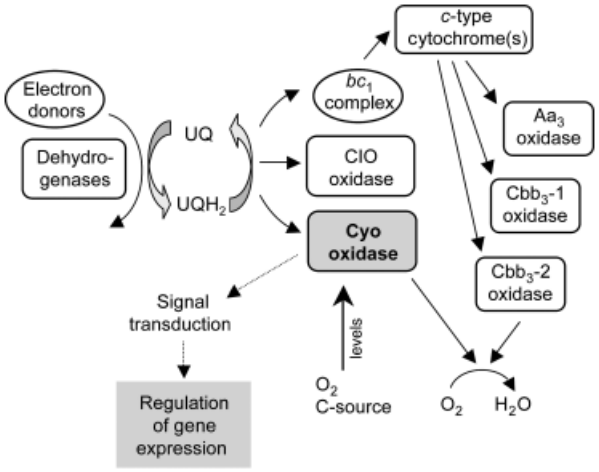

Fig. 5. Proposed aerobic respiratory chain of *Pseudomonas putida*. Compounds such as NADH, lactate or succinate provide electrons to the ubiquinones of the cell membrane (UQ/UQH_2) and are then transferred either to the bc_1 complex or to the Cyo or ClO terminal ubiquinol oxidases. The bc_1 complex feeds the Aa_3, Cbb_3-1 and Cbb_3-2 terminal oxidases. Electrons are finally provided to O_2. The *P. putida* Cyo oxidase is believed to modulate the expression of a large set of genes by a still uncharacterized signal transduction mechanism. *Pseudomonas aeruginosa* contains a similar respiratory chain (Williams *et al.*, 2007), but a role for *P. aeruginosa* Cyo oxidase in signal transduction has not been reported. Note that *P. putida* Cbb_3-1 terminal oxidase corresponds to *P. aeruginosa* Cbb_3-2, while *P. putida* Cbb_3-2 corresponds to *P. aeruginosa* Cbb_3-1 (Ugidos *et al.*, 2008). When O_2 is lacking, *P. aeruginosa*, but not *P. putida*, can use nitrate as an alternative electron donor, because it contains a nitrate reductase that can accept electrons from the ubiquinones and a nitrite reductase that receives electrons via the bc_1/*cytC* proteins.

amino acids and urea as the nitrogen source (Daniels *et al.*, 2010). This finding further supports the importance of the Cyo terminal oxidase in the regulation of carbon and nitrogen metabolism in *Pseudomonas*.

It is at present unknown as to how Cyo transmits the signal that ends up affecting the expression of many genes. However, in other bacterial species, some two-component sensor/regulator systems have been described in which the sensor can detect signals from the electron transport chain, such as the redox state of the quinones or the flow of electrons through a terminal oxidase (Georgellis *et al.*, 2001; Oh & Kaplan, 2001; Elsen *et al.*, 2004). The sensor responds by phosphorylating a response transcriptional regulator that can directly activate or repress the expression of a number of genes. *Pseudomonas putida* may contain similar two-component systems able to receive information from the Cyo terminal oxidase, transforming it into a gene expression response, although this has not been proven so far.

The expression of the genes coding for the Cyo oxidase varies considerably depending on the growth conditions. Expression is high when cells grow exponentially in a complete medium under an ample supply of O_2 and decreases considerably when O_2 is limiting or when cells enter into the stationary phase of growth (Dinamarca *et al.*, 2003; Morales *et al.*, 2006). Interestingly, expression of the *cyo* genes also depends on the carbon source used, being much higher when cells grow at the expense of substrates that induce a CCR response (succinate or amino acids) than when cells assimilate citrate, an organic acid that does not induce CCR on the *P. putida* alkane degradation pathway (Dinamarca *et al.*, 2003). Available information suggests that a correlation exists between the levels of Cyo and the strength of CCR. This agrees with Cyo having two functions: one as a terminal oxidase of the electron transport chain and another one as a component of a global regulation system that senses and transmits information on the activity of the electron transport chain, influencing carbon and nitrogen metabolism. Regulation of the *cyo* genes is probably rather complex. The global regulator ANR inhibits the expression of the *P. putida cyo* genes when O_2 levels are limiting (Ugidos *et al.*, 2008). It is reasonable to predict that other regulators may control Cyo levels in response to the carbon source being used, or to entry into the stationary phase, but these have not yet been identified.

The PTS proteins PtsP (EI^{Ntr}), PtsO (NPr) and PtsN ($EIIA^{Ntr}$)

The most thoroughly studied PTS systems are involved in the uptake and phosphorylation of carbohydrates and, at least in many bacterial species, they also play a central role in controlling the transport and assimilation of nonpreferred (and non-PTS) sugars through inducer exclusion and catabolite repression. However, there is a subset of PTS proteins that are not involved in the uptake of sugars and that is present in many bacteria. These proteins are named PtsP (or EI^{Ntr}), PtsO (or NPr) and PtsN (or $EIIA^{Ntr}$), and are homologous to proteins EI, NPr and EII, respectively (Fig. 6). The *E. coli* PtsP, PtsO and PtsN proteins have been purified and, as for the carbohydrate PTS, phosphate has been shown to be transferred from PEP to PtsP, which then phosphorylates PtsO, which in turn transfers the phosphate to PtsN (Powell *et al.*, 1995; Rabus *et al.*, 1999). Nevertheless, unlike in the case of carbohydrate PTS, there are no known membrane components equivalent to the EIIBCA sugar transport system to which PtsN can transfer the phosphate. Furthermore, because PtsN has not yet been demonstrated to be able to transfer the phosphoryl group to any particular substrate, it is believed that the PtsP/PtsO/PtsN proteins function exclusively in signal transduction and that the phosphorylation state of PtsN provides a regulatory signal. It is worth noting that while the phosphorylation degree of the sugar–PTS components depends on the phosphate drainage by the sugar that is imported and phosphorylated,

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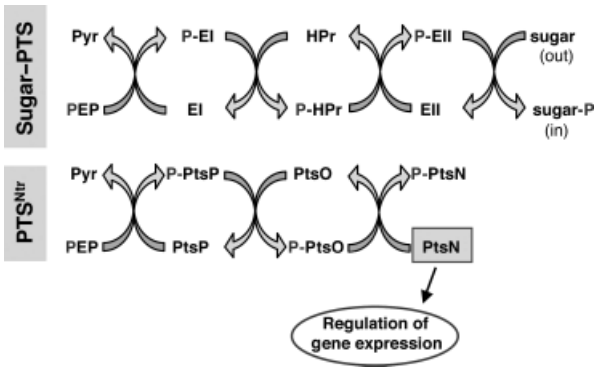

