53 Genetics of Accessing and Exploiting Hydrocarbons

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Abstract: Hydrocarbons abound in the environment and microorganisms are often capable of assimilating and degrading these normally recalcitrant molecules. In order to achieve this, bacteria have developed specific adaptive mechanisms; much of the adaptive response is brought about by transcriptional activation of genes and is controlled by one or two component regulatory systems, global regulators and DNA binding proteins. The expressed gene products are then able to degrade the molecules and often take advantage of the stored energy imparted by the physicochemical properties of the hydrocarbon structure. The response of gene regulators to the presence of hydrocarbons such as toluene in the environment allows initiation or inhibition of transcription, so that the rate of synthesis of metabolically important gene products is adaptively modified. Microorganisms which mount the most appropriate physiological adaptation are then able to proliferate in the changing environment. Here we give a comprehensive overview of adaptive regulation of the TOD and TOL pathways including the involvement of catabolite repression.

1 Introduction

Hydrocarbons, especially benzene and other aromatic compounds, possess a large negative resonance energy. The aromatic hydrocarbons therefore have a high thermodynamic stability which manifests itself in chemical properties very different from those observed for branched hydrocarbons. Next to glucosyl residues, the benzene ring is the most widely distributed unit of chemical structure in nature (Dagley, 1981). Although ubiquitous in nature these aromatic hydrocarbons are of debatable origin. While it is commonly accepted that most are not of biosynthetic origin but are derived from the natural pyrolysis of organic compounds, clearly a substantial quantity of those found in the environment today are of man-made origin. Aromatic hydrocarbons such as benzene, toluene, styrene and the xylenes have been widely used in industry during recent decades and are among the largest volume industrial chemicals produced today. Additionally, polycyclic aromatic compounds are often the starting materials in the production of agrochemicals (pesticides, herbicides and fertilizers), polymers, pharmaceuticals, explosives and a multitude of other everyday products. Predictably, these man-made hydrocarbons have been released into the environment. As aromatic compounds are widely available in nature it is not surprising that microorganisms have evolved capable of degrading these compounds. Due to the current social interest in pollution and the global environment it is not unexpected that there is presently considerable interest in the isolation and characterization of bacteria that are able to thrive on these hydrocarbons.

Bacteria have developed specific adaptive mechanisms in order to deal with the wide range of hydrocarbons of both natural and man-made origin that abound in the environment. Some of these defense mechanisms are post-synthetic, such as the alteration of membrane lipids to prevent entry of hydrocarbons (See Chapter 55, Vol. 2, Part 9); however, much of the adaptive response is brought about by transcriptional activation of genes; the products of which are able to take advantage of the physicochemical properties of the hydrocarbon molecules in question. The response of gene regulators to specific signals (such as the presence of hydrocarbons in the environment) allows initiation or inhibition of transcription, so that the rate of synthesis of metabolically important gene products is adaptively modified. Naturally, microorganisms that are able to tender a successful physiological adaptation are better suited to survive in the changing environment.

Since initial reports in the late 1980s of a bacterium able to thrive in the presence of high concentrations of aromatic hydrocarbons, many other similarly adapted bacteria have been isolated (Inoue and Horikoshi, 1989). Among them, *Pseudomonas putida* DOT-T1E, a microbe that is highly solvent-tolerant, was isolated from a wastewater treatment plant in Granada, Spain (Ramos et al., 1995). This strain can grow in liquid medium with $> 1\%$ (v/v) toluene. The ability of *Pseudomonas putida* DOT-T1E to survive in the presence of hydrocarbons is an inducible phenomenon and the molecular mechanisms underlying this adaptive response and the consequent solvent tolerance in this strain have been extensively studied in our and others laboratories (Busch et al., 2007; Cases et al., 2001; del Castillo and Ramos, 2007; Jurado et al., 2003; Lau et al., 1997; Mosqueda et al., 1999; Ramos et al., 1986, 1987a). *Pseudomonas putida* DOT-T1E adapts to the presence of aromatic hydrocarbons using a series of tools including: (1) the modification of fatty acids and phospholipid head groups in the membrane in order to make it less permeable; (2) synthesis of chaperones that facilitate correct protein folding in the presence of the hydrocarbons; (3) the extrusion of the compounds by means of three efflux pumps namely TtgABC, TtgDEF and TtgGHI and (4) the induction of metabolic pathways that allow the bacteria to use the hydrocarbons as an energy source. This later ecophysiological adaptation is the subject of this chapter.

