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Estudio del mecanismo molecular del sistema de dos componentes TodS/TodT

Memoria que presenta
el Licenciado en Biología
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para aspirar al Título de Doctor

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A mis padres
A las tres rubias
A Carmen

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I. Índice / Index

Abbreviation Index

abbreviation	meaning
aa	amino acid
BTEX	Benzene/Toluene/Ethylbenzene/Xylenes group of contaminants
bp	base pairs
bZIP	basic leucine zipper domain
α-CTD	carboxyterminal α-subunit of RNA-Polymerase
C-terminal	carboxyterminal
CTodS-long	recombinant fragment of TodS comprising aa 452-978
CTodS-short	recombinant fragment of TodS comprising aa 586-976
CTodT	recombinant fragment of TodT comprising aa 154-206
Δ*G*	change in Gibbs free energy
Δ*H*	change in enthalpy
Δ*S*	change in entropy
HK	histidine kinase
HPT	histidine containing phosphotransferase domain
HTH	helix-turn-helix
IHF	integration host factor
ITC	isothermal titation calorimetry
kb	kilobase
LB	Luria-Betani medium
MCP	methyl-accepting chemotaxis protein
MTodS	recombinant fragment of TodS comprising aa 450-585
N-terminal	aminoterminal
NTodS-long	recombinant fragment of TodS comprising aa 1-584
NTodS-short	recombinant fragment of TodS comprising aa 16-449
OCS	one-component system
ORF	open reading frame
PAS	Par-ARNT-Sim domain
REC	N-terminal receiver domain of response regulators
RND	resistance-nodulation-cell domain
RR/R	response regulator / receiver domain
SHK	sensor histidine kinase

abbreviation	meaning
TCA	tricarboxlic acid
TCS	two-component system
TMB	trimethylbenzene
UAS	upstream activator sequence

Figure index

Table index

II. Objetivos / Objectives

1. ***Generación de TodS nativa recombinante y determinación de actividades in vitro de TodS purificada***

2. ***Determinación del sitio de unión de efectores y del perfil de efectores in vitro comparándolo con el perfil in vivo.***

3. ***Determinación del circuito de fosfotransferencia TodS-TodT***

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1. ***Generation of recombinantly expressed, purified, full-length TodS and determination of its activities in vitro.***

2. ***Identification of theTodS effector binding site and determination of the TodS effector profile in vitro comparison to in vivo activities.***

3. ***Determination of the TodS – TodT phosphorylation cascade.***

III. Introducción / Introduction

1. The use of bacteria in bioremediation

The history of human mankind is marked by a depletion of natural resources which gave rise to an increase in the release of waste and pollution into our living habitat. This tendency was particularly pronounced over the last century and it has become obvious that the survival of life on our planet requires a reduction in pollution. Amongst many different alternatives bioremediation, the use of naturally occurring microorganisms to increase the rate of operation of biogenic cycles is an attractive option to fight pollution. The job of microorganisms', in a definition by Carl Oppenheimer, is to "recycle the components of living organisms converting them to nutrient chemicals used by plants in photosynthesis and chemosynthesis". Knowing that microorganisms are the principal actors in earth´s decomposition processes, humans have tried to take advantage of these capabilities not only for the benefit of humans' health and producing elixirs like beer and wine or food like bread and cheese, etc, but also in fighting pollution. Today we know that bacteria can aid in environmental recovery, because we know they can oxidize, bind, immobilize, volatilize or otherwise transform toxic compounds. The growing interest in bacterial bioremediation is due to the fact that it promises to be cheaper and of course environmentally friendlier than other costly chemical or mechanical methods, the so called *muck, suck and truck* technologies that basically consist in taking the pollution elsewhere. Some of these technologies are helpful in avoiding an expansion of contaminated areas, but the real challenge is to re-inject available carbon into the lifecycle. The removal of toxic aromatic hydrocarbons such as toluene, benzene, ethylbenzene and xylenes, the so called BTEX group of contaminants, is beyond other contaminants nowadays subject to studies of a vast scientific community, as we are more and more exposed to them due to industrial activities related to human handling of carbon and oil resources. Nonetheless, these compounds are both of anthropogenic and natural origin and the natural or mankind induced pyrolysis of organic matter is probably the biggest source of these mainly toxic compounds (Dagley, 1971). But why are those compounds so toxic? All these compounds bearing an aromatic benzene ring have high resonance energy and are thermodynamically extremely stable

and therefore recalcitrant. As they have limitede water solubility, they accumulate in living organisms' membranes leading to the removal of membrane proteins and lipids disrupting thereby the membrane potential (Heipieper *et al.*, 1994, Sikkema *et al.*, 1995). Additionally, they are extremely volatile and can have neurotoxic effects through inhalation. The more surprising it was, when scientists found organisms not only living in such a hostile environment as oil spills, but also disrupting aromatic rings using these toxic compounds a carbon and energy source. The initial step in degradation of recalcitrant pollutants, to destabilize the aromatic benzene ring, is the most difficult one. What kind of bacteria manage to disrupt the aromatic ring? Soil is the primary source of bacteria, as the main decomposition and recycling of organic matter happens here. One of the most versatile group of organisms in terms of biochemical resources is the Pseudomonads, gram negative soil-bacteria, prevalently aerobic, although some have been found to be facultative anaerobes. *Pseudomonas* species and closely related organisms have been the most intensively investigated owing to their ability to degrade so many different contaminants. The genome of a few strains of the genus *Pseudomonas* has been completely sequenced and although abundant information on biochemical reactions that metabolize toxic compounds is known, the regulatory mechanisms controlling gene expression are not so well understood. And there is a need to know how the different pathways are regulated and how the organisms respond to changes in environmental conditions, if we want to pursue the aim of effective bioremediation. Because bioremediation strategies that are successful in one location might not work in another, and microbial processes that remediate contaminants in laboratory conditions might not function well in the field (Lovely, 2003). In toluene degradation, up to date there are five aerobic pathways described and they are all characterized by its initial enzymes as they all lead to the same main intermediates: catechol and related compounds (Ruíz *et al.*, 2004; Parales *et al.*, 2008). Those initial enzymes manage to destabilize the aromatic ring by introducing hydroxyl-groups at different C-atoms. Some 20 years ago, the first anaerobic hydrocarbon-degrading bacteria were described, mostly of the denitrifying genus *Aromatoleum* (former genera *Thauera* and *Azoarcus,* Heider, 2007), but also ferric-iron and sulphur-reducing bacteria. All these genera, as

well as methanogenic consortia use the same enzyme to initially attack toluene, yielding after a series of reactions benzoyl-CoA, the central intermediate of anaerobic toluene assimilation. There are thus three characteristic enzymes in toluene metabolism as every aerobic aromatic hydrocarbon degrading microorganism employes a mono- or a dioxygenase for the initial attack and every anaerobic microorganism a benzyl-succinate synthase.

In order to survive and adapt their metabolism microorganisms must have effective sensory/regulatory mechanisms. In our lab, toluene degradation by a highly toluene resistant strain, *P. putida* DOT-T1E is under investigation and the main aim of the work is to understand the regulatory mechanisms involved in tolerance to toluene and in the degradation of this aromatic hydrocarbon. Regulation is driven by the so called One- and Two-Component Systems that will be introduced below.

2. One and Two-component regulatory systems

Unicellular microorganisms, especially those living in the environment, face “life on the edge”, as they are directly exposed to changes in environmental cues which make it primordial to respond in a rapid and effective manner to the prevailing conditions. The studies of how a microorganism is able to detect and to respond to chemical or physical changes in their environment resulted in the discovery of what was called a two-component system (TCS). It was first described by Hall and Silhavy in the year 1981 in *Escherichia coli*; they found that the transcription of the outer-membrane proteins OmpF and OmpC was regulated in response to changes in osmolarity by the sensor EnvZ and the transcriptional regulator OmpR. The minimal components of a TCS were soon found to be a sensor histidine kinase (SHK) and a response regulator (RR). The SHK autophosphorylates upon signal reception and transphosphorylates the cognate RR, which in turn activated exerts its role as typically transcriptional regulator. First insight into the underlying biochemical reactions of a TCS were described by Ninfa and Magasanik (1986) for the NtrB/NtrC system, which controls gene expression in response to nitrogen-source availability. The term “TCS” was coined the same year by Ausubel and co-workers (Nixon *et al.*,

1996) when recognized very conserved aminoacid sequences between the NtrC/NtrB system an other sensory systems in other bacteria. Since then the description of new TCSs grew exponentially with the publication of an increasing amount of genome sequences. The existence of these TCSs seemed to be limited to eubacteria and some archaea, but more recently they have been also found in a very limited number of lower eukaryotes: in fungi, slime molds and plants (*Saccharomyces cerevisiae*, *Dictyostelium discoideum, Neurospora crassa, Arabidopsis thaliana;* Loomis *et al*., 1997, Wurgler-Murphy *et al*. 1997). In complete genome sequences of higher order eukaryotes like the common fruit fly *Drosophila melanogaster* and humans this kind of regulatory systems has not been found.

TCSs are the prevailing mechanism in sensing extracellular stimuli, but transcriptional regulation in response to cytosolic signals is probably dominated by One-Component Systems (OCSs). This statement is based on the study of Ulrich *et al* (2005) who found by analyzing the information encoded in 145 prokaryotic genomes that the large part of specific transcriptional regulation is mediated by OCSs. OCSs are basically composed of two modules, one input or signal reception module and one DNA-binding module. In contrast to TCS, signal transmission between modules is not ATP dependent. Ulrich *et al.* (2005) studied in detail the distribution and co-occurrence of input and output domains in OCSs and TCSs. They detected many more OCSs (~17000) than TCSs (~4000) and a much higher diversity of input and output domains in OCSs. The dominating biological role of both classes of system is transcriptional regulation (~87%) which occurs almost exclusively in response to small ligands (93-96%). TCSs are most frequently transmembrane proteins and respond to extracellular stimuli whereas OCSs are always cytosolic. Although similar in their function, the main advantage of TCSs over OCSs consists in the ability to sense extracellular stimuli, like changes in temperature, O_2/CO_2-concentration, pH, light and nutrient availability. This was proposed to represent an evolutionary advantage of TCSs over OCSs (Ulrich *et al.*, 2005). TCSs were described to regulate a large variety of different biological processes including virulence factors in pathogenic bacteria, antibiotic resistance, chemotaxis, cell differentiation and development steps like in sporulation processes, quorum sensing, nitrogen- and phosphate metabolism and general response to stress

factors (Calva and Oropeza, 2006; Skerker *et al.* 2005, Foussard *et al.*, 2001, Stock *et al.,* 2000)

OCSs consist typically of one input and one output domain (Fig. 1, p. 7). OCSs are characterised by a large structrural and functional diversity. Structural variety is particularly pronounced in the input domain which might reflect the capacity to recognize a diverse range of structurally different ligands. In contrast, TCS not only show variability in their in- and output domains, but also in the domain arrangement of the sensor protein and the number of regulator proteins involved in a phosphorylation cascade. Furthermore, domain duplication is frequently observed in sensor kinases, such as in TodS, the protein under investigation in this thesis.

The prototypal TCS (Fig. 1, p. 7) consists of a Sensor-Histidine Kinase (SHK) with a PAS-type input or sensor domain and one autokinase domain involving ATP hydrolysis and phosphorylation of a His residue, the so called transmitter domain. The SHK has normally two membrane–spanning segments flanking its input domain. The Response Regulator (RR) is composed of an N-terminal receiver domain with a conserved Asp which is phosphorylated by the cognate sensor kinase, and a C-terminal DNA binding domain containing a helix-turn-helix (HTH) motif. The genes encoding both proteins are normally organized on one single operon. The EnvZ/OmpR TCS can be considered as an example of a prototypal TCS as it is considered the most studied system. The SHK EnvZ alters its phosphorylation level in response to changes in osmolarity. The phosphate is then transferred directly from the transmitter domain of EnvZ to the Asp of the receiver module of the RR OmpR. OmpR phosphorylation alters its affinity for its target binding sites located in the *ompF* and *ompC* promoters (Pratt and Silhavy, 1995).

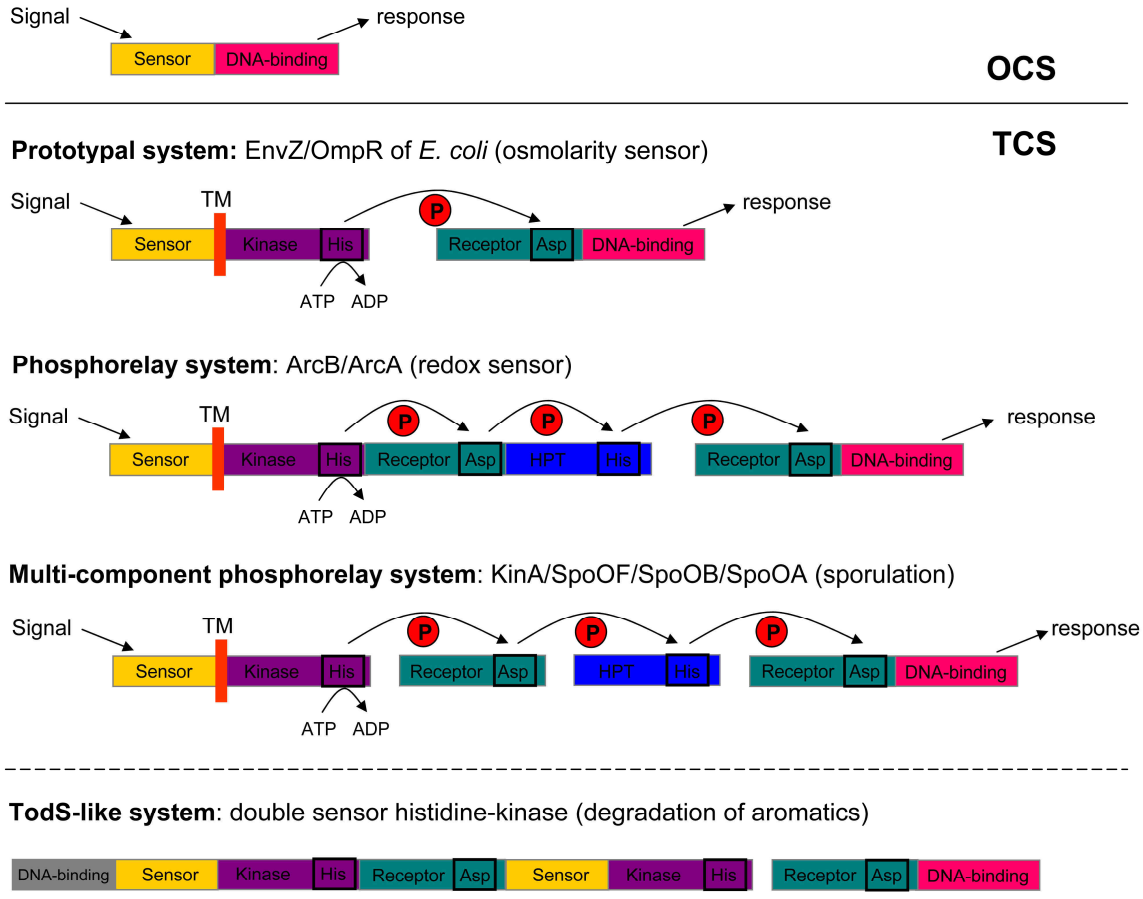

Figure 1. Domain arrangement of OCS and different types of TCS in bacteria. For each class of TCS the most studied sytem is indicated. Domain arrangement of TodS is according to Lau *et al.* (1997). HPT: histidine containing phosphotransfer domain.