Fig. 6. The PTS^{Ntr} system. In the sugar–PTS systems, a high-energy phosphate is provided by PEP and sequentially transferred to the EI, HPr and EII proteins. The transport of the sugar through the cytoplasmic membrane is coupled to its phosphorylation by the phosphorylated EII protein. In the N-related PTS system (the PTS^{Ntr} system), the phosphoryl group flows sequentially from PEP to the PtsP, PtsO and PtsN proteins. These proteins are homologous, respectively, to the EI, HPr and EII proteins. Unlike P-EII, P-PtsN is not involved in the transport of sugars. However, the nonphosphorylated PtsN (and perhaps the phosphorylated PtsN as well) can modulate the expression of some genes by mechanisms that are not fully understood.

no such drainage is known to occur in the case of the PtsP/PtsO/PtsN proteins. It is at present unclear how the phosphorylation state of these proteins is regulated.

The function of this PTS branch, often designated PTS^{Ntr}, is still poorly understood. In many Gram-negative bacteria, including several *Pseudomonas*, the *ptsN* and *npr* genes – but not *ptsP* – are located within the *rpoN* operon, and are cotranscribed with *rpoN* (Reizer *et al*., 1992; Powell *et al*., 1995; Deutscher *et al*., 2006, and references therein). The *rpoN* gene codes for the alternative sigma factor σ^{54}, which is required for the expression of a number of genes involved in the metabolism of carbon and nitrogen, in the synthesis of flagella and in other apparently unrelated functions (Reitzer & Schneider, 2001; Cases *et al*., 2003). In *E. coli* and *P. putida*, expression of the *rpoN* operon, and the amounts of σ^{54} present in the cell, are low and relatively constant under many growth conditions (Powell *et al*., 1995; Jishage *et al*., 1996; Jurado *et al*., 2003). The analysis of mutant strains lacking different components of the PTS^{Ntr} system has provided some hints regarding the role of these proteins (reviewed in Pflüger-Grau & Görke, 2010). Inactivation of *Klebsiella pneumoniae ptsN* leads to increased transcription of *nifH*, a gene involved in nitrogen metabolism that is expressed from a σ^{54}-dependent promoter (Merrick & Coppard, 1989). A similar mutation in *P. aeruginosa* suggested that PtsN may function as a coinducer of some, but not all, σ^{54}-dependent genes (Jin *et al*., 1994). In addition, inactivation of the *E. coli ptsN* gene impairs growth on media containing a poor nitrogen source, whereas growth on rich nitrogen media (glutamine or ammonium) is not affected (Powell *et al*., 1995; Deutscher *et al*., 2006). *Escherichia coli ptsN* null mutants are also very sensitive to growth inhibition by leucine, an effect that could be traced to the need for dephosphorylated PtsN for derepression of the *E. coli ilvBN* operon, which codes for an enzyme required for the metabolism of leucine (Lee *et al*., 2005). On the other hand, PtsP contains an N-terminal extension of 127 amino acids that is absent in EI and that is homologous throughout its length to the N-terminal sensory domains of the NifA proteins, which are transcriptional activators that control nitrogen fixation in several bacteria (Reizer *et al*., 1996). All these observations indicate that the PTS^{Ntr} system may provide a regulatory link between carbon and nitrogen assimilation in bacteria.

In the case of *P. putida*, inactivation of the *ptsN* gene impairs the use of lysine, arginine and glycerol as a carbon source, as well as the use of D- and L-amino acids or dipeptides as nitrogen sources (Daniels *et al*., 2010). This suggests that the *P. putida* PTS^{Ntr} system is involved in the regulation of C/N balance, a role that is further supported by the finding that inactivation of the *ptsO* and *ptsP* genes significantly decreases the ability of cells to accumulate granules of polyhydroxyalkanoates, while inactivation of *ptsN* increases this ability by about 75% (Velázquez *et al*., 2007). These granules are typically formed when there is an excess of carbon relative to nitrogen in the medium, so that part of the carbon is derived to form these storage polymers that can eventually be used when the nutritional conditions are reversed. The mechanism through which the PTS^{Ntr} interferes with the synthesis or the depolymerization of polyhydroxyalkanoate granules is still unclear.

Recent findings have widened the regulatory influence of the PTS^{Ntr} system. The dephosphorylated form of *E. coli* PtsN can interact with the low-affinity K^+ transporter TrkA, inhibiting TrkA-mediated K^+ uptake (Lee *et al*., 2007). In addition, this form of PtsN also stimulates the expression of the genes coding for the high-affinity K^+ transporter KdpFABC (Lüttmann *et al*., 2009). Transcription of these genes is activated by the KdpD/KdpE two-component system. The dephosphorylated form of PtsN can interact with the KdpD sensor kinase, stimulating its kinase activity, which results in increased levels of the phosphorylated response regulator KdpE. It has also been observed that there is a cross talk between the sugar transport PTS and the PTS^{Ntr} system. Therefore, the PTS^{Ntr} system plays an important role in maintaining K^+ homeostasis and links K^+ uptake to carbohydrate metabolism (Lüttmann *et al*., 2009).