2 The TOD Pathway for Catabolism of Aromatic Hydrocarbons

A number of *Pseudomonas putida* strains, including DOT-T1E, can utilize benzene, toluene and ethylbenzene as carbon sources. The enzymatic pathway responsible for converting these aromatic hydrocarbons to TCA cycle intermediates is called the *to*luene *d*egradation (*tod*) pathway; a member of the well characterized cis-dihydrodiol pathways (Finette et al., 1984). The catabolic genes of the TOD pathway form an operon *todXFC1C2BADEGIH* that is transcribed from a single promoter called P_{todX}, located upstream from the *todX* gene (➲ *Fig. 1a*) (Mosqueda et al., 1999). The TOD pathway involves seven enzymatic reactions which are initiated by a multicomponent toluene dioxygenase (encoded by *todC1C2BA*) and finalized by the TodGHI enzymes resulting in the production of central metabolism intermediates pyruvate and acetyl-CoA. Interestingly, the product of the *todX* gene is not a catabolic enzyme but in fact an outer membrane protein; likely a porin through which hydrocarbons may be taken up.

Regulation of TOD pathway expression in *P. putida* is different from many other aerobic toluene-degrading routes in which regulation falls into one of three existing families of aromatic catabolic regulators: (1) LysR transcriptional regulators; (2) σ^{54}-dependent NtrC transcriptional activators, and (3) AraC/XylS activators. The expression of the *tod* catabolic genes is regulated by the gene products of *todST*, which are located downstream of the *tod* genes encoding the pathway enzymes. The *todST* genes form a separate transcriptional unit that is constitutively expressed (➲ *Fig. 1a*). The TodS/TodT proteins have been shown to form a two-component regulatory system which controls the positive regulation of the *tod* operon (Lau et al., 1997). Two-component regulatory systems (TCS) are a key means by which bacteria detect environmental signals that mediate changes in gene expression, cellular behavior (e.g., chemotaxis) and biological processes (e.g., catabolism). These systems typically consist of two proteins (1) an autophosphorylating sensory histidine kinase (HPK) and (2) a response regulator (RR). In the basic model of TCS function, signal perception (physical or chemical) at the input domain of the HPK typically instigates the modulation of the

Figure 1

(a) Organization of the *tod* genes as found on the genome of *Pseudomonas putida* DOT-T1E. The two-component regulatory system *todST* is colored differently in order to distinguish it from the catabolic genes. (b) Schematic, showing the domain organization of the TodS and TodT proteins. PAS-1/2, PAS-type sensor domain; HisK-1/2, histidine kinase domain; RRR, response regulator receiver domain; HTH DNA binding domain. Approximate amino acid locations of the domains in the proteins are indicated. (c) The P*todX* promoter showing the three "TodT boxes" to which phosphorylated TodT is bound; the IHF binding site is shown in orange with IHF bound causing DNA bending and subsequent binding of RNA polymerase at the −10 to + 1 region of the promoter (adapted from Lacal et al., 2008; Lau et al., 1997).

proteins autophosphorylation activity. The phosphate, usually located on a conserved histidine residue at the C-terminal end of the HPK is then transferred to a conserved aspartate residue, typically found at the N-terminus of the RR. Alterations in the functional properties of the phosphorylated response regulators output domain allow control of gene expression at the transcriptional level which is mediated by binding of the RR to promoter sequences. The pronounced diversity which is found in the HPK and their cognate RR proteins allows that the HPKs are able to recognize varied signals and that the RRs can be involved in the regulation of a multitude of different biological processes.

The large (108 kDa) HPK TodS is unusual in that it does not contain membrane spanning domains and possesses multiple protein regions; each of these regions contain a periodic circadian-Ah receptor single-minded protein (PAS) sensor domain and a histidine kinase domain, which are separated by an RR receiver domain (*Fig. 1b*). The signal sensing PAS domains are found in various hydrocarbon sensor proteins and it appears that TodS belongs to a subfamily of HPKs which are involved in the control of catabolic pathways for the degradation of hydrocarbons (Galperin, 2006; Leoni et al., 2003). TodS has 82% and 41% identity respectively with TmoS that controls toluene degradation by the T4MO pathway in *Pseudomonas mendocina* and StyS from *Pseudomonas* sp. strain Y2 which is involved in the degradation of styrene (Ramos-González et al., 2002; Velasco et al., 1998). The TodT protein shows significant amino acid sequence similarity with many response regulators of two-component signal transduction systems. TodT contains an N-terminal input signal domain for accepting the phosphoryl group from TodS and a C-terminal HTH DNA binding domain.