Up to date the molecular mechanisms of domain communication between the two domains in each of the TCS components is still poorly understood (Walthers *et al.*, 2003). In addition, the physiologically relevant environmental signal is unknown for the majority of TCS. This is exemplified by the well studied EnvZ/OmpR system, for which huge efforts were done to identify the signal molecule, which however remains unidentified (Jung *et al.*, 2001).

Apart from prototypal TCS other classes of systems were described which are characterised by a more complex domain arrangement and a more complex mechanism. As a typical feature phosphorelay proteins contain more than two phosphorylatable domains. The additional phosphorylatable domain can either be part of the sensor protein or form individual proteins (Fig. 1). The former type is exemplified by the ArcB/ArcA TCS for control of operons encoding aerobic

respiratory enzymes and is reviewed in Iuchi and Lin (1993) and Georgellis and co-workers (Malpica *et al*., 2006). As for the prototypal TCS the SHK consists of a sensor domain and a transmitter domain containing a phophorytable His residue. The special feature of this type of histidine kinases is possessing, apart from these two domains, an internal receiver domain, whose conserved Asp residue serves as phosphotransfer domain, as well as an C-terminal histidine-containing phosphotransfer (HPT) domain with a conserved His residue, which is the site of phosphorylation. The major difference between a kinase and a HPT domain consists in the fact that the kinase domain has autophosphorylation activity whereas the HPT domain is devoid of this activity, since it can only be phosphorylated by its neighbouring receiver domain (Dutta *et al.*, 1999). In contrast to ArcB, the RR ArcA has the typical two domain arrangement as described above. Kwon *et al.* (2000) revealed in an *in vivo* study with wildtype and mutant alels of *arcB* that the mechanism of ArcB is based on internal phosphotransfer as illustrated in Fig. 1, page 7. Their observations suggested a His-Asp-His-Asp phosphorelay mechanism form ArcB to ArcA: ArcA receiving the phosphoryl group soleyly from the N-terminal His via the Asp in the receiver module and the second His in the HPT domain.

The best-studied example of a TCS with additional phosphorylation sites on additional proteins is the KinA/SpoOF/SpoOB/SpoOA multicomponent phosphorelay system (Fig. 1, p. 7), which controls the cellular development in *Bacillus subtilis* (Fujita and Losick, 2005; Stephenson and Hoch, 2001). Initiation of sporulation is a cellular response to adverse conditions for growth and division. The TCS signal transduction system has adopted various modifications to adapt it probably to process multiple inputs. As for the other systems summarized before, the proteins involved in this signal cascade are in great part identified, but there is no clue as to the nature and identity of the signal(s). ATP was shown to bind to the most N-terminal PAS domain of the SHK KinA, but as there are two more PAS domains for which no ligand has been identified (Stephenson and Hoch, 2001), the authors argue that ATP-binding represents the integration of a co-signal and that the primary signal might bind to the remaining two PAS domains. This system is reviewed in Hoch (1995), but more recent studies in the same group show that there are more

kinases than KinA and KinB involved in transphosphorylation/dephosphorylation of the RR SpoOF, namely KinC, KinD and KinE. All the kinases were expressed during growth and early stationary phase, suggesting that the differential activity observed in growth and sporulation results from differential activation by signal ligands unique to each kinase (Jiang *et al*., 2000)

The TodS/TodT TCS was the first TCS described that participates in regulation of transcription of a degradation pathway for toxic aromatic compounds (Lau *et al*., 1997). Sequence analysis revealed the presence of two segments each containing a HK domain and a PAS- type sensor domain (Fig. 1, p. 7). Both segments are separated by a receiver domain. The two autokinase domains are predicted to be phosphorylated at conserved histidine residues whereas the receiver domain is likely to accept a phosphate at an aspartate residue. The question whether TodS operates by an intramolecular phosphorelay will be addressed in this thesis.

2.1. Classification of Sensor Histidine Kinases and Response Regulators

In bacteria there are typically many different SHKs which interact in most cases in a highly specific manner with their cognate RRs. These TCS are classified in prototypal, two-component or multicomponent phosphorelay depending on the modular structure of the sensor-regulator(s) pairs. Besides, the SHKs and the RRs itself can be classified into different classes according to the aminoacid sequence of the conserved part of the protein, the autokinase core of the SHK and the receiver domain of the RR.

Grebe and Stock (1999) performed a sequence analysis of 348 histidine kinase domains and 298 response regulator receiver domains. Their findings revealed that the histidine kinase superfamily can be divided in distinct subgroups. The comparative sequence analysis with the cognate response regulators demonstrated that a given class of SHKs interacts almost exclusively with a given class of RRs. However, the origin of this specifity is not well understood. It

was proposed that this represents an independent evolution of the subclasses of sensor-regulator pairs.

Members of the SHK superfamily have clusters of highly conserved residues within the autokinase domain that are presumed to play crucial roles in substrate binding, catalysis, and/or structure. They have been termed homology boxes **H**, **N**, **D**, **F**, **G1** and **G2** and serve to define the family (Stock *et al*., 1988; Parkinson and Kofoid, 1992). The **H**-Box contains the histidine phosphorylation site. The **D**-Box is part of the nucleotide binding domain and probably interacts with the amino group of the adenine ring. The protein segment containing the **G**-Boxes fold over bound adenine nucleotide phosphate where they play a crucial role in phosphotransfer reactions (Yang and Inouye, 1993; Stewart *et al*., 1998). The **N**- and **F**-Box are also involved in ATP-binding (Bilwes *et al.*, 2001; Marina *et al.*, 2001; Wolanin *et al.*, 2002). Response regulators receiver domains have previously been classified in terms of their output domains, e.g. OmpR, FixJ, NtrC, etc. (Volz, 1993; Pao and Saier, 1995; Hakenbeck and Stock, 1996). The classification of Grebe and Stock (1999) based solely on the receiver domain sequence analysis coincides with the previous grouping except in a few cases.

The fact that all known eukaryotic kinases fall into a single class, HPK_{1b}, and their receivers fall into a single class, R_B, strongly argues in favour of the idea that the eukaryotic TCS arose from lateral gene transfer (Grebe and Stock, 1999, Wolanin *et al*., 2002).

An approach different to the cluster analysis by Grebe and Stock was used by Kim and Forst (2001). They took a genomic approach to analyse the HK families in bacteria and archaea: phylogenetic analysis, differences in the sequence and organization of the H-Box and kinase domain, as well as secondary structure prediction led to five major HK types. They assigned 92% out of 336 studied HKs to proteins of Type I, predominantly found in bacteria. This type encloses what Grebe and Stock separated into four groups (HPK_{1-4}). The main difference between the two approaches is that the classification of Grebe and Stock separates SHKs of both Type II and Type III into a bacterial and a archaeal group (Type II correspond to $HPK_{5/6}$ and Type III correspond to $HPK_{7/11}$). With their study, Kim and Forst could determine that large bacterial genomes contained several different HK types, while archaeal genomes either

lacked or possessed only one specific type. Type I was predominantly found in eubacteria and Type II and III were ascribed to the archaea *A. fulgidus* and *M. thermoautotrophicum*, respectively. They were probably acquired through a single horizontal gene transfer from bacteria of the same niche.

The classification of HK is mainly done with respect to the conserved boxes within the kinase domain. But the capacity of kinases to sense such a large variety of extra- and intracellular signals does not reside in their enzymatic activities related to the global signalling molecule ATP or respectively phosphate, but in the variability of their sensing domains. In that context the most frequently represented sensor domain is that of the PAS type (acroym from *Drosophila* period clock protein *P*ER, vertebrate aryl hydrocarbon receptor nuclear translocator (*A*RNT), and *Drosophila* single-minded protein (*S*IM) (Nambu *et al*., 1991). PAS domains were shown to monitor changes in light, redox potential, oxygen, small ligands and overall energy level of a cell (Taylor and Zhulin 1999). PAS domains comprise approximately 100-120 aminoacids with one or two highly conserved blocks, the S1 and S2-boxes. The S1-box is the most highly conserved PAS motif and forms the PAS core or active site. The S2-box was earlier defined as the PAC motif, a motif extension in the carboxyl direction (Borgstahl *et al.*, 1995) Larger PAS domains including both conserved boxes were former defined as PAS/PAC motifs, but fall nowadays under the same definition as PAS domains. The PAS domains and their structure-function relationship were reviewed in Taylor and Zhulin (1999). Other but less abundant sensor domains in HKs are the extracellular ligand-binding Cache domain, the cGMP-binding and the GAF domain (Taylor and Zhulin, 1999; Dutta *et al*., 1999; Grebe and Stock, 1999; Hoch, 2000; Mascher *et al.*, 2005)

Analysis of the RR, the ultimate effector of an adaptive response in a regulatory phosphorylation cascade, reveals an even higher functional diversity than the sensor kinases (Galperin *et al*., 2001, Ulrich *et al*., 2005). The vast majority of RR consists of two domains: a conserved N-terminal receiver domain (REC) and a less conserved C-terminal effector or output domain. According to Galperin (2006) RRs with DNA-binding domain acting as transcriptional regulators are the majority (~60%) and can be classified into three main families: OmpR, NarL, NtrC. Transcriptional regulators with other DNA-binding

domains account for another 6%. REC domains can either be coupled to a DNA-binding, enzymatic, RNA-binding, protein- or ligand-binding domain or can even be found as stand-alone REC domain. In Ulrich´s classification (2005), a much higher fraction of DNA-binding RRs (87%) resulted because stand-alone REC domains not combined with other domains as described above and functionally unknown domains were not considered. The most abundant type of DNA-binding domain is the OmpR-like HTH (Helix-Turn-Helix motif) or winged HTH domain typical of the NarL family. REC domains show a highly conserved Asp residue in the centre of an acidic binding pocket, the site of phosphorylation by the cognate HK or small signalling molecules such as acetyl-phosphate, a possible indicator of the global metabolic status of the cell (Wolfe, 2005), or carbamoyl-phosphate, imidazole-phosphate and phosphoramidate (Lukat *et al*., 1992; Wolfe, 2005). Phosphorylation at the REC domain induces frequently dimerization of the RR, a mechanism with a key role in transcriptional regulation, as RR dimers typically bind to their target DNA with higher affinity than monomers as exemplified by OmpR, NarL, LytR and PrrA families (Baikalov *et al*., 1996; Laguri *et al*., 2003; Martínez-Hackert and Stock, 1997; Nikolskaya and Galperin, 2002).

3. Overview of toluene degradation pathways in bacteria: metabolic versatility of the genera *Pseudomonas*

So far five aerobic and one anaerobic toluene degradation pathways have been described in eubacteria (Fig. 2, p. 13). The initial enzymes of each route have lent their names to the pathway. All pathways have in common that they convert toluene into carbon dioxide and water. Three pathways were found to be present in *Pseudomonas* strains, indicating a high metabolic diversity of this genus.

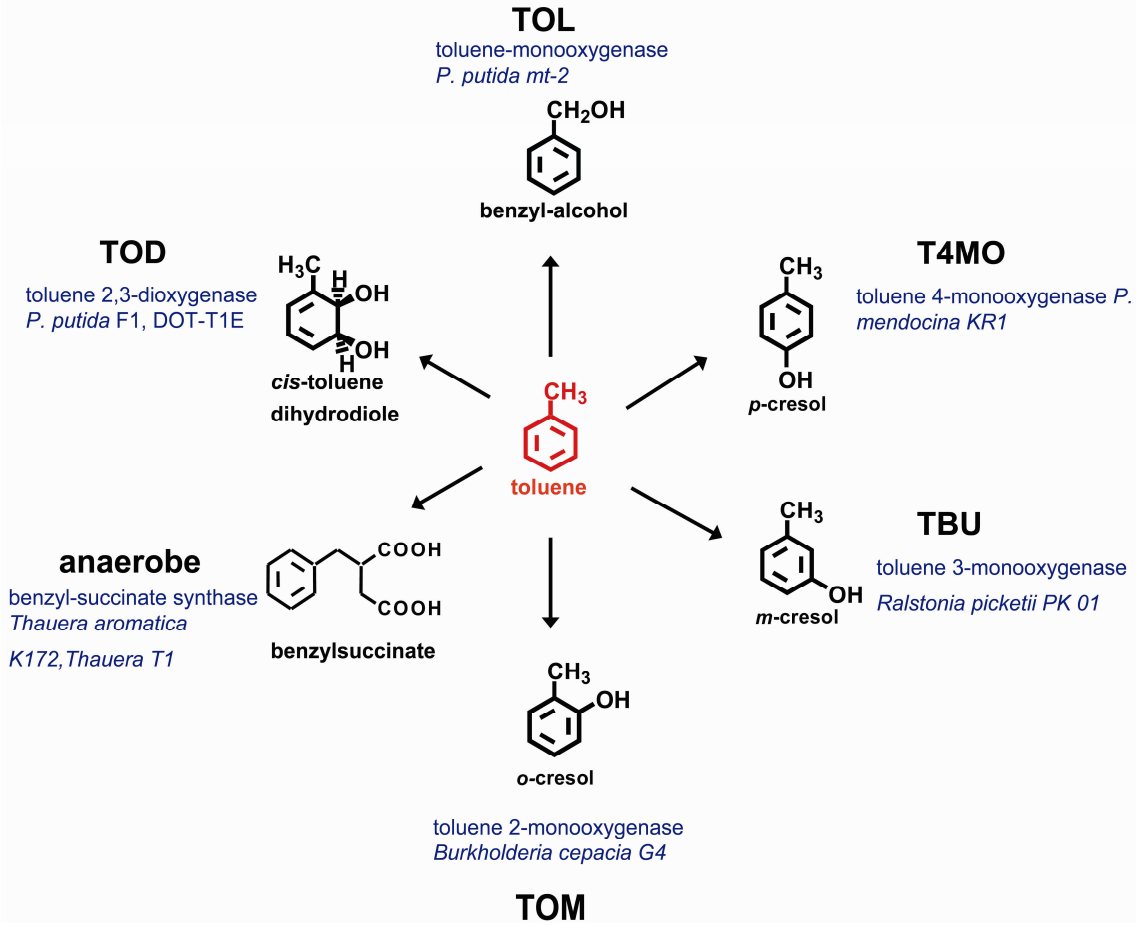

Figure 2. Overview of aerobic and anaerobic pathways of toluene degradation. Indicated are the structures of the initial pathway intermediate, the name of the initial enzyme and the bacterial strain in which these routes are present.