At least in *Pseudomonas*, the regulatory influence of the PTS^{Ntr} system also extends to the expression of some genes in response to signals that can be considered as a CCR effect. This was initially observed for the σ^{54}-dependent *Pu* promoter from the pathway for the assimilation of toluene and

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xylene encoded in the *P. putida* pWW0 plasmid. In the presence of toluene or xylene, the toluene-responsive XylR transcriptional activator induces transcription from the *Pu* promoter. However, induction is downmodulated to different extents by a number of physiological signals. One of them is the presence of glucose or succinate in the medium in addition to toluene (Holtel *et al*., 1994; Duetz *et al*., 1994, 1996). Inactivation of *ptsN* weakens *Pu* repression by glucose or succinate (Cases *et al*., 1999; Aranda-Olmedo *et al*., 2006). The loss of PtsN does not impair glucose consumption, which rules out that weaker repression is an indirect effect caused by decreased transport of the carbohydrate (Cases *et al*., 1999). A site-directed *ptsN* mutant in which residue His-68 (the conserved phospho-acceptor site of this family of proteins) was replaced by an aspartic acid, which mimics the presence of a phosphoryl group at this position, relieved *Pu* inhibition by glucose (Cases *et al*., 1999). However, if alanine replaced His-68, repression was still observed. This supports that the function or activity of PtsN relies on its phosphorylation status. In *P. putida*, two forms of PtsN with different electrophoretic mobilities have been observed *in vivo*, which are believed to correspond to the phosphorylated and nonphosphorylated forms of this protein (Pflüger & de Lorenzo, 2007). Interestingly, although the phosphorylated form of PtsN was detected under all tested growth conditions, the relative levels of the nonphosphorylated form varied drastically depending on the growth phase and the nutrients present in the growth medium, and were higher under repressing conditions (Pflüger & de Lorenzo, 2007). This led us to propose that nonphosphorylated PtsN is the form exerting the regulatory signal.

The importance of phosphorylation in the regulatory role of PTS^{Ntr} is further supported by the observation that inactivation of *ptsO* results in an effect opposite to that of *ptsN* inactivation, because *Pu* activity becomes inhibited even in the absence of glucose (Cases *et al*., 2001b). A double *ptsN ptsO* mutant behaves as the *ptsN* mutant, *Pu* being relieved from the glucose-dependent repressing effect (Cases *et al*., 2001b). Moreover, phosphorylation of PtsO seems to be needed for glucose-mediated repression of *Pu*, because mutation of the conserved His residue at PtsO that presumably serves as a phosphoryl acceptor rendered the protein inactive. It has been proposed that PtsO may modulate PtsN activity, serving as a phospho acceptor for PtsN dephosphorylation. In the presence of glucose, transfer of the phosphoryl group to PtsO would increase the levels of nonphosphorylated PtsN, the form that is believed to mediate *Pu* repression. Upon glucose depletion, phosphate would be transferred back to PtsN (Cases *et al*., 2001b). The physiological signals that bring about phosphorylation/dephosphorylation of the PtsN enzyme are not totally understood. However, there are several indications that the levels of glucose metabolites in *P. putida*, and in particular those of 2-keto-3-deoxy-6-phosphogluconate (KDPG), are critical for glucose-mediated catabolite repression, and may directly or indirectly affect the phosphorylation of PtsN (Velázquez *et al*., 2004; del Castillo & Ramos, 2007; Kim *et al*., 2009).

A proteomic approach revealed that *P. putida* PtsN is involved in other regulatory events in addition to the glucose-induced catabolite repression (Cases *et al*., 2001a). In cells grown in a complete medium with no glucose added, inactivation of the *ptsN* gene modified the levels of almost 9% of the protein spots detected (108 out of 1117). Complementation with a wild-type *ptsN* gene *in trans* reverted the effect for only 30% of the spots, suggesting that the Ω*Km* cassette used to inactivate the *ptsN* gene had a polar effect on downstream genes (e.g. in *ptsO*). In the absence of PtsO, the PtsN protein in the complemented strain would be in its nonphosphorylated form (see Fig. 6). Therefore, it could be speculated that the nonphosphorylated PtsN may regulate the expression of the complemented spots (30% of those affected by the lack of PtsN), while the noncomplemented spots would be regulated by the phosphorylated PtsN. This possibility has not been explored further. In a wild-type background, addition of glucose to the complete medium reduced the levels of 22% of the spots, although inactivation of the *ptsN* gene relieved this glucose-induced repression in only six cases. This suggests that PtsN is indeed a global regulator, but is not a major player in the extensive inhibition of gene expression that takes place when glucose is added to *P. putida* cells growing in a complete medium. It should be noted, however, that PtsN may repress the expression of other catabolic pathways that are not induced when cells grow in a complete medium because the pathway substrates are absent (e.g. pathways for aromatic compounds). These cases would have not been detected in the work reported. In other words, the PTS^{Ntr} system may participate in the catabolite repression of more genes than those currently known.

Specific mechanisms

In some specific cases, the presence of a repressing carbon source directly interferes with the induction of a catabolic pathway without the need for global regulatory factors. The *clcABD* operon of plasmid pAC27, identified in a *P. putida* strain, encodes the genes necessary for the assimilation of 3-chlorocatechol via 2-chloro-*cis*,*cis*-muconate. Expression of these genes is induced by the transcriptional activator ClcR in the presence of 2-chloro-*cis*,*cis*-muconate, which acts as an effector. Induction by ClcR is inhibited when cells grow in the presence of succinate, citrate or fumarate. *In vitro* assays showed that fumarate and 2-chloro-*cis*,*cis*-muconate compete for binding to ClcR, so that when the

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effector pocket is occupied by fumarate, ClcR is unable to activate transcription (McFall *et al.*, 1997). The effect of fumarate was concentration dependent and reversible. Because fumarate is a component of the TCA cycle, its binding to ClcR provides a direct connection between the pools of an intermediate metabolite and the repression of a catabolic pathway for a nonpreferred substrate.

Catabolic pathways can be regulated by one or several distinct CCR systems

The examples known to date suggest that *Pseudomonas* global regulatory systems interfere with the induction of catabolic pathways in several ways. In some cases, regulation is directed to downmodulate the levels of the specific transcriptional regulator of the pathway. In other cases, global control does not modify the levels of the regulator, but interferes with its ability to regulate transcription. Translational regulators, such as the Crc protein, can inhibit the expression of the specific transcriptional regulator of the pathway, of the transporters required to internalize the substrate, of key pathway enzymes or combinations of these. In addition, the induction of many catabolic pathways depends on the combined activity of several global regulators. Some of these regulators respond to CCR signals, while other ones respond to physiological signals that cannot be rigorously considered a CCR effect. It is the combination of all these factors that ultimately determines the induction levels achieved. For this reason, the following sections describe the regulation of some catabolic pathways that are influenced by more than one global control system, including the effect of regulators that respond to the compounds being consumed (a CCR signal) and of factors that respond to other physiological signals.