Initially expression of the *tod* operon was shown to be induced in the presence of toluene, styrene, benzene, ethylbenzene, and *m*-xylene. More recently it has been shown that the TodS/TodT two-component regulatory system is able to recognize a wide range of signal molecules and that the mechanism of P_{todX} induction also involves DNA-bending proteins (Busch et al., 2007; Lacal et al., 2006). The TodS protein has a low basal level of autophosphorylation, which in the presence of certain hydrocarbons increases causing a stimulation of TodS phosphotransfer to TodT. The phosphorylated TodT then functions as a positive regulator. The P_{todX} promoter is referred to as an "extended promoter" because it has a well defined-10 consensus sequence but no -35 consensus (Domínguez-Cuevas and Marqués, 2004). The TodT protein binds to three so called "TodT boxes" which are centered at base pairs-106, -85 and -56 of the P_{todX} promoter, quite distant from the RNA polymerase binding site. Consistent with the involvement of integration host factor (IHF) in the bending of DNA at the P_{todX} promoter is the fact that IHF is able to bind between base pairs-24 and -75 of the promoter. In the absence of IHF the expression from P_{todX} is very low (Lacal et al., 2008). The current model of transcription activation of P_{todX} is; the increased autophosphorylation of TodS caused by the presence of certain aromatic hydrocarbons allows an increase in the transphosphorylation of TodT. Phosphorylation of TodT leads to conformational changes that alter its interaction with the RNA polymerase, the contact between phosphorylated TodT and the RNA polymerase is brought about by IHF, which introduces a DNA bend that favors the required interactions. Formation of the TodT-*P*/IHF/RNA polymerase activation complex induces transcription of the *tod* operon (*Fig. 1c*).

The complex nature of the two component regulation of *tod* operon expression has recently been augmented by the discovery that TodS can bind both agonists (such as toluene, benzene and styrene) and antagonist (such as *o*-chlorotoluene and *o*-xylene) molecules at the same N-terminal TodS effector binding site. Surprisingly, the affinities for both agonists and

antagonists are similar however; binding of an antagonist molecule does not stimulate autophosphorylation of TodS and the subsequent induction of the P_{todX} promoter (Busch et al., 2007).

3 The TOL Pathway for Catabolism of Aromatic Hydrocarbons

The pWW0 TOL plasmid of *Pseudomonas putida* encodes the information for the catabolism of toluene and xylenes, which is organized in two operons generally referred to as the upper and *meta* operons. *Fig. 2* shows the genetic organization of the catabolic operons and the complex array of regulatory mechanisms involved in controlling their expression. The genes of the upper operon encode the enzymes for oxidation of the lateral alkyl chain of toluene and xylenes to their corresponding carboxylic acids. The genes for the *meta*-cleavage pathway encode the enzymes for oxidation of benzoate and toluates to Krebs cycle substrates. Regulation of the TOL plasmid catabolic pathway for the degradation of toluene and xylenes is a model system exemplifying both specific and global regulation in response to hydrocarbons. Expression of the catabolic operons involves the TOL plasmid-encoded XylR and XylS regulators, a set of sigma factors (σ^{70}, σ^{54}, σ^{32}, and σ^{38}), and the DNA-bending proteins, IHF and HU. When bacteria are growing in the presence of toluates (alkylbenzoates) only the *meta*-regulatory loop functions, however, when bacteria grow on xylenes both the upper and *meta*-catabolic pathways are expressed due to the action of the cascade loop (Ramos et al., 1987b, 1997).

When bacteria containing the TOL plasmid are growing in the presence of xylenes the transcriptional control is directed by the XylR regulator. Briefly, this regulator drives transcription from the *Pu* promoter in front of the *xylUWCMABN* genes, which constitute the