3.1. The anaerobic pathway

The first bacterium demonstrated of being capable of anaerobic degradation of aromatic compounds was described in 1987 by Tschech and Fuchs. The bacteria tentatively identified as denitrifying *Pseudomonads* were later renamed as *Thauera aromatica K172,* since sequencing of 16S-rDNA revealed a close relationship to the isolates of *Thauera selenatis* (Rabus and Widdel, 1995, Anders *et al*., 1995). It was demonstrated that the initial attack on the toluene ring was the addition of fumarate to the methyl-group by the benzyl-succinate synthase (Fig. 2 and Fig. 3, p. 15), encoded by *bssABC* to yield (R)-benzyl-succinate (Biegert and Fuchs, 1995, Biegert *et al*., 1996). (R)-benzylsuccinate is then metabolized via a series of reactions to benzoyl-CoA, the central intermediate in anaerobic metabolism of aromatic compounds (Boll *et al.,* 2002,

Gibson and Harwood, 2002, Leutwein and Heider, 1999, Egland *et al.*, 1997, Spormann and Widdel, 2000). The genes responsible for the β-oxidation-like transformation of benzylsuccinate to benzoyl-CoA, were located on the operon *bbsABCDEFGHI* (Leutwein and Heider, 2001). The capability to transform benzylsuccinate to benzoly-CoA is defined as *bbs* activity. The benzoyl-CoA reductase is encoded by the four genes *bcrCBAD*. All genes for the so-called benzoyl-CoA pathway were found to be clustered on the *T. aromatica* K172 chromosome. In a homologous strain *Thauera aromatica* T1 genes encoding a TCS were identified upstream of the benzyl-succinate synthase genes which were termed *tutC1B1* (Coshigano 2000, Coshigano and Young, 1997; Coshigano *et al*., 1998, Leuthner and Heider, 1998; Achong *et al*., 2001). TutC1 corresponds to a shortened version of TodS (~108 kDa) since it has only one histidine kinase domain and resembles TdiS (~63 kDa) encoded in the operon *tdiSR* of *Azoarcus* sp. strain T and *Thauera aromatica* K172, which are also able of degrading toluene through the anaerobic pathway. Leuthner and Heider (1998) suggested that the TdiS-type protein could be specific for anaerobic toluene metabolism, whereas TodS would be involved in aerobic metabolism only. In *T. aromatica* T1, another TCS called TutCB was described and the sensor TutC was found to share 50 % sequence identity with TodS of *P. putida* F1 and DOT-T1E. However, it has not been established whether TutC1B1 controls the anaerobic pathway and TutCB the aerobic toluene catabolism. Shinoda *et al*. (2004) reported that *Thauera* sp. strain DNT-1 has the ability to grow on toluene as the sole carbon source both under aerobic and anaerobic conditions. The authors found homologous genes to the toluene 2,3-dioxygenase (*tod*) and benzylsuccinate synthase (*bss*). The *tod* genes were expressed only under aerobic conditions and the *bss* genes under both, aerobic and anaerobic conditions. How this strain modulates the expression of two different initial pathways of toluene degradation according to oxygen availability is an intriguing but yet open question.

In addition, other denitrifying bacteria such as *Azoarcus* sp. EbN1 are able to utilize toluene as carbon source (Rabus and Widdel, 1995, Rabus *et al.*, 2005, Kube *et al.*, 2004), as well as iron- and sulphate-reducing bacteria such as *Geobacter metalireducens* (Kane *et al.*, 2002) and *Desulfobacula toluolica* (Beller and Spormann, 1997, Rabus and Heider, 1998). All of them show BBS

activity induced by toluene and benzylsuccinate in strains K172 and T1 (Hermuth *et al*., 2002), and the initial step in toluene degradation.

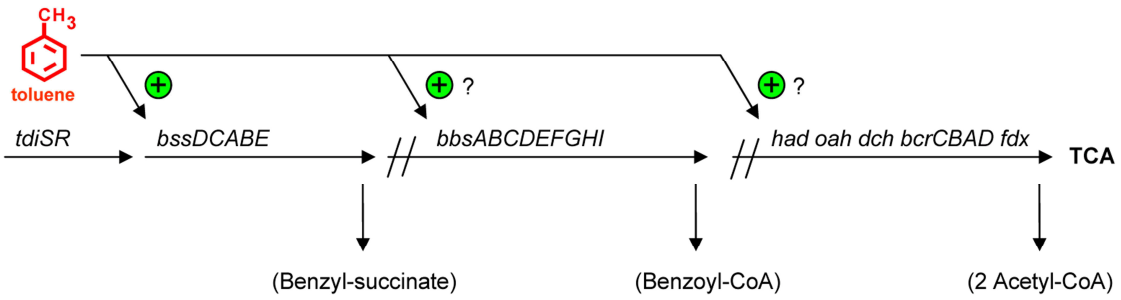

Figure 3. Operon architecture and regulation of the anaerobic toluene pathway. Intermediate products are shown in brackets. Figure adapted from Parales *et al.* (2008)

3.2. The TBU pathway

The TBU pathway was first reported in *Ralstonia picketii* PKO1 (Kukor and Olsen, 1991). The genes coding for the benzene, ethylbenzene, toluene, phenol and *m*-cresol degradation are located on the chromosome (Kukor and Olsen, 1990). The initial attack on the toluene ring leads to *meta*-cresol (Fig. 2, p 13, and Fig. 4, p. 16), indicating the use of a toluene 3-monooxygenase. In contrast, more recent evidence suggested that a toluene 3-monoxygenase oxidized preferentially the toluene ring in the *para* position, which is in conflict with the data indicating that *meta*-cresol and not *para*-cresol is a growth substrate and only *m*-cresol an effector which activates transcription of the 3-monooxygenase in *Ralstonia picketii* PKO1 (Fishman *et al*., 2004). Apart form this controversy, there is no conflict between the *para*-oxidizing T4MO pathway in *P. mendocina* KR1 and the pathway described here, as *P. mendocina* KR1 uses further the β-ketoadipate (*ortho*-) and *R. picketii* PKO1 the *meta*- ring cleavage pathway.

The genes that encode the enzymes for the degradation pathway are encoded in three operons: *tbuA1UBVA2C* encoding the six structural genes of the toluene 3-monooxygense, *tbuD* encoding phenol/cresol hydroxylase and further downstream the *tbuWEFGKIHJ* operon encoding enzymes of the *meta*-cleavage pathway for the conversion of catechol and methylcatechols to TCA cycle intermediates (Fig. 4, p. 16). TbuT is the transcriptional activator of all three operons in the presence of toluene, benzene, ethylbenzene and also by

the chlorinated aliphatic hydrocarbon trichloroethylene (Kukor and Olsen, 1990). In addition, TbuX was proposed to be a facilitator of toluene entry into the cell (Kahng *et al*., 2000).

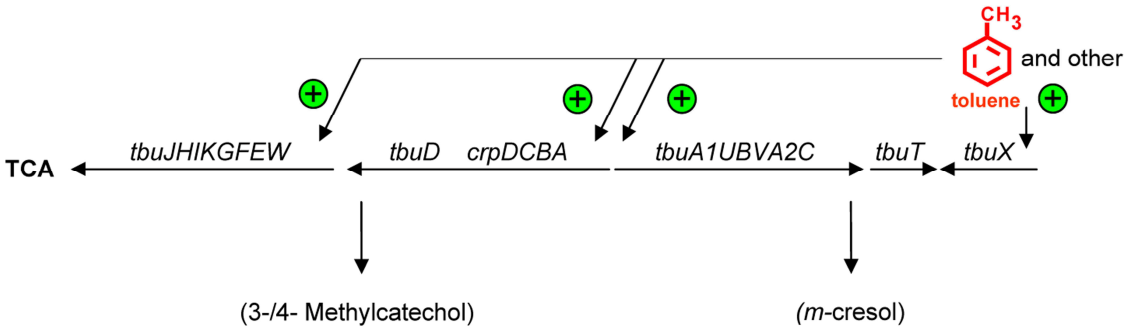

Figure 4. Operon architecture and regulatory mechanism of the *tbu* pathway for toluene degradation. Intermediate products are shown in brackets.

Although TbuT is similar to other XylR/NtrC family members like XylR and DmpR, it is significantly different in the effector binding domain (domain A), which can also be activated by aliphatic hydrocarbons and not only aromatics. The aminoacid sequence of TbuT shows around 45% identiy with XylR, much less then among other family members (85-99.5%). The expression of the *tbuT* gene and the *tbuA1UVBA2C* operon is linked by read-through transcription from the toluene 3-monooxygenase promoter P*tbuA1*. Therefore, the expression levels of *tbuT* and the monooxygenase genes are linked to each other in a positive feedback mode: silent in the absence of toluene and high when an effector molecule is present. Upstream the P*tbuA1* promotor (-24/-12, σ^{54}-dependent) a region homologous to the XylR-binding site at the Pu promoter was found to be essential for the transcription, since a mutant with a deletion of that region showed no transcriptional activity (Byrne and Olsen, 1996). Genes encoding additional regulators are located upstream the P*tbuA1* promotor, located between both catabolic operons of the TBU pathway. TbuS protein probably interacts with the effectors phenol or *m*-cresol and acts as a transcriptional activator of the *meta*-pathway operon, repressed in the absence of effectors by TbuS (Olsen *et al.*, 1994). Additionally, there is evidence that effectors interact with TbuR, which gives rise to a complex which probably

activates transcription of the phenol/cresol hydroxylase encoded by *tbuD* (Olsen *et al*., 1994).

3.3. The TOM pathway

This pathway was initially described in *Pseudomonas cepacia* (Shields *et al*., 1991). This strain has been renamed twice, first to *Burkholderia cepacia G4* and then to *Burkholderia vientanamiensis* G4. The strain was first isolated by Nelson *et al.* in 1987. The initial step of the TOM monooxygenase pathway involves a unique toluene-*ortho*-monooxygenase reaction. (Fig. 2, p. 13, Shields *et al*., 1989, 1991). All of the toluene degradation genes in *B. vietnamiensis* G4 are located on a large, self-transmissible plasmid designated TOM (pBriE04 on the genome data bank, Shields and Francesconi, 1996; Shields *et al*., 1995). The *tomA1A2A3A4A5* operon encodes the toluene 2-monoxygenase which catalyses the synthesis of 3-methylcatechol. The following steps follow probably the *meta*-cleavage pathway as the complete genome sequence and experimental evidence revealed the presence of *meta*-cleavage genes downstream the genes encoding the toluene 2-monooxygenase (Shield *et al*., 1989, 1991). Furthermore, a gene encoding a problable regulator was identified further downstream and shares 53% identity with TbuT of *R. picketii* PKO1 and 48 % with PcuR of *P. mendocina* KR1 (see below, O´Neill *et al*., 1998).

The TOM pathway was shown to be constitutively expressed, although the putative regulator TmbR was found on the plasmid coding the TOM operon. Additionally, the degradative plasmid of around 108 kb carrying the TOM pathway genes harbours also a catechol 2,3-dioxygenase, what enables the strain to oxidize trichloroethylene, toluene, *m*-cresol, *o*-cresol, phenol, and methyl-catechol. Up to date, no clear notion exists on the regulatory elements involved (Shields *et al.*, 1995).

3.4. The T4MO pathway

P. mendocina KR1 was isolated from the Colorado River in Texas from an algal-bacterial mat, in first instance was found of being able to degrade toluene

and chlorocatechol (Klecka and Gibson, 1981). The initial attack on the toluene ring is an oxidation in the *para* position to form *p*-cresol (Fig. 2, p.13, and Fig. 5), the enzyme catalyzing this reaction, toluene 4-monooxygenase, is encoded in the *tmoXABCDEF* operon (Whited and Gibson, 1991a, Pikus *et al*., 1996). Subsequent oxidation of the methyl-group to form 4-hydroxybenzoate is brough about by the enzymes encoded in the *pcuBAXC* operon. (Whited and Gibson, 1991b). Protocatechuate is the result of this second ring hydroxylation by the PobA1 enzyme and serves as the substrate for an *ortho* ring cleavage (Whited and Gibson, 1991b). This is the only pathway which does not make use of a *meta*-cleavege in the metabolization of toluene. The operons *pcuBAXC* and *pobA1* are not clustered with the *tmo* genes and are independently regulated by PcuR and PobR1, respectively, (Ben-Bassat *et al*., 2003, Ramos-González *et al*., 2002) which act as positive regulators in the presence of the intermediates which are substrates or products of the enzymes encoded in the operon they regulate. The T4MO pathway itself is regulated by the TmoS/TmoT TCS which is homologous to the TodS/TodT TCS in *P. putida* F1 and DOT-T1E.There are further similarities with the TOD pathway regulatory system, as the sequence of the P_{tmoX} promoter and *tmoX* gene show analogy to the P_{todX} promotor and *todX* gene (Ramos-González *et al*., 2002).

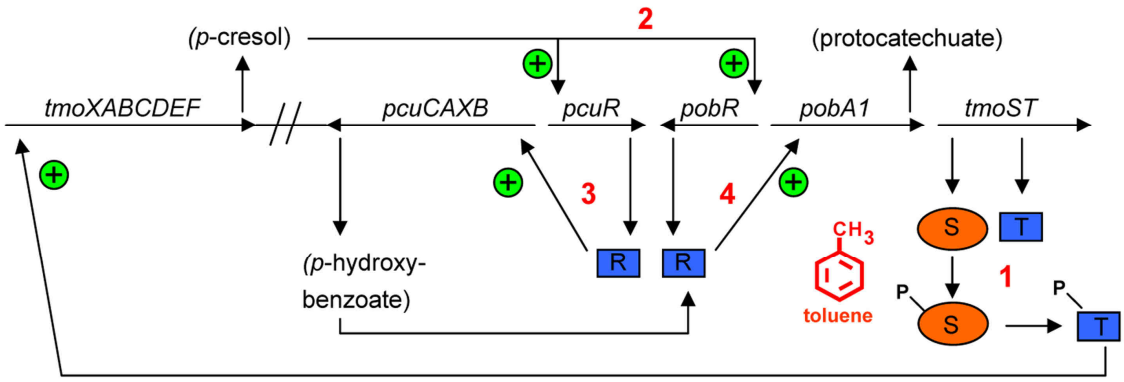

Figure 5. Operon architecture of the T4MO pathway of toluene degradation. The order of degradation steps and regulatory mechanism are marked in red numbers (1-5). Intermediate products are shown in brackets.

The product of the toluene hydroxylation by the toluene 4-monooxygenase, *p*-cresol (Yen *et al*., 1991), is the substrate of the enzymes of the second catabolic operon under control of the transcriptional regulator PcuR, which is transcribed divergently (Fig. 5). PcuR is a member of the XylR/NtrC subfamily of

transcriptional regulators and has homology with TbuT from *R. pickettii* PKO1 acting as an activator of the *pcuCAXB* operon transcription in the presence of effector *p*-cresol, *p*-hydroxybenzylalcohol or *p*-hydroxybenzylaldehyde, which are produced by the enzymes encoded in the *pcu* operon (Ben-Bassat *et al*., 2003). The third operon encodes a *p*-hydroxybenzoate hydroxylase PobA1 and is regulated by PobR1 (Ramos-González *et al*., 2002), a member of the AraC/XylS subfamily. But the regulatory mechanisms underlying the control of these three operons are scarcely known.

3.5. Strains with no specific toluene degradation pathway

Burkholderia sp. JS150 was obtained from Tyndall Air Force Base at Panama City, USA (Spain and Nishino, 1987). Haigler *et al.* (1992) described in this strain three different dioxygenases, various monooxygenases and four ring cleavage pathways, including the *ortho* and *meta* pathways described in the other toluene degrading organisms. Genes coding for a toluene/benzene 2-monooxygenase (*tmbABCDEF*), with high sequence homology to the toluene 2-monooxygenase in *B. vietnamiensis* G4, as well as a regulatory gene (*tbmR*) were identified. Interestingly, the genes coding for a toluene 4-monooxygenase were localized in the region between the operons *tmbABCDEF* and *tbmR*. Furthermore, at least two monooxygenases were needed to produce *p*-cresol, as the toluene 4-monooxygenase on its own was unable to generate this compound (Johnson and Olsen, 1997). Two other monoxygenases encoded by the operons *tbc1ABCDEF* and *tbc2ABCDEF* as well as a dioxygenase pathway were shown to be induced simultaneously in the presence of toluene (Haigler *et al.*, 1992). All metabolic clusters involved in the toluene metabolism were shown to be located on a large catabolic plasmid (Kahng *et al.*, 2000).