The alkane degradation pathway encoded in the *P. putida* OCT plasmid

The OCT plasmid, originally identified in *P. putida* GPo1 (formerly *Pseudomonas oleovorans* GPo1), encodes all genes required for the assimilation of *n*-alkanes containing between 3 and 13 carbon atoms (C_3–C_{13} alkanes). The genes of this pathway are grouped into two clusters: *alkBFGHJKL* and *alkST* (van Beilen *et al.*, 1994, 2001; Rojo, 2009; see Fig. 7). When no alkanes are available, the *alkBFGHJKL* operon remains silent while the *alkST* genes are transcribed at low levels from promoter *PalkS1*, a promoter that relies on σ^S-RNA polymerase (Canosa *et al.*, 1999). AlkS binds to a site that overlaps this promoter, downmodulating its activity, but allowing for a low expression. In the presence of alkanes, AlkS transforms into a transcriptional activator, inducing the expression of its own gene, and that of *alkT*, from a promoter named *PalkS2* located 38 nt downstream of *PalkS1*. This leads to an increase in the levels of AlkS, which

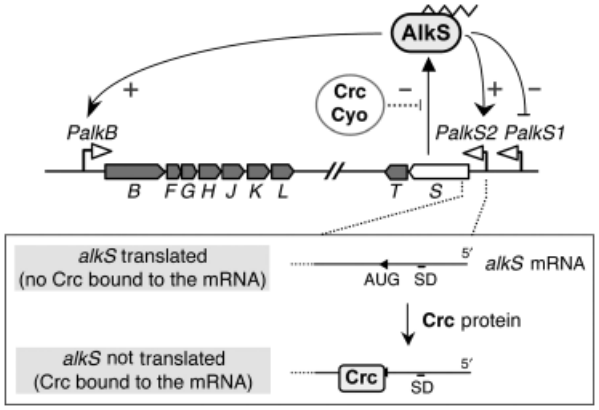

Fig. 7. Regulation of the alkane degradation pathway encoded in the OCT plasmid. The genes are grouped in two clusters, *alkBFGHJKL* and *alkST*. When alkanes are absent, *alkS* is expressed from promoter *PalkS1*. AlkS binds to a site that overlaps *PalkS1*, negatively modulating this promoter, but allowing for a low expression. In the presence of alkanes, and from its binding site at *PalkS1*, AlkS activates transcription from promoter *PalkS2*, located 38 bp downstream from *PalkS1*. This leads to self-amplification of AlkS expression and to a simultaneous activation of promoter *PalkB*, from which the *alkBFGHJKL* genes are transcribed. The activation of promoters *PalkS2* and *PalkB* by AlkS is inhibited by a CCR process that is mediated by the Crc and Cyo proteins. AlkS is an unstable protein present in limiting amounts. Crc and Cyo reduce AlkS levels below those needed for a full induction of the *PalkS2* and *PalkB* promoters. The Crc protein binds to a specific site at the *alkS* mRNA that is adjacent to the AUG translation initiation codon, and inhibits *alkS* translation by impeding the assembly of the initiation complex. It is unclear how Cyo interferes with AlkS function, but it also decreases the activity of the *PalkS2* and *PalkB* promoters. 'SD' indicates the Shine–Dalgarno sequence.

can then activate the expression of the *alkBFGHJKL* operon from promoter *PalkB* (Kok *et al.*, 1989; Panke *et al.*, 1999; Canosa *et al.*, 2000). AlkS recognizes C_5–C_{10} *n*-alkanes as effectors (Sticher *et al.*, 1997).

Activation of promoters *PalkB* and *PalkS2* by AlkS is negatively modulated by a dominant global control when cells grow in a complete medium containing alkanes or in a minimal salts medium containing alkanes and other alternative carbon sources such as amino acids, succinate or lactate (Yuste *et al.*, 1998; Staijen *et al.*, 1999; Canosa *et al.*, 2000). Compounds such as citrate, pyruvate or glycerol, which are also metabolized, do not exert this inhibitory effect. Repression is particularly strong (about 70-fold as measured with a *PalkB-lacZ* transcriptional fusion and $>$ 12-fold by measuring mRNA levels) during exponential growth in a complete medium, where amino acids are the carbon source used, and rapidly fades away when cells enter into the stationary phase. The repression exerted by succinate or lactate in a minimal salts medium is milder, in the range of 4- to 5-fold (Yuste *et al.*, 1998). Repression is not related to the growth rate that each carbon source supports.

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Continuous cultures in which the source of nitrogen was provided at limiting concentrations, but that contained succinate in excess as the carbon source, showed a clear catabolite repression effect. However, repression was not observed in cells growing at the same growth rate in a medium containing a limiting concentration of succinate, all other nutrients being in excess (Dinamarca *et al*., 2003). Therefore, it is the presence of an excess of succinate that inhibits the expression of the alkane degradation pathway, not the growth rate *per se*.

When cells grow in a complete medium, the negative control inhibiting the expression of the alkane degradation genes depends on the additive effects of the Crc protein (Yuste & Rojo, 2001) and of signals arising from the Cyo terminal oxidase (Dinamarca *et al*., 2002, 2003). However, when cells grow in a minimal salts medium containing succinate as the carbon source, the role of Crc is small and the inhibitory effect derives mainly from the Cyo terminal oxidase (Yuste & Rojo, 2001; Dinamarca *et al*., 2003).

As explained in detail in the section on the Crc regulator, this protein binds to a specific site at the 5′-end of the *alkS* mRNA, inhibiting its translation (Moreno *et al*., 2007, 2009b). This generates a strong decrease in the levels of the AlkS transcriptional activator, an unstable protein present in limiting amounts even under inducing conditions (Yuste & Rojo, 2001). Attaining AlkS levels below those required for maximal induction of the pathway simultaneously inhibits transcription of the *alkST* and *alkBFGHJKL* operons. In support of this idea, a moderate overproduction of the AlkS protein from a heterologous promoter relieves all the CCR observed in a complete medium (Yuste & Rojo, 2001). This explains why inactivation of the *crc* gene indirectly leads to a sixfold increase in *alkS* mRNA levels in cells growing in a complete medium (Yuste & Rojo, 2001). The Cyo terminal oxidase also inhibits the expression of the *alkST* and *alkBFGHJKL* genes, because inactivation of the *cyoB* gene leads to a sevenfold increase in the levels these mRNAs in cells growing in a complete medium (Dinamarca *et al*., 2002). However, the molecular mechanism involved is at present unclear.