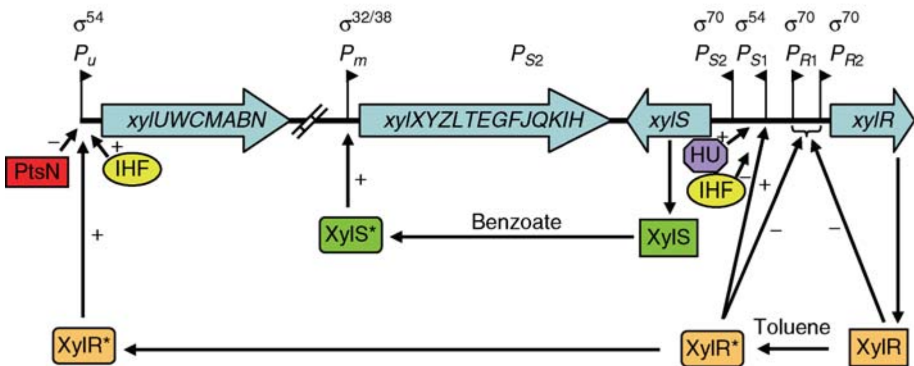

Figure 2

Regulation of the TOL pathway. The genes encoded in the upper pathway (*xylUWCMABN*) and the meta-pathway (*xylXYZLTEGFJQKIH*) are indicated along with those of the regulatory genes *xylS* and *xylR*. The actions of the regulatory proteins on given promoters are indicated by the arrows and either a minus sign for repression or a plus sign for induction. Sigma factors required for expression are indicated above each promoter. The roles of DNA binding proteins IHF and HU along with PtsN are indicated as discussed in the text, activated XylR and XylS are shown with an asterisk (adapted from Aranda-Olmedo et al., 2006).

upper operon, for conversion of toluene and *p*- and *m*-xylenes into the corresponding benzoates, as well as from the *Ps1* promoter to increase expression of the *xylS* gene (*Fig. 2*). Subsequently, this results in the induction of the *meta*-cleavage pathway operon for the oxidation of benzoates to Krebs cycle intermediates. However, the full picture of the adaptive response of bacteria containing the pWW0 TOL plasmid to the presence of aromatics hydrocarbons is extremely complex: Two σ^{70}-dependent promoters, Pr1 and Pr2 upstream of the *xylR* gene allow the expression and production of XylR. However, when cells are growing in the presence of toluene; XylR binds directly with the hydrocarbon and becomes activated, the activated XylR* is then able to drive transcription from the *Pu* promoter of the upper pathway (Delgado and Ramos, 1994). Activation of the upper pathway *Pu* promoter requires IHF and also σ^{54} RNA polymerase. The *xylS* gene which is constitutively expressed at low levels from its *Ps2* promoter is further induced by the activated XylR* and a DNA bending protein (HU) acting together to up-regulate transcription of the *xylS* gene from a second σ^{54} dependent promoter named *Ps1*. Naturally, the *Pr1/2* and *Ps1/2* promoters are located in the intergenic region between the divergently transcribed *xylR* and *xylS* genes. This proximity further complicates the regulation because when XylR* binds to the upstream activator sequences for Ps1 of *xylS* it blocks access of RNA polymerase to its own Pr1 and Pr2 σ^{70}-dependent promoters; hence reducing its own expression. In the presence of benzoates the *meta*-pathway is rendered fully operational because *xylS* is only transcribed from the constitutive XylR-independent promoter (*Ps2*). Benzoate interacts with the XylS protein which leads to the transcriptional activation of the *Pm* promoter in the *meta*-cleavage pathway.

4 Catabolite Repression and Expression of the TOL Plasmid Genes

On top of the previously discussed interplay of specific plasmid regulators, σ factors and DNA bending proteins, catabolite repression plays a key role in the control of the expression of the TOL plasmid pathways. In fact, the expression of TOL plasmid operons is incorporated into the overall metabolic control in *Pseudomonas putida* because both *Pu* (the upper pathway promoter) and *Ps1* (the *xylS* promoter) are subject to catabolite repression. It follows that as a consequence of catabolic repression control on *xylS*, expression of the *meta*-cleavage operon is also indirectly subjected to catabolite repression. Catabolite repression control is generally defined as the ability of an organism to preferentially metabolize one carbon source over another when both carbon sources are present in the organism's environment. However, the catabolite repression observed in *P. putida* is normally referred to as crossed catabolite repression because the bacteria are able to use two carbon sources (e.g., glucose and toluene) simultaneously. This type of control is similar to that for *Klebsiella oxytoca* which can use both sucrose and glycerol simultaneously (Piñar et al., 1998), but is in stark contrast to the strict catabolite repression of *E. coli* when it preferentially consumes glucose before moving on to use lactose.

Interestingly, transcription from the *Pu* promoter of the upper TOL pathway (normally mediated by RNA polymerase with σ^{54}) is repressed when the bacteria are grown in the presence of alternative carbon sources. This can be clearly seen when cells are grown in the presence of both acetate and *m*-xylene; the bacteria contain significantly lower levels of the upper pathway proteins than the same cells grown in the presence of *m*-xylene as the sole carbon source. A similar repression can be seen when cells are grown with *o*-xylene

because it is unable to induce expression of the TOL catabolic systems when bacteria are grown in the presence of an excess of nutrients.