The regulator TbmR of *Burkholderia* sp. strain JS150 forms part of the XylR/NtrC subclass, too. The TmbR protein of strain JS150 is borne on the same plasmid as the gene clusters for a toluene 2-monooxygenase and a toluene 4-monooxygenase and was shown to regulate the expression of both monooxygenases. Both monooxygenases were active and their expression is induced by TbmR in the presence of the same effectors (toluene, benzene, and chlorobenzene). The toluene 4-monooxygenase was even induced in the

presence of phenolic compounds, despite the fact that these were not substrates for the enzyme. This incongruity between effector and substrate profiles may indicate that TmbR was originally the regulator for the toluene 2-monooxygenase pathway and was then recruited to control the expression of the 4-monooxygenase. Also, hydroxylated intermediate products induced monooxygenase activity, generating a positive feedback loop to maximize 2-/4-monooxygenase expression (Johnson and Olsen, 1997).

P. stutzeri OX1, isolated from a wastewater treatment plant (Baggi *et al.*, 1987), is another organism without a defined catabolic pathway for toluene degradation. It has a toluene-*o*-xylene monooxygenase, encoded by the operon *touFEDCBA*, highly similar to the toluene 4-monooxygenase of *P. mendocina* KR1, which is able to catalyze the transformation of toluene to all three cresol isomers, which are further converted to 3-methylcatechol or 4-methylcatechol through the enzymes encoded in the *touKLMNOP* operon. These compounds enter then the *meta*-cleavage pathway leading to the production of TCA – cycle intermediates. The master regulator is TouR.

TouR is another NtrC-like activator, which controls the activity of the σ^{54}-dependent P_{tou} promoter of the catabolic operon encoding the toluene-*o*-xylene monooxygenase. In this regulatory system a new phenomenon called gratuitous activation was observed (Solera *et al*., 2004), a feature that has not been described previously for the σ^{54}-dependent regulatory circuits. The P_{tou} promoter transcription can be activated in a growth-phase dependent fashion in the absence of TouR effectors. Gratuitous activation occurred at the onset of the stationary phase which is characterised by a depletion of carbon sources. This indicates that the phenomenon of gratuitous induction is also subject to global regulation, in this case triggered by carbon starvation. A model for the *tou* regulatory circuit was established by the same authors: TouR activates transcription in the presence of phenolic effector compounds or in the absence of effector upon carbon limiting conditions. This gratuitous activation ensures a basal activity sufficient to convert non-effector compounds into phenolic intermediates that, in a positive feedback manner would increase transcription regulated by TouR.

3.6. The TOL pathway

The TOL pathway was first described in *P. putida mt-2,* isolated from soil in Japan (Nakazawa, 2002, Nozaki *et al*., 1963) and its genes are located on a large transmissible plasmid termed TOL or pWWO plasmid (Williams and Murray, 1974, Wong and Dunn, 1974; Williams *et al.*, 2004). It is probably the most extensively studied aromatic degradation pathway so far, and it shows a complex and diverse regulatory network. The initial step in toluene degradation is carried out by a toluene monooxygenase hydroxylating the methyl-side chain (Fig. 2, p. 13, and Fig. 6), which is encoded by the TOL plasmid *upper* operon *xylUWCAMBN*. Toluene is finally converted to benzoate through additional steps carried out by the benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase (Bühler *et al.*, 2002).

The TOL plasmid lower or *meta* pathway, encoded by the operon *xylXYZLTEGFJQKIH,* convert benzoate to catechol and further to TCA cycle intermediates acetyl-CoA and pyruvate. The *xylS* and *xylR* regulatory genes downstream of the *meta* operon and these two genes are divergently transcribed. Their products regulate the *meta* and *upper* pathways, respectively.

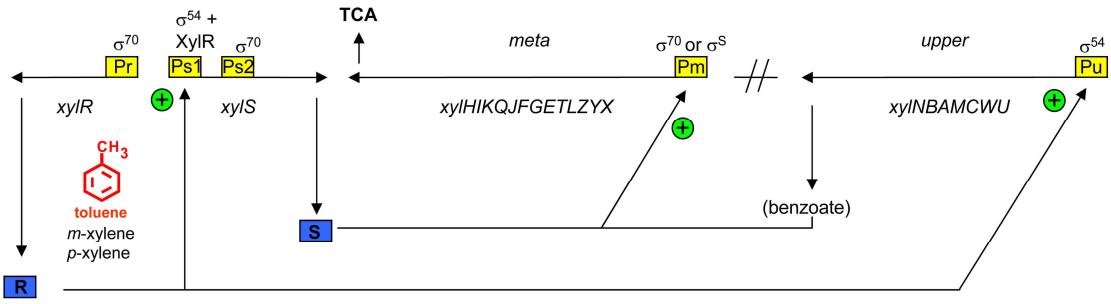

Figure 6. Operon architecture of the TOL *upper* and *meta* pathway and its regulatory elements. Intermediate products are shown in brackets.

The well-established regulation of both, the *upper* and *meta* pathways is described as follows:

In the presence of a substrate molecule such as toluene or derivatives thereof (Abril *et al*., 1989; Delgado and Ramos, 1994), both catabolic operons are co-ordinately expressed to ensure a complete degradation to TCA intermediates. The XylR protein, name-giving of the XylR/NtrC- family of enhancer-binding proteins (EBP) (Inouye *et al*., 1988; Kustu *et al*., 1989; North *et al*., 1993, review

on transcriptional regulators in aromatic degradation pathways: Tropel and van der Meer, 2004) controls the expression from the σ^{54}-dependent promoters Pu and Ps1. Pu regulates transcription of the *upper* pathway and transcription from Ps1 increases the synthesis of XylS protein driving the transcription of the *meta* pathway genes.

XylR has the four characteristic domains of the NtrC family: the N-terminal effector binding A-domain linked to the catalytic C-domain via the central Q-linker domain and the C-terminal DNA-binding D-domain containing a HTH motif. The A-domain was shown to interact with the effectors as mutants in this domains altered effector specifity (Abril *et al.*, 1989; Delgado and Ramos, 1994; Delgado *et al*., 1995; Garmendia *et al*., 2001). This A-domain probably acts as an intramolecular repressor in signal transmission through the Q-linker to the central catalytic domain involved in ATP-binding and hydrolysis (Devos *et al.,* 2002; Garmendia and de Lorenzo, 2000, Pérez-Martín and de Lorenzo, 1995; Shingler and Pavel, 1995; Wikstrom *et al.*, 2001) According to the model proposed by Pérez-Martín and de Lorenzo to explain the activation of Pu by XylR, upon ATP-binding to the C-domain the regulator multimerizes bound to its upstream activator sequence (UAS), followed by ATP hydrolysis, triggering the σ^{54}-dependent transcription of Pu, allowing XylR to detach and dissociate again probably after every round of transcription. Results indicating that the XylR protein is not permanently linked *in vivo* to its UAS, mostly unoccupied at all growth stages (Valls and de Lorenzo, 2003) support this idea.

Pu is a σ^{54} –dependent class of promoter and as such shows the typical organization: a -12/-24 recognition sequence of σ^{54}–RNA-Polymerase and a XylR UAS between positons -120 and -175. The Integration host factor (IHF) binds between positions -52 and -79 and was shown to have a dual role: the DNA-bending activity to bring the XylR in contact with the RNA-Polymerase and the recruitment of the latter one to its binding site. This activity was associated to another upstream element, reminiscent of the so called α-carboxy terminal domain (CTD)-binding UP elements of σ^{70}-dependent promoters. The recruitment was determined by the correct positioning of the UP-like element with respect to -12/-24 binding site (Bertoni *et al.*, 1998).

XylR is the main regulatory element of the upper pathway, but the *meta* pathway requires two regulatory elements: the Pm promoter/RNA-Polymerase pair and the XylS/effector molecule pair. As stated above, when XylR senses an effector molecule, the transcription of *xylS* raises as XylR controls the Ps1 promotor. This involves that XylS is expressed at basal levels by the activity of the constitutive σ^{70}–dependent promoter Ps2 when cells are growing in the absence of substrate molecules of the TOL pathway. But only in the presence of intermediates of the upper pathway like benzoate or 3-methylbenzoate XylS becomes active and promotes transcription from the *meta* pathway promoter Pm. XylS belongs to the XylS/AraC family of transcriptional regulators involved in carbon metabolism, pathogenesis or response to alkylating agents. These proteins show typically only two domains: a less-conserved effector binding- and dimerization domain at the N-terminal and a highly conserved DNA-binding domain, normally located at the C-terminal. Mutational analyses of effector binding domains allowed to identify key residues involved in effector recognition (Ramos *et al.*, 1997, Ruíz and Ramos, 2002), in contacting the α-CTD of the RNA-polymerase, connecting the N- and C-terminal domains and in protein dimerization (Ruíz *et al.*, 2003). Furthermore, mutations in the C-terminal domain containing two HTH motifs showed that highly conserved aminoacids have a structural role, rather than in direct interactions with the DNA. A recent study (Dominguez-Cuevas *et al*., 2008a) with an extensive series of epistasis assays combining mutant Pm promoters and single substitution mutants revealed a bipartite binding of a XylS monomer to the promoter sequence. Pm exhibits a bipartite DNA-binding motif consisting of two boxes, called A and B. They identified the aminoacids which established contacts to the two boxes A and B of the promotor. The bipartite motif is repeated at the Pm promoter so that one of the XylS monomers binds to each of the repeats. This bipartite motif is repeated and each of the monomers binds to each of the repeats. This study further identifies aminoacids involved in contacting the phosphate backbone of the DNA and suggests therefore that not only the direct interaction aminoacid-nucleotide but also the secondary structure of the DNA is of relevace in Pm regulation. The authors propose a model of XylS dimer head-to-tail conformation binding the boxes A and B in order to allow the N-terminal subunit to directly interact with the RNA-Polymerase. The direct repeats are located

between -70/-56 and -49/-35, as characteristic in this family of one-component regulators, the proximal repeat overlapping with the -35 region recognized by the RNA-Polymerase (González-Pérez *et al*., 1999). This overlap may facilitate interactions between the RNA-Polymerase and XylS. As in the case of XylR, XylS exerts a second role: the RNA-Polymerase recruitment to the Pm promoter, as the rate of isomerization of RNA-Polymerase from closed to open complexes increased in the presence of effectors. XylS also undergoes dimerization upon effector binding (Ruíz *et al*, 2003) via its N-terminal domain and the effector 3-methylbenzoate induces an intramolecular derepression by conformational changes altering direct interactions between the N- and C-terminal domains and favouring DNA binding (Domínguez-Cuevas *et al*., 2008b).

There is also evidence that the global bacterial metabolism participates in the regulation of this pathway. Since the first description of the pathway it has always been observed, that the expression of the TOL pathway is regulated by the available carbon-sources (Duetz *et al.*, 1994, 1996; Holtel *et al.*, 1994; Hugouvieux-Cotte-Pattat *et al.*, 1990; Marqués et al, 1994; Worsey and Williams, 1975). It was observed, that the XylR dependent Pu and Ps1 promotors were silent during the exponential growth phase in rich medium and were only active at the end of the exponential growth phase, a behaviour described as exponential silencing (Cases *et al.*, 1996). In minimal media on the contrary, the expression from Pu and Ps1 was immediate. When carbon was the rate-limiting factor, both promoters where induced at maximum levels (Duetz *et al.*, 1996) and under excess of carbon the system was repressed. A preferential and sequencial use of different carbon sources tested in minimal medium was observed, a phenomenon described in *E. coli* as carbon catabolite repression, where CPR exerts its role as global regulator. In *Pseudomas putida* mt-2 several other hypothesis were established concerning the nature of carbon catabolite repression such as by the action of the alarmone ppGpp (Carmona *et al.*, 2000). However, determinants were identify involved in catabolic repression of the pWW0 plasmid through a series of mutants of various “global players” described in *Pseudomonas* (Cases *et al.*, 1999; Aranda-Olmedo *et al.*, 2005). They showed that *crc* (catabilte repression control) was highly expressed under minimal medium growth conditions and TOL pathway expression inhibited. In a

crc^{-} background the repression in minimal media was partially relieved. PtsN, the IIA^{Ntr} phosphotransferase present in the *rpoN* gene cluster, is involved in the glucose-dependent repression of Pu transcription. A p*tsN*$^{-}$ mutant was also shown to de-repress transcription, but none of both mutants could totally relieve this repression (Cases and de Lorenzo, 1999; Aranda-Olmedo *et al.*, 2005). The mechanism by which PtsN exerts its inhibition on *Pu* in the presence of glucose is not yet known, but it involves other proteins of the PTS family such as PtsO (Powell *et al*., 1995; Cases *et al.*, 2001). The molecular mechanism underlying each of these processes remains to be elucidated.

3.7. The TOD pathway

The TOD (toluene dioxygenase) pathway was first described in *Pseudomonas putida* F1 and all the structural and regulatory genes identified by Gibson *et al.* (1968, 1970). This pathway employs in the first step the toluene 2,3-dioxygenase (Fig. 8, p. 26; Zylstra and Gibson, 1989; Zylstra *et al*., 1988) The activity of the dioxygenase renders toluene *cis*-dihydrodiol (Fig. 2, p. 13, and Fig. 7) that is converted into 3-methylcatechol by the *cis*-dihydrodiol dehydrogenase, encoded by *todD.* The resulting intermediate is then further oxidized through the activity of the catechol 2,3-dioxygenase (*todE*), the 2-hydroxy-6-oxohepta-2,4-dienoate hydrolase (*todF*), the 2-hydroxypent-2,4-dienoate hydratase (*todG*) and the 4-hydroxy-2-oxovalerate aldolase (*todH*) to yield acetaldehyde and pyruvate to acetaldehydegenes. *todI* encodes a acetaldehyde dehydrogenase converting acetaldehyde to acetyl-CoA (Fig. 8, p. 26).

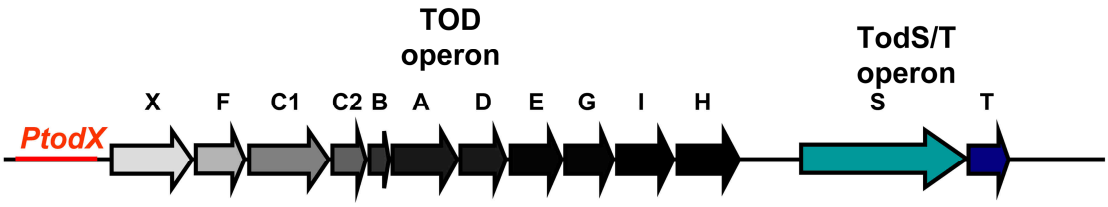

Figure 7. Architecture of the operons *todXFC1C2BADEGIH* and *todST*. *tod*: the toluene 2,3-dioxygenase comprises genes *todC1C2BA*, the *meta* pathway genes are *todFEGIH* and *todX* is of unknown fuction (putative facilitator protein for toluene entry). *todST*: *todS* encodes the sensor kinase and *todT* the cognate response regulator.