The toluene/xylene (TOL) degradation pathway encoded in the pWW0 TOL plasmid of *P. putida*

There are several plasmids that encode similar pathways for the degradation of toluene and related compounds. Despite being different in many other characteristics, they are collectively known as TOL plasmids. The best studied is the pWW0 plasmid, identified in *P. putida* mt-2 (Williams & Murray, 1974; Greated *et al*., 2002). The genes coding for the enzymes required to transform toluene or xylenes into benzoate or methylbenzoates, respectively, are clustered in the so-called '*upper* operon', while those specifying the enzymes that convert the benzoate or methylbenzoates into Krebs cycle intermediates are grouped into the '*meta* operon' (see Fig. 8; reviewed in references Ramos *et al*., 1997; Greated *et al*., 2002). Two regulatory genes, *xylR* and *xylS*, located downstream of the *meta* operon, encode the specific transcriptional regulators that activate the expression of the pathway genes. Benzoate or methylbenzoates, the substrates of the *meta*-pathway enzymes, induce transcription of the *meta* operon by serving as effectors of the XylS transcriptional regulator, which activates promoter *Pm*. Conversely, toluene and xylenes, which are transformed to benzoate or methylbenzoates by the *upper* pathway enzymes, induce transcription of the *upper* operon from promoter *Pu* by interacting with the XylR transcriptional activator. Interestingly, toluene and xylenes also induce expression of the *meta* operon without the need for their conversion into benzoate or methylbenzoates. This occurs because *xylS* can be expressed from two promoters. Promoter *Ps2* provides for a low and constitutive expression of *xylS* that leads to XylS protein levels that can activate *Pm* only in the presence of the effectors benzoate or methylbenzoates. However, in the presence of toluene or xylene, XylR activates the expression of *xylS* from another promoter, named *Ps1*, which considerably increases *xylS* transcription and generates an mRNA that is translated 10 times more efficiently than that originating at *Ps2*. This leads to an overproduction of XylS, which reaches levels high enough to activate *Pm* even in the absence of the effectors benzoate or methylbenzoates (Inouye *et al*., 1987; Ramos *et al*., 1987; Gallegos *et al*., 1996; González-Pérez *et al*., 2004). The *xylR* gene, in turn, is expressed from two contiguous σ^{70}-dependent promoters: *Pr1* and *Pr2*. Although XylR itself negatively controls the activity of these promoters, the final expression levels are high (Inouye *et al*., 1985).

Induction of the TOL pathway by the substrates of the upper pathway is inhibited to different extents under several growth conditions. Repression is primarily directed to reduce XylR activation of the *Pu* and *Ps1* promoters, which are both recognized by σ^{54}-RNA polymerase. Initial studies showed that induction of the pathway genes was severely inhibited during exponential growth in a complete medium, although repression was relieved upon entry into the stationary phase (Hugouvieux-Cotte-Pattat *et al*., 1990; de Lorenzo *et al*., 1993). This was referred to as 'exponential silencing' (Cases *et al*., 1996). However, several observations indicated that repression is due to components of the growth medium, rather than to the growth phase. On the one hand, inhibition was not observed when using a 'spent Luria–Bertani medium', which is a complete medium that has already supported growth, which is then filtered, pH adjusted and sterilized (Marqués *et al*., 1994). In addition, when cells were grown in a minimal salts medium containing the aromatic substrates as the carbon source, induction was strong and immediate (Hugouvieux-Cotte-Pattat *et al*.,

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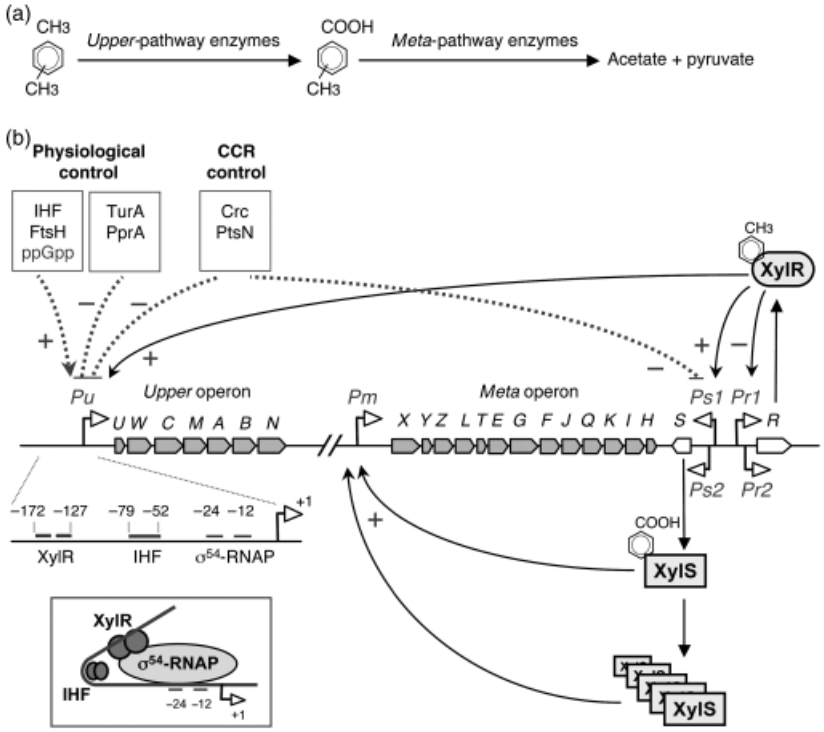

Fig. 8. Regulation of the toluene/xylene degradation pathway encoded in the pWW0 TOL plasmid. (a) The enzymes coded by the genes in the '*upper*' operon sequentially transform toluene or xylenes into benzoate or methylbenzoates, respectively. These are then transformed into acetate and pyruvate by the enzymes encoded in the '*meta*' operon. (b) Regulation of the *upper* and *meta* operons. Structural genes are indicated in grey, and the regulatory genes *xylS* and *xylR* are depicted in white. The *Pu*, *Pm*, *Ps1*, *Ps2*, *Pr1* and *Pr2* promoters are indicated. The activating (+) or repressing (−) effects of XylR and XylS are indicated by black lines. To activate *Pm*, XylS should either bind to an aromatic effector or be expressed at high levels. The DNA region containing the σ^{54}-dependent promoter *Pu* is expanded to show the position of the binding sites for σ^{54}-RNA polymerase, IHF and XylR (coordinates are indicated relative to the *Pu* transcription start point). The proposed geometry adopted by the transcription initiation complex is shown below. The action of global regulators on the expression of the *Pu* and *Ps1* promoters is indicated by grey dotted lines. Crc and PtsN respond to different independent CCR signals, while the action of IHF, FtsH, ppGpp, TurA and PprA responds to distinct physiological signals.