Cyclic AMP (cAMP) is the signal molecule for catabolite repression in Enterobacteriaceae; the levels of cAMP vary depending on the concentration of glucose and other sugars in the growth environment. However, in *P. putida* the cAMP levels remain stable despite the growth conditions, indicating that cAMP is not involved in catabolite repression in this Pseudomonad. Catabolite repression in *P. putida* instead incorporates at least five different regulators CyoB, Crc, Crp, RelA and PtsN, this quality greatly increases the complex nature of the repression system. In fact, in a *P. putida ptsN* mutant background the expression from the upper operon *Pu* promoter is de-repressed in the presence of glucose suggesting that in a wildtype strain the repressing carbon source (in this case glucose) causes PtsN to prevent XylR* from binding to the upstream activator sequences at *Pu* (Aranda-Olmedo et al., 2006).

Several recent pieces of work investigating the phenomenon of glucose repression of the TOL *Pu* upper pathway operon promoter from both the physiological and molecular points of view have clarified the players involved in catabolite repression (del Castillo and Ramos, 2007). *Pseudomonas putida* catabolizes glucose to pyruvate via the Entner-Doudoroff pathway using a different set of enzymes from those used in either glycolysis or the pentose phosphate pathway. Genetic evidence has suggested that catabolites of the pathway signal carbon source repression of the *Pu* promoter of *P. putida*. It has been found that in *P. putida* 6-phosphogluconate (6PG) is synthesized by three converging pathways; (1) the glucose dehydrogenase (Gcd) route, (2) the glucokinase (Glk) route and (3) via direct phosphorylation of gluconate mediated by gluconokinase (del Castillo and Ramos, 2007). Interestingly, toluene and glucose have regulatory effects on each others metabolism in wild-type *P. putida* and also mutant strains which are lacking in either the Gcd or Glk pathways. Glucose causes repression of toluene metabolism by signaling through 2-dehydro-3-deoxygluconate-6-phosphate and the PtsN global regulator, whilst toluene causes repression of glucose metabolism via the Glk pathway with the help of the Crc regulator protein. The Crc protein appears to be acting as a switch for the Glk pathway because in *crc* mutant backgrounds basal glucokinase levels are not repressed in the presence of toluene.

5 Conclusions and Research Needs

There are two principal modes of specific transcriptional regulation in bacteria which are associated with either one-component systems or two component systems (TCSs) (Ulrich et al., 2005). One component systems are formed by a single protein such as XylS or XylR, which generally contain a sensor and a DNA-binding domain. In the prototypal one-component system the binding of signal molecules to the sensor domain alters the affinity of the protein for a promoter region giving rise to a modulation of transcriptional activity. As detailed above TCSs are more complex and their function is based on covalent protein modification. Compared to one component systems TCSs increase a bacteria's ability to thrive in more complex environments; such as aquatic or soil environments (Ashby, 2004). The probable evolutionary advantage lies in the capacity of a TCS to detect and modulate cell metabolism, motility and behavior in response to extra-cytosolic signals, whereas one component systems generally respond to cytosolic signals. Indeed, 88% of TCS sensor kinases were predicted to be transmembrane proteins. However, the currently available data for

TodS and sequence analysis of its homologues indicate that they form part of the 12% of kinases which are located in the cytoplasm where they sense signals.

The reason why some hydrocarbon degradation pathways are regulated by one component systems and others by TCS is unknown, but clearly both systems are efficient in the control of their corresponding gene circuits and the regulation of expression of toluene catabolic pathways seems to be extremely complex. The current knowledge of pathways such as TOD and TOL allows a quite complete picture of the processes involved in this type of ecophysiological adaptation; however, there are many questions that remain unanswered. For example, what is the true functional role of the *todX* gene product? Does the structurally unusual TodS protein interact with membrane proteins to accept signals indicating the presence of hydrocarbons? Are global regulatory proteins such as Crc, Crp, PtsN or CyoB involved in the regulation of the *tod* operon? What are the conformational changes brought about in TodT following phosphorylation that allow the operon to be expressed and are TCS connector proteins (Mitrophanov and Groisman, 2008) involved in modulation of the signal? In the TOL plasmid story how is the glucokinase pathway controlled in the presence of aromatic hydrocarbons? Is there a specific chemical signal or is the control imparted directly by Crc or indirectly via transcriptional modulation of other regulators? Naturally, answers to these questions will allow a more complete and thorough understanding of the adaptive response of bacteria to the presence of aromatic hydrocarbons in their environment.

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