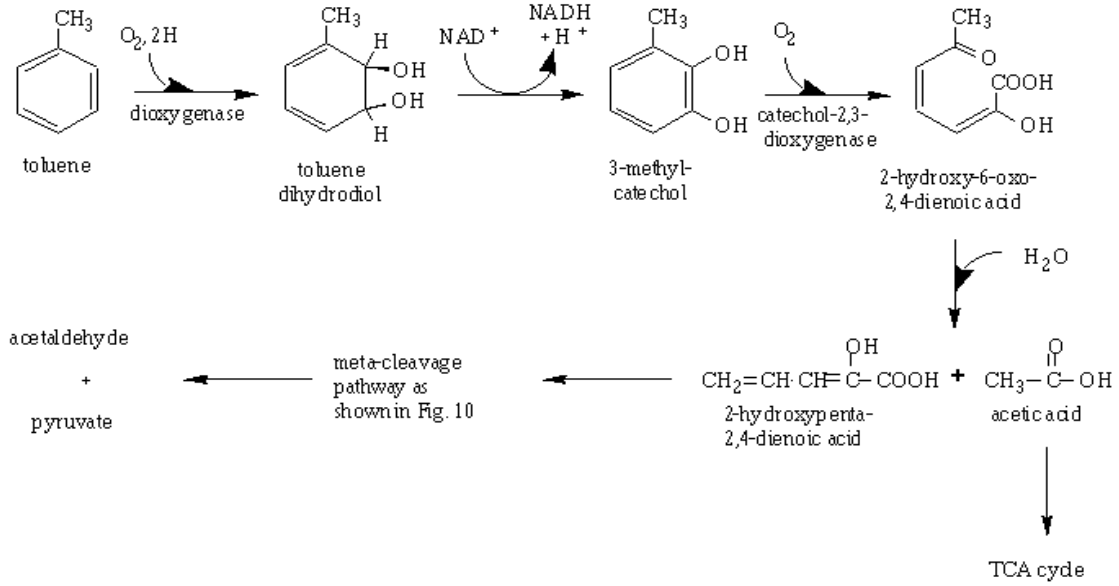

Figure 8. Toluene 2,3-dioxygenase pathway intermediates. For operon architecture, see Fig. 7., p. 25.

All enzymes of the toluene 2,3-dioxygenase pathway are located on one single 10.3 kb operon *todXFC1C2BADEGIH* (Fig. 7, p. 25) Lau *et al*., 1994, 1997, Menn *et al*., 1991, Mosqueda *et al*., 1999, Wang *et al*., 1995, Zylstra and Gibson, 1989, 1991, Zylstra *et al*., 1988). No plasmids have been detected in *P. putida* F1, and the genes encoding the toluene degradation pathway are located on the chromosome of the recently completed sequence of *P. putida* F1 (US DOE Joint Genome Institute, completed 2007/06/04, genbank: CP000712). Like strain F1, up to 18 genomes of the genus Pseudomonas have already been sequenced, indicative of the rising interest in their metabolic versatility. The whole TOD operon was initially described in its genetic organisation by Zylstra *et al.* (1988) and later completed by Lau *et al.* (1994). The TOD pathway was shown to be induced in the presence of toluene and other aromatic compounds as initially described by Finette and Gibson (1988) and Wang *et al.* (1995). Mosqueda *et al.* (1999) described the *tod* pathway in *P. putida* DOT-T1E.

3.7.1. The TodS/TodT Two-Component System

The expression of the *tod* genes is regulated by a two-component system (TCS) (Lau *et al.*, 1997, Mosqueda *et al*., 1999), encoded by the *todST* operon, which

is located downstream with respect to the catabolic genes. This operon was found both in *P. putida* F1, originally isolated from a polluted creek in Urbana, USA (Gibson *et al.*, 1968) and *P. putida* DOT-T1E, isolated in a wastewater treatment plant of Granada, Spain (Ramos *et al.*, 1995). Selective interruption of *todS* and *todT* showed the essential role of both genes in *tod* activation, as there was no transcription of any *tod* genes in the absence of either *todS* or *todT*. (Lau *et al*., 1997, Mosqueda *et al.*, 1999)

The TodS/TodT TCS was first described in *Pseudomonas putida* F1 as the activator of the TOD pathway (Lau *et al*., 1997). The *todST* genes downstream of the *todX* operon (Fig. 7, p. 25) are transcribed in the same direction. The 2934 bp ORF *todS* encodes a 978-residue protein with a molecular mass of 108.01 kDa and the 618 pb ORF *todT*, codes for a 206-residue protein with a molecular mass of 23.03 kDa. The start codon was suggested to overlap with the stop codon of *todS* in the configuration ATGA (Lau *et al.*, 1997). Lau *et al.* predicted the domain architecture depicted in Fig. 2 (p. 13) and suggested based on analogy with known systems, that H190 and H760 are autophosphorylation sites of the two HK domains. Conserved aa D458, D500, S530 and L552 defined the internal response regulator receiver (RRR) domain. At the N-terminus of TodS they postulated a bZIP motif with a leucine zipper extending from aa 43 to 71. BZIP motifs are common among transcriptional factors of eukaryotes but rarely described in prokaryotes (Hurst, 1994; Kerppola and Curran, 1991) and function as DNA-binding and dimerization domains. They typically consist of a region with several basic residues and an adjacent heptad repeat of leucines involved in dimerization. The sequence in between the putative RRR domain and the C-terminal HK domain was assigned as a putative oxygen-sensing region (Sos) sharing seven invariant aa with the heme-binding domain of the *R. meliloti* FixL protein.

Lau *et al.* (1997) synthesised a 45-residue peptide (TodSbs) including aa 1-42 of TodS to test binding to the intergenic region of *todHS* (Fig. 7, p. 25), where they localized a pseudopalindromic sequence identical to the recognition sequence of the bZip motif containing transcription factors Fos and Jun (5'-TGACTCA). At the C-terminal end of the synthetic peptide the sequencer Gly-Gly-Cys was added with the idea that the oxidation of two neighbouring cysteine residues favours a dimeric state of the peptide. The authors reported specific

DNA-binding activity of the synthetic TodSbs peptide to the *todHS* intergenic region with a loss of DNA-binding when the disulfide bonds where reduced; supporting the idea that dimerization of the peptide is necessary for DNA-peptide interaction. No experimental data on full-length TodS were reported.

The RR TodT was expressed as heterologous protein and was shown to bind specifically to the *todX* promoter region of the *tod* pathway genes (Lau *et al.*, 1997). The authors localized a TodT-binding site centered at -105 to -106 from the *todX* transcriptional start point and speculated that TodT binds as a dimer due to the dyad symmetry of the binding region.

In *Pseudomonas putida* DOT-T1E, the homologous TodS/TodT TCS was described in 1999 by Mosqueda *et al*. DNA sequence analysis revealed an almost identical operon arquitecture as described in *P. putida* F1 (Lau *et al.*, 1997) for both the *tod* catabolic operon genes and the *todST* operon. An insertional inactivation mutant in the *todC1C2* genes coding the α and β subunits of the toluene dioxygenase confirmed the essential role of the *tod* genes in toluene metabolism (Mosqueda *et al.*, 1999). Enzymatic studies indicated that the *tod* genes were only expressed in the presence of the aromatic compounds tested such as toluene, styrene, ethylbenzene, *m*-xylene and benzene. The transcriptional initiation point from the *todX* gene could be localized, regardless of the inducer used, 60 bp upstream of the A of the first ATG. The -10 and -35 regions of the *todX* promoter showed no significant homology to other promoters and the RNA Polymerase/sigma factor involved remained elusive. On the other hand the expression of the *todST* genes was constitutive at low levels regardless of the growth conditions, and the transcription initiation point was located 25 bp from the A of the first ATG.

Lee and co-workers isolated *P. putida* F1 strains adapted to assimilate new substrates such as n-propylbenzene and n-butylbenzene (Choi *et al.*, 2003). They conducted sequence analyses of the adapted strain and compared it to the originally described sequences for F1 (Lau *et al.*, 1997). The authors verified whether the genetic adaptation leading to a broadening of the substrate range was within the structural or the regulatory genes. The authors concluded that mutations in TodS were the origin of this adaptation, as there were no differences in sequence within key structural genes of the TOD pathway. The TodS mutations were located in the RRR- and PAS2-domains as well as in their

boundaries (see Fig. 9, p. 33, and Choi *et al.*, 2003). The exact function of those aminoacids in TodS activation requires further investigations. The same group isolated another strain (*P. putida* KL47, Lee *et al.*, 2006) in which only one aminoacid change in the PAS1- domain was deduced to be responsible for the constitutive expression of *todST* genes at high levels, what in their opinion might provide strain KL47 with a means of adaptation of the *tod* catabolic operon to various aromatic compounds.

The TodS/TodT proteins share high sequence identity with TCS shown to be involved in the regulation of toluene and styrene degradation (Table 1): TmoS/TmoT (Ramos-González *et al.*, 2002), TutC/TutB (Coshigano and Young, 1997; Leuthner and Heider, 1998), StyS/StyR (O'Leary *et al.*, 2002; Panke *et al.*,1999; Santos *et al.,* 2000; Velasco *et al.*, 1998). Furthermore, other microoganisms capable of degrading aromatic compounds possess TCS homologous to TodS/TodT. It is therefore possible that these also regulate the expression of the corresponding catabolic genes. The apparent functional link between the TodS/TodT type of TCS with a regulation of organic solvent degradation remains to be established.

strain	Proteins	Sequence identity (%)		regulatory role
		TodS	TodT	
Pseudomonas putida F1	TodS/TodT	99	100	aerobic toluene degradation (TOD)
Pseudomonas mendocina KR1	TmoS/TmoT	84	86	aerobic toluene degradation (TMO)
Thauera aromatica K172	TutC/TutB	50	53	anaerobic toluene degradation
Methylibium petroleiphilum st. PM1	Mpe_A0812/ Mpe_A0811	47	54	presumed to be involved in aromatic degradation
Pseudomonas flourescens ST	StyS/StyR	43	48	styrene degradation
Dechloromonas aromatica RCB	Daro_3813/ Daro_3812	43	48	presumed to be involved in aromatic degradation
Pseudomonas Y2	StyS/StyR	42	48	styrene degradation

Table 1. The family of TodS/TodT-like TCS. Indicated are sequence identities of other systems with respect to TodS/TodT in *P. putida* DOT-T1E as well as its identified or presumed regulatory role. For a sequence alignment of the corresponding sequences refer to p. 82, suppl. material.

3.7.2. The Response Regulator TodT

The domain prediction analysis of TodT using the SMART algorithmn (Schultz *et al.*, 1998) revealed conserved aminoacids typically found in response regulator N-terminal receiver domains forming part of an acidic binding pocket.

D57 was predicted to be the phosphoacceptor. The C-terminal part of TodT was predicted to contain a HTH DNA-binding domain. Both domains are connected by a Q-linker which is likely to play an important role in the the signal transmission from the N- to the C-terminal domain. P_{todX} promoter anatomy revealed that it belongs to the set of -10 extended promoters, to the NarL family of activators (Lacal *et al.*, 2007). A typical feature of this promoter type is the absence of a defined -35 region for σ^{70}- dependent binding, which is often compensated by the presence of additional regulatory elements like IHF in case for P_{todX} promoter.

TodT was shown to bind to two pseudoplindromic sites centered respectively at base pairs -107 (box 1) and -85 (box 2) and to an additional site which corresponds to a half of the above palindromic sequences. This site is centered at -57 (box 3) of P_{todX} in *Pseudomonas putida* DOT-T1E. (Lacal *et al*. 2006, 2007). TodT, in contrast to other family members which dimerize upon phosphorylation and prior DNA-binding (Leoni *et al*., 2005, Rampioni *et al.*, 2008) was shown to be a monomer both in a phosphorylated and a non-phosphorylated form (Lacal *et al.*, 2007). Each of the half-sites of the pseudopalindromes at -113/-98 (TodT box1) and -92/-77 (TodT box 2) as well as the half-palindrome were shown to be the target of a single TodT monomer, although a dimerization upon binding could not be excluded (Lacal *et al.*, 2008). Mutational analysis revealed that TodT binding to all three boxes has an activatory effect on transcription. The most important site was identified as box 1 as there was nearly no transcriptional activity of a mutant promotor with a mutated box 1. No TodT binding to the promoter could be observed *in vitro* when box 1 was mutated, which is consistent with the idea of a sequential and cooperative binding of TodT which is initiated at box-1. The affinity of TodT to individual half-palindromes, entire palindromes and combination of multiple TodT binding sites was determined and the results were consistent with an inter- and intrabox positive cooperativity (Lacal *et al*. 2007, 2008).

The far upstream location of the TodT boxes indicates that activation requires DNA-bending. Integration host factor (IHF) was found to bind between the upstream TodT boxes and the -10 hexamer region centered at -38 region and plays a crucial role in the activation process, as a significant decrease in transcriptional activity was observed in an IHF deficient background.

Additionally, interaction of TodT with the α-CTD subunit of the RNA-Polymerase was shown to stimulate transcriptional activity, consistent with the data obtained for other promoters, such as *mtr*, regulated by TyrR (Yang *et al*., 1997) and the TOL plasmid *Pm* promoter (Ruíz *et al*., 2003).

Mutation of conserved aminoacid D57, presumed to be the phosphoacceptor did not alter significantly the affinity of TodT with respect to the promotor boxes, although no transcriptional activity could be measured with TodTD57, revealing an essential role of D57 in TodT activity, as previously described in *P. putida* F1 (Lau *et al*., 1997). The DNA-binding behaviour of a truncated version of TodT containing the DNA-bindig domain only (C-TodT) and the full-length protein were found to be similar, indicating that the molecular determinants for the positively cooperative binding are located in this domain. However, no transcriptional activity was observed when TodT was replaced by the DNA-binding domain. It was therefore proposed that phosphorylation of TodT causes protein-conformational changes, which may favour interaction with the RNA-Polymerase (Lacal *et al*., 2007). Furthermore, TodT but not C-TodT was shown to bend DNA between boxes 2 and 3 (Lacal *et al*., 2008) indicating a role of the N-terminal domain in inducing this DNA bend.

In short, the authors concluded that i) positive cooperative DNA-binding is independet of the the N-terminal domain. However, this domain is essential for the formation of the DNA-kink, ii) phosphorylation is essential in promoter activity but does not alter the binding affinity and iii) transcription form P_{todX} requires productive interactions between TodT monomers, IHF and RNA-polymerase in a complex for transcription to occur.

3.7.3. The Sensor kinase: double sensor histidine kinase TodS

TodS is a large and complex sensor kinase and TodT the cognate response regulator which regulates the expression from the P_{todX} promotor. The initial description of the TodS/TodT TCS (Lau *et al.*, 1997) contained experimental data on purified TodT, but not on TodS. As a consequence, the data available on TodS at the beginning of this thesis did not exceed much a domain prediction as presented in figure 2.

Sequence analysis of TodS using the SMART algorithm (Schultz *et al.*, 1998) showed 5 defined domains as illustrated in figure 9 (p. 33): two histidine kinase domains (HK), preceeded both by a PAS-type sensor domain and a central response regulator receiver domain (RRR) separating both SHK segments. Lau *et al*. (1997) predicted a bZIP motif at the N-terminus and showed that a peptide comprising this motif bound to DNA. However, domain annotation algorithms predicted that the N-terminal part of TodS forms a PAS domain (aa 40-155). Additionally, sequence analysis of TodS with an algorithm for the detection of bZIP motifs (2ZIP, Bornberg-Bauer *et al*., 1998) gave no positive hit. BZIP motifs were shown to adopt an α-helical structure (Lupas and Gruber, 2005). However, sequence analysis of TodS with secondary structure prediction algorithms indicated the the N-terminus adopts an α/β-structure, typically observed for PAS-domains (Taylor and Zhulin, 1999 and secondary structure prediction of TodS, suppl. material, p. 84/85)

Both HK domains show the characteristic highly conserved sequence blocks of the HK superfamily (see p. 10): **H**, **N**, **G1**, **F**, **G2**. Both HKs domains share 24% sequence identity. According to the classification of Grebe and Stock (1999) the N-terminal HK domain (HK1) belongs to the HK_{1a} subfamily and the C-terminal HK domain (HK2) to the HK_4 subfamily. The fact that both HKs belong to different subfamilies is most probably indicative for a domain-recruitment and not a gene-duplication event. This highly complex nature of the SHK TodS is unusual among the SHK superfamily, as even the so-called hybrid SHKs described so far only show one HK domain and not two as TodS. Thus, two scenarios were possible at the beginning of this thesis: a) the two segments each containing a PAS and a HK domain work independently, receiving different signals and serving potentially different RR or b) all the domains are functionally linked to one specific response, possibly integrating different incoming signals in one common response.