1990; de Lorenzo *et al.*, 1993; Marqués *et al.*, 1994). Furthermore, when added to a minimal salts medium containing benzyl alcohol (an *upper* pathway substrate that also serves as an inducer), some carbon sources such as glucose, gluconate, lactate or acetate inhibited the activation of promoter *Pu* in batch cultures (Holtel *et al.*, 1994). Addition of casamino acids, or of yeast extract (a mixture of amino acids and vitamins), also led to the repression of *Pu* and *Ps1* promoter activity (Marqués *et al.*, 1994). This was interpreted as indicating that induction of the upper pathway genes is subject to CCR. A clear proof that the induction of the TOL pathway is inhibited by CRC was obtained by growing cells in continuous cultures containing minimal salts medium and controlling the growth rate (dilution rate) and the concentration of the sources of carbon, nitrogen, phosphorous or sulphur supplied (Duetz *et al.*, 1994, 1996, 1997). Induction of the *upper* pathway enzymes by *o*-xylene (a nonmetabolizable inducer) or by *m*-xylene (a metabolizable inducer) was inhibited when a repressing carbon source such as succinate was provided at nonlimiting concentrations. However, inhibition did not occur when succinate was supplied at limiting concentrations, all other nutrients being in excess. Limiting the concentrations of the sources of nitrogen, phosphorous or sulphur, while providing succinate in excess, did not relieve repression. The succinate-induced repression led to a clear decrease in the mRNA levels originating at the *Pu* and *Ps* promoters. Repression of the *meta*-pathway enzymes also occurred, but was much milder. Repression occurred at both high and low growth rates (controlled by the dilution rate), as long as the carbon source (succinate) was provided in excess (Duetz *et al.*, 1996). These results showed not only that induction of the TOL pathway is subject to a strong CCR effect, but also that repression is not due to the growth rate itself, but rather due to the presence of an excess of a repressing carbon source such as succinate. The authors proposed that CCR could be due to the energy status of the cell, which is high when an anabolic substrate is limiting but the carbon source is in excess, and low when growth is limited by the availability of carbon.

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The repression exerted on *Pu* activity by succinate in cells growing in continuous cultures is relieved by 50% upon inactivation of the *ptsN* gene, while inactivation of the *crc* gene has no effect (Aranda-Olmedo *et al.*, 2006). A similar result was observed when succinate was replaced by glucose. The absence of PtsN had very little influence on *Ps1* promoter activity. Therefore, the phosphorylation state of the PtsN enzyme is related to the CCR exerted by succinate or glucose in cells growing in a minimal salts medium, while the role of the Crc protein is low or undetectable under these conditions. However, if cells are grown in a batch culture containing minimal salts medium, *o*-xylene and yeast extract, a clear repression effect was observed both on the *Pu* and on the *Ps1* promoters, and in the two cases, repression depended on both PtsN and Crc (Aranda-Olmedo *et al.*, 2005). Inactivation of the *cyoB* gene had a very small effect on *Pu* and *Ps1* repression, suggesting that the Cyo terminal oxidase has little influence on the induction of these promoters. Analysis of *Pu* and P_R promoter occupancy *in vivo* suggested that PtsN interferes with the ability of XylR and *o*-xylene to activate transcription from promoter *Pu*, perhaps by impeding – directly or indirectly – the binding of the regulator to its site at the DNA or its interaction with σ^{54}-RNA polymerase (Aranda-Olmedo *et al.*, 2005). The experiments reported did not clarify how Crc inhibits transcription from promoter *Pu*.

An interesting study showed that, when a *P. putida* strain containing the pWW0 plasmid is grown in a minimal salts medium containing glucose and toluene, cells simultaneously assimilate both compounds, although the two carbon sources exert a CCR effect on the expression of the genes required to assimilate the other compound (del Castillo & Ramos, 2007). Toluene decreased glucose metabolism (glucokinase activity) about twofold, while glucose inhibited induction of the TOL pathway by about threefold, a clear case of crossed catabolite repression. The growth rate was very similar when cells used just glucose or just toluene, or both simultaneously, suggesting that the two compounds are equally good carbon sources. The effect of toluene on glucose metabolism was observed to depend on the Crc regulator and was directed to inhibit the expression of enzymes of the glucokinase branch that transforms glucose into 6-phosphogluconate, while gluconate metabolism was not affected (see Fig. 2). The CCR induced by glucose on the expression of the toluene degradation genes relies mainly on the PtsN protein in response to an increase in the levels of KDPG, an intermediate of glucose metabolism (del Castillo & Ramos, 2007). It should be mentioned that, although toluene allows for good growth of *P. putida* containing the pWW0 plasmid, it also has a clear toxic effect, because it damages cell membranes and proteins, and induces a stress-response programme that has an energetic cost (Domínguez-Cuevas *et al.*, 2006).