Another interesting feature, which distinguishes TodS from most of the SHK described, is that it does not contain regions of sufficient hydrophobicity to suggest transmembrane regions as it is normally the case in other SHKs (Lau *et al.*, 1997). The large part of SHKs are transmembrane proteins containing typically two, but frequently more transmembrane domains. As a consequence there are only SHKs could be produced as active, full-length protein and most

biochemical studies available on SHKs used recombinant individual domains. Therefore, the generation of active, full-length protein was a primary objective of this thesis.

TodS was predicted to contain three phosphoaccepting residues, which are H190 of HK1, D500 of the RRR domain and H760 of HK2 (Fig. 9, and multi-alignment with TmoS/TutC/Mpe_A0812/StyS(ST)/Daro_3813/StyS(Y2) in suppl. material, p. 82) The well-studied hybrid SHK systems as ArcB (Fig. 2, p. 13) contain also three phosphorylatable residues which were shown to be involved in an internal phosphorelay (Fig. 2, p 13). However, the domain architecture of TodS and hybrid sensor kinases differ significantly: (i) TodS contains two autokinase domains whereas the hybrid kinases one kinase and one HPT domain, (ii) TodS contains two sensor domain whereas the hybrid kinases possess a single domain of this type. Therefore another objective of this thesis is to establish whether TodS also employs a phosphorelay mechanism.

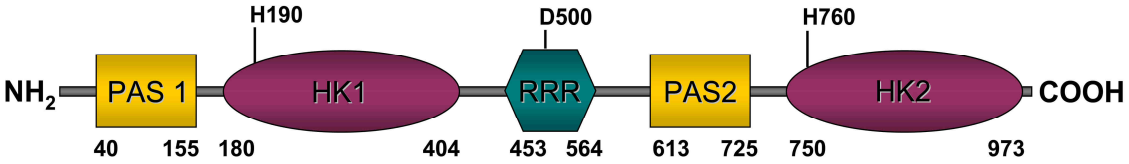

Figure 9. Domain architecture of TodS accoring to SMART domain prediction software. In numbers the aminoacid position

Although the implication of many TCS in global or specific regulatory networks has been established in vivo, the physiological signal molecules which interact with the SHK remain unknown for the majority of the cases. The TOD pathway metabolizes toluene, benzene and ehtylbenzene and we hypothezise that these pathway substrates are amongst the effectors which bind to TodS. Another related question concerns the site of the molecular interaction of effector molecules, since two sensor domains have been predicted (Fig. 9, p. 33).

Apart from these questions on the mechanism of action of TodS other, more general issues related to sensor kinases, await to be addressed. One such issue concerns the fact that TodS has five domains whereas the prototypal kinases possess typically only two domains. What is thus the advantage of having such a complex sensor protein? Another issue concerns the fact that several routes for the metabolization of toluene are regulated by one-

component systems whereas others by two component sytems (see above). The physiological relevance of these differences is unclear at the moment. The study of the molecular mechanism of TodS which I envisage in this thesis might enable us to shed light into these more general issues which are likely to further our understanding of transcriptional regulation in a soil bacterium.

IV. Resultados / Results

Capítulo 1: Actividades basales de la sensor-quinasa TodS

The TodS-TodT two-component regulatory system recognizes a wide range of effectors and works with DNA-bending proteins

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La sensor-quinasa TodS y el regulador de respuesta TodT forman un tipo de sistema de dos componentes que hasta ahora no había sido descrito a nivel bioquímico y molecular. TodS tiene la peculiaridad de estar formada por dos dominios histidín-quinasa completos con sus respectivos dominios PAS y separados por un dominio receptor interno. Este sistema regula la expresión de los genes de la ruta de la tolueno dioxigenasa (TOD). A pesar de que sólo son sustratos de la ruta el tolueno, el benceno y el etilbenceno, el perfil de efectores de TodS es mucho más amplio. Estudios *in vitro* determinaron que TodS tiene una actividad basal de autofosforilación, que en presencia de efectores como el tolueno, que se une con alta afinidad (*Kd*: 684 $\pm$ 13 nM), aumenta de 5 a 7 veces. Este aumento se traduce en aumento de la transfosforilación hacia TodT. Al poseer TodS dos dominios PAS, se generaron dos versiones truncadas, separando los dominios receptores de señal. Ensayos calorimétricos revelaron que el tolueno sólo se une al dominio N-terminal.

TodT, transfosforilado por TodS, se une a dos regiones muy similares del promotor P_{todX} del la ruta TOD en las bases -107 y -85. En la activación de la transcripción juega un papel importante IHF, que interacciona con el ADN entre los sitios de unión de TodT y la región de -10. En ausencia de IHF la actividad de transcripción disminuye 8 veces, lo cual se confirma a través de ensayos de transcripción *in vitro*. Se establece un modelo funcional en el que IHF favorece la interacción entre TodT y la subunidad α de la ARN-polimerasa, permitiendo una eficiente activación de la transcripción.

The TodS–TodT two-component regulatory system recognizes a wide range of effectors and works with DNA-bending proteins

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The TodS and TodT proteins form a previously unrecognized and highly specific two-component regulatory system in which the TodS sensor protein contains two input domains, each of which are coupled to a histidine kinase domain. This system regulates the expression of the genes involved in the degradation of toluene, benzene, and ethylbenzene through the toluene dioxygenase pathway. In contrast to the narrow substrate range of this catabolic pathway, the TodS effector profile is broad. TodS has basal autophosphorylation activity *in vitro*, which is enhanced by the presence of effectors. Toluene binds to TodS with high affinity (K_d = 684 ± 13 nM) and 1:1 stoichiometry. The analysis of the truncated variants of TodS reveals that toluene binds to the N-terminal input domain (K_d = 2.3 ± 0.1 μM) but not to the C-terminal half. TodS transphosphorylates TodT, which binds to two highly similar DNA binding sites at base pairs −107 and −85 of the promoter. Integration host factor (IHF) plays a crucial role in the activation process and binds between the upstream TodT boxes and the −10 hexamer region. In an IHF-deficient background, expression from the *tod* promoter drops 8-fold. *In vitro* transcription assays confirmed the role determined *in vivo* for TodS, TodT, and IHF. A functional model is presented in which IHF favors the contact between the TodT activator, bound further upstream, and the α-subunit of RNA polymerase bound to the downstream promoter element. Once these contacts are established, the *tod* operon is efficiently transcribed.

Pseudomonas | sensor kinase | toluene dioxygenase | transcriptional regulator

Many *Pseudomonas putida* strains are able to use benzene, toluene, and ethylbenzene as the sole carbon and energy source through the toluene dioxygenase (TOD) pathway (1). In this pathway, the aromatic hydrocarbons are oxidized to their corresponding substituted catechols, which are further metabolized to Krebs cycle intermediates (1, 2). The catabolic genes of the TOD pathway form the operon *todXFC1C2BADEGIH*, which is transcribed from a single promoter called P_{todX}, located upstream from the *todX* gene (1–3). The *todST* genes are found downstream and form an independent operon that is expressed constitutively (2, 3).

TodS and TodT have been proposed to form a two-component regulatory system (TCS) that regulates the *tod* catabolic operon in *P. putida* F1 (3). TodT shows all of the characteristics of a response regulator, whereas sequence-based domain predictions indicate that the 108-kDa TodS belongs to a family of sensor histidine kinases that have not been studied at the biochemical level. TodS is predicted to comprise two supradomains, each containing a PAS/PAC sensory domain and a histidine kinase domain. The supradomains are separated by the receiver domain of a response regulator. In contrast to other histidine kinases, TodS apparently lacks transmembrane regions (3). The mode of action of this previously unrecognized type of histidine kinase has yet to be established. On the basis of moderate sequence similarity with the heme-binding oxygen sensor FixL, the C-terminal supradomain of TodS was proposed to be involved in oxygen sensing (3). Choi *et al.* (4) isolated TodS mutants that recognized aromatic compounds that are not effectors of the wild-type protein. Mutations were scattered along the C-terminal half of TodS.

The TodT protein was shown to bind to the Tod box centered at base pair −107 in the P_{todX} promoter (3). This site is located far from the downstream RNA polymerase-binding site. The P_{todX} promoter exhibits a well defined −10 base pair region, although there is no base pair −35 consensus region, consistent with the fact that P_{todX} belongs to the "extended promoter" type that resembles *P. putida* σ^{70}-dependent promoters (5). Because TodT binds far from the RNA polymerase-binding site, we hypothesize that DNA bending is required for transcription activation, which implies that the activation mechanism involves a direct contact between TodT and the RNA polymerase. This type of enhancer-activation system is well documented for regulators of the NtrC family working in conjunction with RNA polymerase/σ^{54} (6, 7).

This study was undertaken to characterize this previously unrecognized TCS. We first addressed questions related to signal sensing and transmission, such as the definition of the effector profile of TodS, the thermodynamic characteristics of the interaction of TodS with aromatic hydrocarbons, the modulatory effect of effector binding on the autophosphorylation activity of TodS, and the detection of TodS–TodT transphosphorylation activity. We then explored the interaction of TodT with P_{todX} and the involvement of DNA-bending proteins such as IHF in the mechanism of transcriptional activation. A functional model for transcription activation mediated by this TCS is discussed.

Results

P. putida DOT-T1E grows on toluene, ethylbenzene, and benzene. We had previously generated *todT* and *todS* mutants of this strain, which failed to grow on the above hydrocarbons as the sole carbon source (2). The chromosomal *todT* mutation was complemented by the *todT* gene supplied in trans in pJLC1. However, the *todS* mutant was complemented by pMIR66 (bearing *todST*) only. These results indicated that both TodS and TodT are needed for induction of the TOD pathway.

P. putida KT2440 does not have the *tod* genes on its chromosome and cannot grow on toluene. We transferred pMIR77 (P_{todX}::'*lacZ*) to this strain, and, regardless of the presence of toluene, no β-galactosidase expression took place. When KT2440 (pMIR77) was transformed with pMIR66, induction took place in response to toluene, and β-galactosidase levels reached 4,500 Miller units. However, when the plasmid bore a mutation in *todS* (pJLC1) or *todT* (pJLC2), no induction from P_{todX} occurred (Table 1). There-

Conflict of interest statement: No conflicts declared.

Abbreviations: CTD, C-terminal domain; CTodS, C-terminal TodS fragment; IHF, integration host factor; NTodS, N-terminal TodS fragment; TCS, two-component regulatory system; TOD, toluene dioxygenase.

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MICROBIOLOGY

Table 1. Bacterial strains and plasmids used in this study

Strains/Plasmids	Relevant characteristics	Refs.
E. coli DH5αF′	F′/*hsdR17, recA1, gyrA*	28
E. coli BL21 (DE3)	F^-, *ompI*, $hsdS_B$ (r^-_B m^-_B)	28
P. putida DOT-T1E	Tol^+, wild type	29
P. putida DOT-T1E *todST*	DOT-T1E, *todST*::Km, Tol^-	30
P. putida DOT-T1E Δ*todT*	DOT-T1E, *todT*::Km, Tol^-	This work
P. putida KT2440	Tol^-, Cm^R	31
P. putida KT2440-IHF3	KT2440, *ihfA*::Km	31
pBS*aphA*	Ap^R, *aphA3* cassette from pU18K	32
pET-28b$^+$	Km^R, protein expression vector	28
pET11c-ihfABhis	Ap^R, *ihfA* and His-tagged *ihfB* genes	33
pMIR77	Tc^R, P_{todX}::′*lacZ*	30
pJLC77	Tc^R, mutant P_{todX}::′*lacZ*	This work
pMIR66	Gm^R, containing the *todST* genes	30
pJLC1	pMIR66, *todS*::*aphA3*	This work
pJLC2	pMIR66, *todT*::*aphA3*	This work
pJLTodT	pET28b containing *todT* gene	This work
pTodS	pET28b containing *todS* gene	This work
pCTodS	pET28$^+$: todS; encoding CTodS	This work
pNTodS	pET28$^+$: todS; encoding NTodS	This work
pREIIα	Ap^R, *ropA*	19, 20
pREIIα-Δ235	Ap^R, RpoA Δ235 residues	19, 20
pREIIα-289A	Ap^R, codifies RpoAL289A	19, 20
pREIIα-291A	Ap^R, codifies RpoAK291A	19, 20

Ap^R, Cm^R, Gm^R, Km^R, Rif^R, Tc^R indicate resistance to ampicillin, chloramphenicol, gentamicin, kanamycin, rifampicin, and tetracycline, respectively. Tol^+ and Tol^- indicate either that the strain grows or fails to grow on toluene, respectively.

fore, although *P. putida* codifies for a large number of TCSs encoded in its chromosome (8, 9), our results indicate that the TodS–TodT interaction is highly specific and that the Tod regulatory proteins cannot be replaced by proteins of any other TCS.

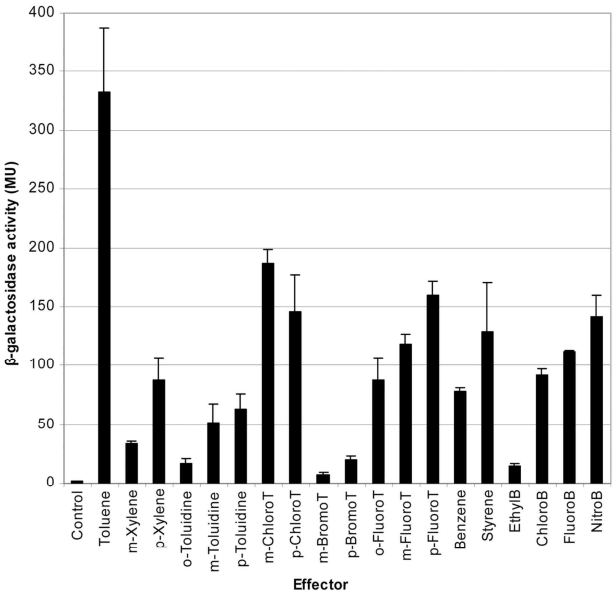

Fig. 1. Induction of P_{todX} by TodS–TodT in response to a wide range of aromatics. *P. putida* DOT-T1E bearing pMIR77 (P_{todX}::′*lacZ*) was grown on M9 medium with 1 mM of the indicated effector. When turbidity of the cultures was 0.8, β-galactosidase activity was determined. Tested compounds that did not induce were as follows: *o*-xylene, *m*- and *p*-ethyltoluene; *o*-, *m*-, and *p*-nitrotoluene; *o*-chloro-, *o*-, *m*-, and *p*-iodotoluene; propyl-, butyl-, and isobutylbenzene; 1,2,3-, 1,3,5-, 1,2,4-trimethylbenzene; and benzamide. T, toluene; B, benzene.

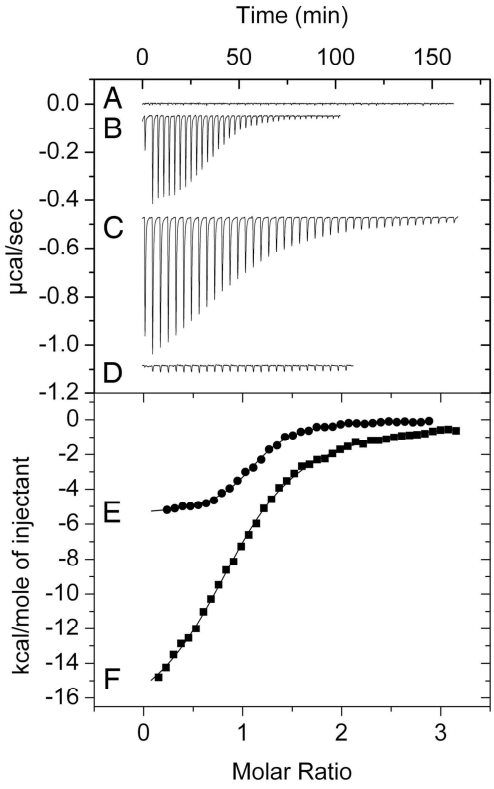

Fig. 2. Isothermal titration calorimetry data for the binding of toluene to TodS and its recombinant fragments NTodS and CTodS. (*Upper*) Heat changes are shown. (*Lower*) Integrated peak areas are shown. (*A*) Titration of buffer with 1 mM toluene. (*B*) Titration of 15 μM TodS with 1 mM toluene. (*C*) Titration of 12 μM NTodS with 0.8 mM toluene. (*D*) Titration of 8 μM CTodS with 1 mM toluene. Integrated peak areas are shown for heat changes in *B* (*E*) and *C* (*F*).