Induction of the *Pu* promoter is further modulated by several factors that, although do not respond to a true CCR signal, they adapt the activity of the TOL pathway to the physiological context of the cell. An interesting case is that of the integration host factor (IHF), which plays several roles in *Pu* promoter activity. The XylR transcriptional regulator binds to sequences that are located rather far from the σ^{54}-RNA polymerase, between positions -127 and -172 relative to the transcription start point of *Pu* (see Fig. 8). IHF binds to a site located between that of XylR and that of σ^{54}-RNA polymerase, and strongly bends the DNA, generating a promoter geometry that facilitates the contacts between XylR and σ^{54}-RNA polymerase (Pérez-Martín *et al.*, 1994; Bertoni *et al.*, 1998). In addition, IHF stimulates the recruitment of σ^{54}-RNA polymerase to the *Pu* promoter by facilitating the interaction of the C-terminal domain of the RNA polymerase α subunit with an UP-like promoter element located upstream of the IHF site (Bertoni *et al.*, 1998; Carmona *et al.*, 1999; Macchi *et al.*, 2003). The lack of IHF abolishes *Pu* promoter activity (Calb *et al.*, 1996; Pérez-Martín & de Lorenzo, 1996a; Valls *et al.*, 2002). Interestingly, during exponential growth in a complete medium, the levels of IHF protein are low and the IHF-binding site at promoter *Pu* is essentially unoccupied. However, upon entry into the stationary phase, the levels of IHF increase drastically, which allows binding to its site at *Pu* and induction of the promoter (Valls *et al.*, 2002). Because the mRNA levels of the *ihfAB* genes vary little upon entry into the stationary phase (Yuste *et al.*, 2006), the regulation of IHF expression is probably post-transcriptional, although this idea has not been validated experimentally. When cells grow in a minimal salts medium, the levels of *ihfAB* mRNAs are also low, although those of the IHF protein have not been analysed (Aranda-Olmedo *et al.*, 2005). Under these growth conditions, induction of promoter *Pu* by toluene is very efficient, suggesting that IHF levels are high enough to allow for an efficient promoter activity.

Pu promoter activity decreases significantly in cells lacking FtsH (Carmona & de Lorenzo, 1999), a protein that behaves as a protease and as a chaperone, maintaining quality control over some proteins and aiding the assembly, operation and disassembly of some protein complexes (Neuwald *et al.*, 1999; Ito & Akiyama, 2005). FtsH seems to act on the XylR protein (Sze *et al.*, 2002). To activate transcription, XylR and other σ^{54}-dependent regulators oligomerize and form higher-order structures, a process that requires ATP hydrolysis (Porter *et al.*, 1993; Pérez-Martín & de Lorenzo, 1996b). Perhaps FtsH aids the assembly and/or disassembly of these complexes. It should be noted, however, that FtsH is required for the activity of some, but not all, σ^{54}-dependent regulators (Carmona & de Lorenzo, 1999; Sze *et al.*, 2002).

Finally, three additional factors affect *Pu* promoter activity, namely ppGpp, TurA and PprA. The alarmone ppGpp

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moderately stimulates transcription from *Pu* (Carmona *et al.*, 2000). This alarmone mediates a global response elicited by nutritional limitation and physicochemical stress, favouring the transcription of genes important for starvation survival at the expense of those required for growth (Magnusson *et al.*, 2005). Under rapid growth in a complete medium, the levels of ppGpp are very low, but increase significantly upon entry into the stationary phase, when the preferred amino acids have been consumed, or during growth in a minimal salt medium with a poor carbon source (Sze & Shingler, 1999; Sze *et al.*, 2002). Therefore, ppGpp levels are high precisely under those conditions in which *Pu* promoter activity is also high. The effect of ppGpp is to interfere with the competition between the housekeeping sigma factor, σ^{70}, and the alternative σ^{54} for binding to the core RNA polymerase, which is available in limited amounts; the outcome is that ppGpp increases the effective concentration of σ^{54}-RNA polymerase in the cell, thus favouring the transcription of σ^{54}-dependent promoters (Laurie *et al.*, 2003; Bernardo *et al.*, 2006; Szalewska-Palasz *et al.*, 2007). There is indeed a correlation between the extent to which ppGpp affects transcription from σ^{54}-dependent promoters and the innate affinity of the promoters for σ^{54}-RNA polymerase (Bernardo *et al.*, 2006).

The regulatory proteins TurA and PprA affect *Pu* promoter activity by binding to the promoter. TurA, which belongs to the MvaT/H-NS family of proteins, represses *Pu* activity both *in vivo* and *in vitro*. Its effect is moderate under optimal growth conditions (i.e. growth in a complete medium at 30 °C), but increases substantially when the growth temperature is decreased to 16 °C (Rescalli *et al.*, 2004). It has been proposed that TurA may limit *Pu* activity under suboptimal environmental conditions, adjusting the expression of the TOL pathway to cell needs. The PprA protein, in turn, binds to a site that overlaps with the XylR DNA-binding sequences, inhibiting *Pu* activity by competing with XylR for binding to DNA (Vitale *et al.*, 2008). PprA is a member of the LytTR family of two-component response regulators. It is at present unclear which are the signals to which PprA responds. However, the examples of IHF, ppGpp, TurA and PprA, show that the activity of the *Pu* promoter is finely tuned and integrated within host physiology not only by regulators that respond to CCR signals, but also by other factors that respond to environmental, nutritional or physicochemical cues.

The phenol degradation pathway encoded in the *P. putida* pVI150 plasmid

The *P. putida* CF600 plasmid pVI150 encodes a complete pathway for the degradation of phenol and methylated phenols (Powlowski & Shingler, 1994). All catabolic genes are clustered on a single operon, the *dmp* operon, which is transcribed from the *Po* promoter (Shingler *et al.*, 1992, 1993). This promoter is recognized by σ^{54}-RNA polymerase and is positively regulated by the DmpR transcriptional activator in the presence of phenol or methylated phenols. DmpR is structurally and mechanistically very similar to the XylR transcriptional activator of the pWW0 plasmid. Transcription of the *dmp* operon from promoter *Po* is also controlled by nutritional and environmental cues that are dominant over the specific regulator DmpR (Sze *et al.*, 1996; Sze & Shingler, 1999). In spite of the clear similarities between the *Po*/DmpR and the *Pu*/XylR systems, the way in which they integrate and respond to physiological signals is surprisingly different. When cells grow in a minimal salts medium, the addition of phenol generates an immediate activation of promoter *Po*, an induction that is not affected by the simultaneous presence of other carbon sources such as glucose, gluconate or succinate (Sze *et al.*, 1996). However, when grown in a complete medium, or in a minimal salts medium containing casamino acids, the response of promoter *Po* to phenol is strongly repressed and delayed until cells reach the stationary phase of growth. In the *Pu*/XylR system, the delayed induction in a complete medium was traced to the activity of Crc, the levels of IHF and, to a lower extent, to the FtsH protein and the ppGpp levels. In the case of the *Po*/DmpR system, the role of Crc is still unknown. FtsH and PtsN are known to play no role in the activity of the *Po*/DmpR system (Sze *et al.*, 2002). IHF plays different roles at *Pu* and *Po* promoters, but it is needed in both cases. As promoter *Pu*, the *Po* promoter has an IHF-binding site located between the DmpR and the σ^{54}-RNA polymerase sites. At *Pu*, IHF favours the recruitment of σ^{54}-RNA polymerase to the promoter (Bertoni *et al.*, 1998; Carmona *et al.*, 1999; Macchi *et al.*, 2003), while at *Po*, IHF facilitates or stabilizes the open complexes formed upon transcription activation by DmpR (Sze *et al.*, 2001). At least *in vitro*, σ^{54}-RNA polymerase has a similar affinity for the *Pu* and *Po* promoters when IHF is present, but when IHF is absent, the affinity for *Pu* is significantly lower than that for *Po*. This would explain why the recruitment effect of IHF is less important for *Po* than for *Pu*. The activity of the DmpR/*Po* system is strongly dependent on ppGpp, whose levels are low during exponential growth in a complete medium and increase in the stationary phase; in fact, ppGpp levels are responsible for the observed repression of *Po* induction in cells grown in a complete medium (Sze & Shingler, 1999; Bernardo *et al.*, 2006). The lack of ppGpp does not affect the levels of DmpR or of σ^{54} considerably (Sze & Shingler, 1999). A detailed analysis of the role of ppGpp showed that this alarmone is needed to increase the levels of σ^{54}-RNA polymerase, which are critical for DmpR/*Po* activity (Laurie *et al.*, 2003; Bernardo *et al.*, 2006, 2009; Szalewska-Palasz *et al.*, 2007).

To further understand the different properties of the XylR/*Pu* and DmpR/*Po* systems, the regulators and

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promoters of each pair were swapped in artificial constructs containing the DmpR site upstream of the *Pu* promoter and the XylR site upstream of promoter *Po*. The DmpR/*Pu* artificial system was responsive to phenol, as expected, and strongly dependent on ppGpp, but independent of the FtsH and PtsN proteins. On the contrary, the XylR/*Po* system responded to toluene and was sensitive to FtsH and PtsN, and less so to ppGpp. This indicates that the global regulatory factors that link the activity of the DmpR/*Po* and XylR/*Pu* systems to the nutritional and physiological status of the cell predominantly act by exploiting the properties of the XylR and DmpR regulators, rather than those of the promoters (Sze *et al*., 2002).

Concluding remarks

Available evidence suggests that the role of CCR is to optimize metabolism to attain an efficient growth at a minimum energetic cost. Therefore, CCR helps to improve the ability of metabolically versatile free-living bacteria to compete in their natural habitats. CCR is only observed when the preferred carbon source is present at concentrations that do not limit growth, or when at least is present in excess relative to other nutrients. It can be argued that microorganisms most frequently live in habitats where the carbon sources are present in limiting amounts. However, a versatile bacterium could benefit not only from traits that allow it to scavenge nutrients present at very low concentrations, but also from regulatory networks that allow to optimize metabolism when, perhaps occasionally, the microorganism gains access to nutrients present at concentrations that allow a nonrestricted growth. It is important to note that CCR systems influence metabolism precisely under conditions that allow growth (when there is an excess of carbon sources), and it is under these conditions that optimizing growth and proliferation can be useful to outgrow other microorganisms that are potential competitors for the resources available. In this sense, it will be interesting to investigate whether the inactivation of genes responsible for CCR in *Pseudomonas* strains leads to a reduction in their bacterial fitness and in their ability to compete with other microorganisms. At least for the Crc regulator, available information suggests that this is the case (Moreno *et al*., 2009a).

CRC has been studied mainly under laboratory conditions, and there are very few data on its real influence when cells grow in their natural environments. It has been reported that the exudates from some plant roots can reduce the phenanthrene-degrading ability of *P. putida* ATCC 17484, a bacterium that can grow associated with plant roots (Rentz *et al*., 2004). This is an interesting observation that suggests that catabolite repression may be operative under natural conditions in which the concentrations of carbon sources are not high, although perhaps the local concentrations, or the scarcity of other nutrients (the C/N ratio, for example), create conditions that trigger the repressive response.

Understanding the molecular mechanisms underlying CCR is important to know how metabolism is regulated and also to learn how bacteria degrade compounds in the environment. This is particularly true for compounds that are difficult to degrade and that tend to accumulate in the environment, creating pollution troubles. Hydrocarbons, which frequently pose important pollution problems, are a relevant example of nonpreferred compounds for most bacteria. Therefore, optimizing bioremediation strategies and the design of tailor-made biocatalysts can benefit from a detailed understanding of the CCR regulatory networks and of the molecular mechanisms involved.

In addition, in several bacterial species, CCR systems can influence the expression of genes that are relevant for virulence (reviewed in Görke & Stülke, 2008). It should be recalled that virulence traits are frequently directed to facilitate access to new sources of nutrients. This has not been investigated in detail in *Pseudomonas*, but there are some examples that link the expression of some *P. aeruginosa* virulence factors to the regulation of metabolism. It has been mentioned that inactivation of the gene coding for the Crc regulator in *P. aeruginosa* impairs twitching motility and biofilm formation, two traits that are important for the colonization of human tissues (O'Toole *et al*., 2000). In addition, deregulation of glucose metabolism, and in particular of the *zwf* gene coding for glucose-6-phosphate dehydrogenase, facilitates the adaptation of *P. aeruginosa* to survive in the lungs of cystic fibrosis patients, probably through an increase in the production of the exopolysaccharide alginate (Silo-Suh *et al*., 2005). This adaptation makes the *zwf* gene insensitive to CCR, although the molecular details underlying this effect are still unknown. The mucus produced in the lungs of cystic fibrosis patients is a nutritionally rich environment that contains several amino acids, glucose and lactate, and allows *P. aeruginosa* to grow to high cell densities (Palmer *et al*., 2005, 2007). In this medium, alanine, proline, arginine, glutamate and aspartate are preferentially used over other carbon sources such as glucose or lactate. Although it is not yet clear how this substrate selection takes place, it may be a case of a natural habitat (the host fluids) where CCR could be regulating metabolism.

In summary, CCR networks affect many aspects of *Pseudomonas* metabolism and of the interaction of the bacteria with the environment. A more complete comprehension of the molecular mechanisms underlying the CCR in *Pseudomonas* is essential for the development of efficient biocatalysts and bioremediation strategies and to understand how pathogenic *Pseudomonas* infect and interact with the host.

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