Effector Profile of the TodS–TodT System. The range of effectors recognized by catabolic pathway regulators is typically larger than the range of substrates metabolized by the pathway (10–13). To identify the range of aromatic compounds that activate transcription from P_{todX} we measured induction by using the P_{todX}::*lacZ* fusion. Basal activity was 1.9 ± 0.7 Miller units, but the addition of benzene to the culture led to an almost 50-fold increase in activity (Fig. 1). Then we analyzed benzene derivatives with different substituents. The data indicated that a short lateral chain, such as in toluene, increased the activation of P_{todX} significantly, whereas longer alkyl chains resulted in a significant decrease in the inducing capability of P_{todX}. This finding is exemplified by ethylbenzene, which was a poor effector, whereas *n*-propylbenzene showed no inducer activity (Fig. 1). Chloro-, fluoro-, and nitrobenzene significantly induced expression from P_{todX} (Fig. 1).

Because toluene was the best tested effector, we evaluated different substituents in the aromatic ring at C2, C3, and C4. A number of substituents were permissible at positions 3 and 4 in the aromatic ring of toluene, allowing for methyl, amino, fluoro, bromo, and chloro substitutions (Fig. 1). In contrast, in position 2, only fluoro and amino substituents were permitted. This result evidenced that the regulator profile was significantly broader than the substrate range accepted by the pathway.

Isothermal Titration Calorimetry. Purified His-tag TodS protein was used to determine the thermodynamic parameters for the binding of toluene and ethylbenzene. Fig. 2 shows that the heat changes

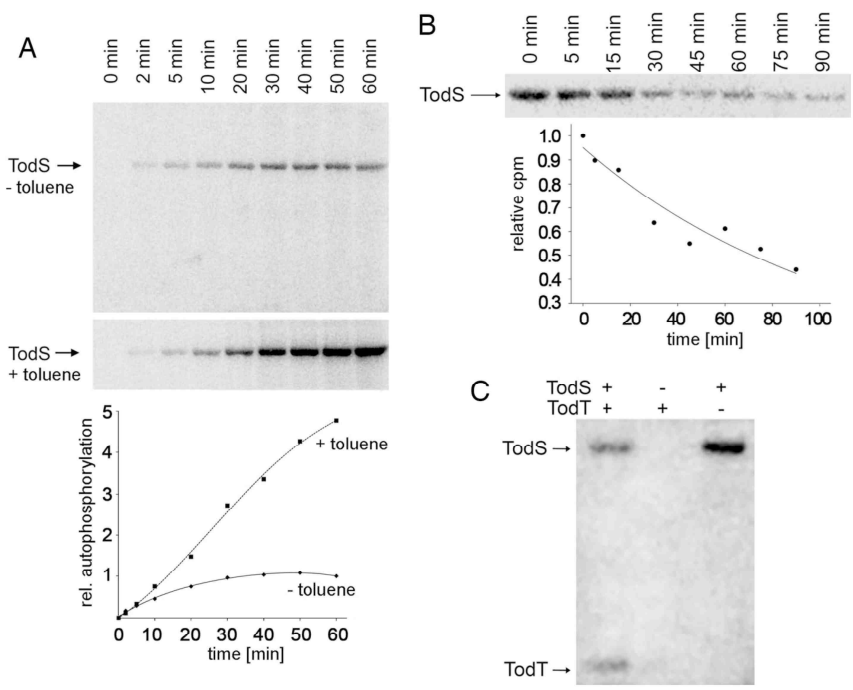

Fig. 3. The catalytic properties of TodS. (*A Upper*) Autophosphorylation in the presence and absence of toluene. Experiments were carried out in parallel, and the graph (*A Lower*) shows the densitometric analysis of the gels. (*B*) Dephosphorylation kinetics. (*C*) Transphosphorylation to TodT. Conditions for these assays are described in *Materials and Methods*.

obtained for the injection of toluene into buffer were negligible. Binding of toluene to TodS (Fig. 2*B*) was driven by favorable enthalpy ($\Delta H = -5.5 \pm 0.1$ kcal/mol) and entropy ($T\Delta S = 2.9 \pm 0.1$ kcal/mol) changes. Binding was tight, with a dissociation constant of 684 ± 13 nM, and the TodS:toluene binding stoichiometry was 1:1. Ethylbenzene binds to TodS in a similar fashion ($\Delta H = -3.6 \pm 0.1$ kcal/mol; 1 kcal = 4.18 kJ) but with lower affinity ($K_d = 3.1 \pm 0.2$ μM).

Because TodS contains two PAS/PAC-type input domains, to identify the toluene-binding domain, two truncated variants of TodS were created. The N-terminal TodS fragment (NTodS), comprising residues 1–584, contained the N-terminal PAS/PAC-histidine kinase domains and the receiver domain of a response regulator, whereas the C-terminal TodS fragment (CTodS), comprising residues 452–978, contained the receiver domain of a response regulator followed by the PAS/PAC-histidine kinase domains located at the C terminus of TodS. We purified both TodS variants and found that toluene bound to NTodS with a very favorable enthalpy change ($\Delta H = -18.3 \pm 0.2$ kcal/mol), an affinity of 2.3 ± 0.1 μM, and a 1:1 stoichiometry (Fig. 2*C*), whereas purified CTodS did not interact with the effector (Fig. 2*D*).

Modulation of Basal Autophosphorylation Activity of TodS by Toluene and Rate of Dephosphorylation. A typical property of histidine kinases is the capacity to autophosphorylate in the presence of ATP. TodS was incubated with 50 μM [γ-^{32}P]ATP, and samples were taken at different times and analyzed (Fig. 3*A Upper*). Densitometric analysis revealed that phosphorylation in the absence of effectors reached a plateau at ≈30 min, indicative of an equilibrium between phosphorylation and dephosphorylation (Fig. 3*A Bottom*). In a parallel assay, phosphorylation was done in the presence of 100 μM toluene. After 50 min of incubation, autophosphorylation activity was ≈5 times as high as the basal level (Fig. 3*A*).

In a subsequent experiment, TodS was autophosphorylated in the presence of toluene, and, after 50 min, a 500-fold molar excess of unlabeled ATP was added. Samples were taken at different times and analyzed by SDS/PAGE to measure the rate of dephosphorylation. TodS dephosphorylates with a half-life of ≈70 min (Fig. 3*B*), which is similar to the half-life of other sensor kinases (14). As mentioned above, TodS contains two sensor domains that probably sense two different signals. In this study, we demonstrate that the addition of a single signal, i.e., toluene, suffices to activate this regulatory system.

TodS–TodT Transphosphorylation Activity. The *in vivo* data suggested that TodS–TodT forms a TCS. Attempts were thus made to detect transphosphorylation *in vitro*. Purified TodS and TodT and a mixture of both proteins were incubated in the presence of radioactive ATP for 50 min. As illustrated in Fig. 3*C*, when alone, TodS was autophosphorylated by ATP, which was not the case for TodT when it was alone. However, a band corresponding to TodT appeared in the mixture of both proteins, demonstrating phosphorylation through TodS. This phosphorylation was confirmed by the fact that the band corresponding to TodS had become fainter (Fig. 3*C*).

IHF Participates in the Activation of P_{todX}. Lau *et al.* (3) demonstrated that the TodT-binding box was located far upstream from the RNA polymerase-binding site and was centered at base pair −107 with respect to the transcription start point. This finding implies that activation requires DNA bending to allow productive contacts between the transcriptional regulator and RNA polymerase bound in the downstream element. IHF is involved in DNA bending and in the activation and repression of a number of promoters in which the regulator-binding site is located far from the transcription start point (6, 7). A potential IHF site (AAAAACAATA) was detected between −43 and −34 in P_{todX}, which matched the consensus defined for IHF (6). To study the involvement of IHF in transcription activation, we transferred pMIR66 and pMIR77 into the wild-type KT2440 strain and its isogenic IHF mutant strain and measured the expression from P_{todX} in response to toluene. In both backgrounds, basal expression was similarly low (≈2 Miller units). In response to toluene in the culture medium, β-galactosidase levels in KT2440 reached values of ≈4,500 units; however, induction in the IHF mutant strain was 1/8 of that found in the wild type, which supports a direct role for IHF in the activation of P_{todX}.

MICROBIOLOGY

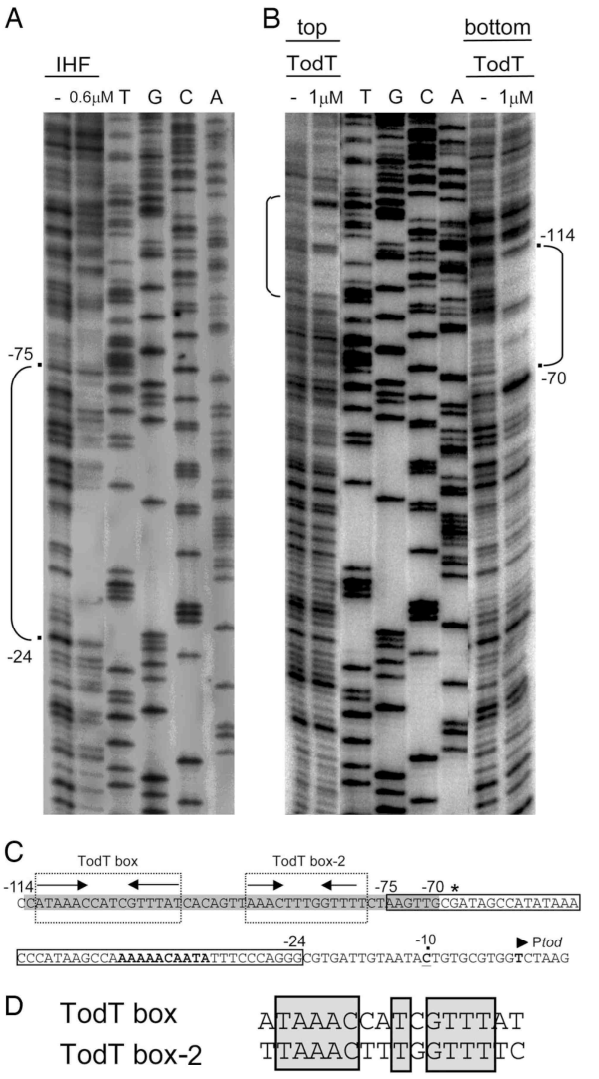

Fig. 4. Identification of TodT and of IHF-binding sites at P_{todX}. DNase I footprints of IHF (*A*) and TodT (*B*). Brackets indicate the protected area. (*C*) Sequence of P_{todX}. The gray shading indicates the region protected by TodT. The dotted outline boxes indicate the two TodT-binding sites. Arrows represent palindromic sequences; the asterisk marks a hyperreactive G; sequences matching the IHF consensus sequence are in bold. The narrow box indicates the fragment protected by IHF. The transcription start site is indicated by an arrowhead, and the −10 base pair position in the promoter is marked with a dot and underlined. (*D*) Sequence alignment of the two TodT binding sites. Conserved nucleotides are boxed.

To verify the role of IHF, we constructed pJLC77 carrying a P_{todX}* mutant with an altered IHF site fused to *'lacZ*, and β-galactosidase activity was measured in *P. putida* DOT-T1E in the exponential phase after induction with toluene. Activity levels with the mutant promoter were 8-fold lower than those obtained with the wild-type promoter (data not shown).

IHF Binds to P_{todX} *in Vitro*. To confirm that IHF binds P_{todX} *in vitro*, we carried out EMSA with increasing IHF concentrations and a 352-bp ^{32}P-labeled P_{todX} promoter or the P_{todX}* with an altered IHF site. No binding of IHF to the mutant P_{todX}* promoter was found (data not shown). In contrast, 100 nM IHF retarded 80% of the

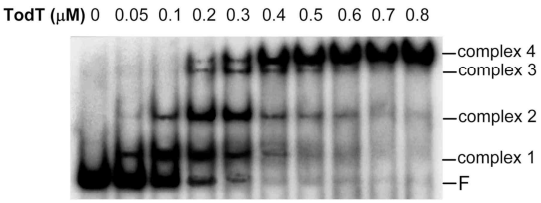

Fig. 5. Specific binding of TodT to P_{todX} EMSA for the binding of TodT to a 352-bp DNA fragment containing the P_{todX} promoter. F, free DNA.

wild-type P_{todX} promoter. From the EMSA, we estimated that the apparent affinity of IHF for its target was ≈70 nM (data not shown). DNase I footprint assays revealed that IHF protected the −24 to −75 base pair region of P_{todX} (Fig. 4 *A* and *C*), which includes the region identified *in silico*.

Redefining the TodT-Binding Region in P_{todX}. We used EMSA to study the binding of TodT to P_{todX} (Fig. 5). We observed four bands corresponding to TodT–DNA complexes, which might be indicative of the existence of multiple TodT-binding sites. To identify the TodT-binding site(s), DNase I footprint assays were carried out with 1 μM TodT. As shown in Fig. 4 *B* and *C*, the region between base pairs −114 and −70 was protected by TodT. This fragment contained the TodT box centered at base pair −107, as described in ref. 3. However, there was clear evidence that protection went beyond this site. Inspection of the sequence downstream from the TodT box revealed a second palindromic fragment, termed "TodT box-2" (Fig. 4*C*). Sequence alignment of the two TodT boxes revealed a high degree of identity (Fig. 4*D*), and we hypothesize that TodT box-2 represents a second binding site for TodT. The consensus sequence between the two TodT boxes (AAACNNT-NGTTT) can be considered a specific TodT-binding motif.

Activation from the P_{todX} Promoter Requires Contacts with the α-Subunit of RNA Polymerase. A number of positive transcriptional regulators interact with the α-subunit of RNA polymerase (15, 16). To test whether this interaction occurs in the case for TodT, experiments were carried out in *Escherichia coli* with defects in the α-subunits bearing pMIR66 and pMIR77. This approach was based on an experimental set-up in which most of the RNA polymerase pool contained a mutant subunit (15–17). The assays were designed to primarily detect a negative effect on transcription. We first tested activation in a mutant background in which α-C-terminal domain (CTD) lacked 235 residues and found that activity from P_{todX} was ≈50% of that in the wild type. This observation is consistent with data obtained for other promoters, such as *mtr* regulated by TyrR (18), the TOL plasmid P_m promoter (17), and the *rha* promoter of *E. coli* (15). Subsequently, we used a series of point mutants exhibiting alanine substitutions (19, 20). TodT-dependent transcription from P_{todX} was significantly affected in a background in which α-CTDL289A or α-CTDK291A mutants were overproduced, because activity decreased by ≈40% with respect to the wild type. Overproduction of α-CTD carrying other mutations did not affect the level of expression from P_{todX}. Therefore, the drop in activity in the two α-CTD mutants seems to result from decreased interactions of α-CTD with TodT. This finding is in agreement with previous studies showing that the patch of residues around position 289 are involved in interactions between the α-CTD and the MetR regulator of *E. coli* and the bacteriophage P2 Ogr protein (21).

***In Vitro* Transcription Assays.** The results obtained *in vitro* and *in vivo* indicated that expression from P_{todX} requires that TodT be activated by TodS through transphosphorylation in response to toluene. Expression from P_{todX} also involves DNA bending in a process assisted by IHF. To provide unequivocal evidence, we performed

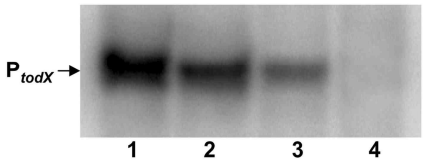

Fig. 6. *In vitro* transcription from P_{todX}. Transcription assays were performed as described in *Materials and Methods*. The assay performed in the presence of 1.5 μM TodS and TodT, 50 nM IHF, and 150 μM toluene is shown in lane 1. In lane 4, TodTD57A replaced TodT. IHF and toluene were omitted in lanes 2 and 3, respectively.

in vitro transcription assays with linear P_{todX} DNA. Fig. 6 shows that efficient transcription from P_{todX} took place in the presence of RNA polymerase, TodS, TodT, IHF, and toluene (Fig. 6, lane 1). However, when TodS, TodT, or toluene was omitted, hardly any or no transcription from P_{todX} occurred (for absence of toluene, see Fig. 6, lane 3). In the absence of IHF, expression from P_{todX} was ≈20–30% of that observed in the complete system (Fig. 6, lane 2). To further confirm that the phosphorylated TodT protein is the activating form of the response regulator, we replaced, in the *in vitro* transcription assay, the TodT protein by a mutant variant that retained the ability to bind target DNA but in which the phosphoaccepting Asp-57 was replaced by alanine. When this mutant regulator was used in *in vitro* transcription assays, no transcript was seen (Fig. 6, lane 4).

Discussion

Soil bacteria sense and respond to environmental cues through a number of TCSs (9). Analyses of annotated bacterial genomes revealed that soil bacteria possess ≈50 TCSs per genome, which is far greater than the average for other bacteria (22). The soil bacterium *P. putida* KT2440 was shown to have 93 TCSs (8). The large number of structurally similar proteins in a single organism raises the question of specificity of each of these regulatory systems. Here we report assays with *todS* and *todT* mutants that demonstrate that there is no functional interaction between either of the two proteins with other histidine kinases or with transcriptional regulators. We have concluded that the interaction of TodS with TodT is highly specific.

The TOD catabolic enzymes metabolize benzene, toluene, and ethylbenzene. These substrates were found to induce P_{todX} (Fig. 1); however, they induce it to a different degree. Toluene was the most efficient effector of all of the molecules tested, benzene was found to be a good inducer, and the inducing effect of ethylbenzene was modest. Our results showed that there was a high degree of tolerance to different substituents on the aromatic ring and that substitutions in the 3 and 4 positions were better tolerated than substitutions at position 2. This finding suggests that TodS is a protein with broad effector-recognition properties, which is unusual for sensor histidine kinases.

The affinity of TodS for toluene (≈0.7 μM) is significantly higher than the binding of effector molecules to other histidine kinase sensors. For instance, CitA has a K_d of 5.5 μM for the binding of citrate (23), and NarX and PhoQ have apparent affinities of ≈35 μM for nitrate and ≈300 μM for Mg^{2+} ions, respectively (24, 25). The high affinity of TodS for toluene assures that bacteria activate transcription and use it as a carbon source at very low concentrations, as expected for a volatile chemical.

TodS contains two signal-sensing domains. Isothermal titration calorimetry showed that toluene bound to NTodS with an affinity of 2.3 ± 0.1 μM, whereas no binding was observed with CTodS. This observation provided direct proof that toluene binds to the N-terminal sensor domain. At first sight, our data may appear inconsistent with the findings by Choi *et al.* (4), who showed that TodS mutants within the C-terminal half had a wider effector specificity range. Although we demonstrated that toluene binds to the N-terminal supradomain, an indirect role of the C-terminal region in toluene sensing is likely. This proposal is deduced from the larger enthalpy changes observed for toluene binding to NTodS ($\Delta H = -18.3 \pm 0.2$ kcal/mol) than for the wild-type TodS ($\Delta H = -5.5 \pm 0.1$ kcal/mol).

On the basis of sequence similarity to the heme-binding, oxygen-sensing domain of FixL, it was suggested that the C terminus of TodS was also a heme-containing sensor domain (3). Heme-binding proteins, recombinantly produced in *E. coli*, copurify with heme and show characteristic absorption peaks between 400 and 600 nm (26). Spectral analysis of purified and active TodS showed no maximum peaks in this wavelength range, indicating that it is unlikely that TodS exhibits a heme group.

The TodS protein has a low but measurable basal level of autophosphorylation. We showed that the addition of toluene increases autophosphorylation and results in TodS phosphotransfer to TodT. Phosphorylated TodT functions as the positive regulator. This phenomenon has been corroborated in *in vitro* transcription assays, because, in the absence of TodT or in the presence of mutant TodTD57A, no transcription took place (Fig. 6).

EMSA studies revealed the presence of four retarded bands, indicating that multiple TodT monomers interact with their target DNA. The footprint analyses revealed the existence of two binding sites of similar size and sequence (Fig. 4). The four bands observed in EMSA may be the result of the binding of one TodT monomer per half site in each of the two TodT boxes identified here. Our EMSA and footprint results differ from those originally reported by Lau *et al.* (3), which showed only two retarded bands corresponding to only one TodT binding site. This discrepancy could be due to the fact that, in the initial study, a recombinant TodT-GST fusion protein was used (3). Because GST is similar in size to TodT, this fusion protein may have caused steric hindrance, preventing other TodT molecules from binding to the proximal Tod box-2.

Expression from P_{todX} in an IHF-deficient background was very low and probably reflected the limitations imposed by the absence of the bending protein. Indeed, IHF binds to P_{todX} and protects the region between base pairs −24 and −75, which is consistent with IHF-mediated DNA bending, allowing a direct interaction between TodT bound to the −85 to −107 boxes and RNA polymerase bound to the downstream promoter site (6). On the basis of the observation that expression from P_{todX} decreased in mutant backgrounds that express RpoA variants, the structure of α-CTD was resolved and shown to be compactly folded and to contain four α-helices (27). TodT seems to interact with residues in α-helix 3 (residues 286–292), a subset of residues also identified in interactions with other positive regulators (21).

Our results support the following model for the mechanism of transcription activation from P_{todX} by the TodS–TodT TCS. TodS exhibits basal autophosphorylation activity, which increases in the presence of toluene. Increased autophosphorylation increases the rate of transphosphorylation of TodT, which is persistently bound to its target sequences in the P_{todX} promoter. Phosphorylation of TodT probably induces conformational changes, which allow or alter the interaction with RNA polymerase in a process assisted by IHF. Once the RNA polymerase–IHF–TodT–*P* scaffold is formed, the *tod* operon is read.

Materials and Methods

Bacterial Strains, Culture Media, and Plasmids. The bacterial strains and plasmids used are shown in Table 1 (28–33). *E. coli* strains were grown at 30°C in LB or 2× YT medium (for recombinant protein expression) with shaking. *P. putida* was grown on M9 minimal medium with glucose.

β-Galactosidase Assays. To quantify the expression from the TOD pathway promoter, we used pMIR77 [P_{todX}::*lacZ* (30)] and a mutant P_{todX}* promoter carrying an altered IHF site (AAAGAGGATA,

MICROBIOLOGY

mutations are underlined) in pJLC77 (P_{todX}*::*lacZ*). Bacterial strains were grown on M9 minimal medium with the appropriate antibiotics without or with the inducer. β-galactosidase activity was determined in permeabilized cells (34).

Construction of *todS* and *todT* Mutants in pMIR66. The *todS* and *todT* genes in plasmid pMIR66 were disrupted by using the *aphA3* kanamycin (Km)-resistance cassette, which produced no polar effects (32). The resulting plasmids were called pJLC1 (*todS*:*Km*) and pJLC2 (*todT*:*Km*).

Overexpression and Purification of TodT, TodS, CTodS, NTodS, and IHF. *todS*, *todT*, and the *todS* mutant variants were cloned independently in pET28b$^+$ to yield plasmids pTodS, pJLTodT, pCTodS, and pNTodS, which allow the overexpression of these proteins with a His-6 tag. These proteins were purified by using HisTrap columns (Amersham Biosciences). IHF was purified after overexpression of the *ihfA* and *ihfB* genes, as described by Ilves *et al.* (33).

EMSA. A 352-bp DNA fragment containing the P_{todX} promoter was amplified by PCR from pMIR77 and end-labeled with ^{32}P as described. Approximately 2 nM labeled DNA (≈1.5 × 10^4 cpm) was incubated with the indicated amounts of purified TodT or IHF for 30 min at 30°C in 10 μl of binding buffer [50 mM Tris·HCl, pH 8.0/100 mM KCl/1 mM DTT/10 mM $MgCl_2$/10% (vol/vol) glycerol] containing 20 μg/ml poly(dI-dC) and 200 μg/ml BSA. Electrophoresis in PAGE was done as described in ref. 35.

DNase I Footprint. The DNA fragment and buffer used for EMSA was also used for footprinting assays. Approximately 10 nM (≈10^5 cpm) labeled probe was incubated in the presence and absence of IHF (0.6 μM) or TodT (1 μM). Reactions were incubated for 30 min before treatment with DNase I (10^{-5} units/μl). Then DNA was precipitated, suspended in 10 mM Tris/0.1 mM EDTA (pH 8), heated at 90°C for 3 min, and analyzed by using 6.5% (wt/vol) PAGE.

***In Vitro* Transcription Assays.** Reactions (20 μl) were performed in 25 mM Tris·HCl/9 mM $MgCl_2$/100 mM KCl (pH 7.5) containing 50 nM σ^{70}-holoenzyme, 20 units of RNasin (Promega), and 5 nM linear P_{todX} DNA template (680 bp). The mixtures were incubated for 10 min at 30°C before the addition of 0.1 mM ATP, CTP, and GTP, 0.05 mM UTP, and 3.6 μCi of [α-^{32}P]UTP (10 μCi/μl) (1 Ci = 37 GBq). After a 20-min incubation, the reactions were chilled at 4°C, and 4 μl of formamide sequencing dye was added. Samples were separated in a 6.5% (wt/vol) PAGE.

Isothermal Titration Calorimetry. Measurements were done on a VP microcalorimeter (Microcal, Amherst, MA) at 25°C. TodS was dialyzed against 50 mM Tris·HCl/250 mM KCl/2 mM $MgCl_2$/2 mM DTT/0.1 mM EDTA, pH 7.5. One millimole toluene and ethylbenzene solutions were made up in dialysis buffer. The titrations involved 1.6-μl injections of toluene or ethylbenzene into a solution of 12–15 μM TodS. Titration curves were fitted by a nonlinear least-squares method (ORIGIN software; Microcal) to a function for the binding of a ligand to a macromolecule (36).

In Vitro* Phosphorylation Assays.** ***Autophosphorylation of TodS. Assays were done in 50 mM Tris·HCl, pH 7.5/200 mM KCl/2 mM $MgCl_2$/0.1 mM EDTA/10% (vol/vol) glycerol/10 mM DTT. The autophosphorylation assay was performed at 24°C with 85 pmol purified TodS in a final reaction volume of 100 μl in the presence and absence of toluene (100 μM). Reactions were initiated by adding radiolabeled ATP (5 nmol ATP containing 1 μCi [γ-^{32}P]ATP), and 7.5-μl samples were removed at different times. The reaction was stopped by adding 2× SDS sample buffer and transferring onto ice. All samples were run on SDS/7.5% (wt/vol) PAGE.

Dephosphorylation of TodS. Protein was autophosphorylated in the presence of 100 μM toluene, as detailed above, for 50 min. A 500-fold molar excess of ATP was added, and samples were removed at different times before analysis by SDS/PAGE.

Transphosphorylation of TodS–TodT. A mixture of TodS (11 μM) and TodT (55 μM) was incubated in a final volume of 20 μl, as described above, for autophosphorylation of TodS. In parallel, TodS and TodT were individually submitted to the same treatment. After 50 min, samples were analyzed by SDS/10% (wt/vol) PAGE.

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Capítulo 2: Perfil de efectores de TodS *in vivo* e *in vitro*

The bacterial TodS sensor kinase interacts with agonists and antagonists.

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El sistema de dos componentes TodS/TodT regula la expresión del promotor P_{todX} en *Pseudomonas putida* DOT-T1E, siendo TodS la sensor quinasa responsable de detectar tolueno. Como respuesta aumenta el nivel de fosforilación de TodS, una señal que transmite a TodT, la cual activa la transcripción de los genes de la ruta TOD. Se ha establecido el perfil de efectores de la sensor quinasa TodS completando el perfil *in vivo* con ensayos de β-galactosidasa y estableciendo un perfil *in vitro* utilizando ensayos de autofosforilación y de microcalorimetría. Al relacionar los datos obtenidos en los ensayos de unión de efectores con microcalorimetría con la capacidad de los efectores de modular la actividad de autofosforilación y de inducir expresión *in vivo*, se distinguieron dos tipos de efectores: a) agonistas, compuestos que se unen a TodS, estimulan la autofosforilación e inducen la expresión génica y b) antagonistas, compuestos que se unen al mismo sitio que los agonistas, pero que no modulan la autofosforilación ni inducen la expresión de los genes de la ruta TOD. El estudio revela que el tolueno es un agonista muy efectivo, pero que derivados con sutituyentes en posición *ortho* en el anillo aromático, como el *o*-xileno, aunque se une con una afinidad muy similar a la del tolueno, no son capaces de activar la transcripción y por tanto son antagonistas. Se propone que es la estimulación de la fosforilación y la capacidad de TodS de transmitir este aumento de actividad a TodT y no el hecho de que se una o no un compuesto el factor determinante en la activación de la transcripción. Análisis mutacional de los aminoácidos F46, I74, F79 e I114 indicaba que estos residuos están implicados en la unión de efectores y que probablemente formen parte del bolsillo de unión de efectores, para el cuál se propone un modelo. Estos datos indican que la determinación de la actividad inhibitoria de antagonistas puede ser una alternativa prometedora en el diseño de inhibidores específicos para procesos regulados por sistemas de dos componentes.

Bacterial sensor kinase TodS interacts with agonistic and antagonistic signals

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The TodS/TodT two-component system controls expression of the toluene dioxygenase (TOD) pathway for the metabolism of toluene in *Pseudomonas putida* DOT-T1E. TodS is a sensor kinase that ultimately controls *tod* gene expression through its cognate response regulator, TodT. We used isothermal titration calorimetry to study the binding of different compounds to TodS and related these findings to their capacity to induce gene expression *in vivo*. Agonistic compounds bound to TodS and induced gene expression *in vivo*. Toluene was a powerful agonist, but *ortho*-substitutions of toluene reduced or abolished *in vivo* responses, although TodS recognized *o*-xylene with high affinity. These compounds were called antagonists. We show that agonists and antagonists compete for binding to TodS both *in vitro* and *in vivo*. The failure of antagonists to induce gene expression *in vivo* correlated with their inability to stimulate TodS autophosphorylation *in vitro*. We propose intramolecular TodS signal transmission, not molecular recognition of compounds by TodS, to be the phenomenon that determines whether a given compound will lead to activation of expression of the *tod* genes. Molecular modeling identified residues F46, I74, F79, and I114 as being potentially involved in the binding of effector molecules. Alanine substitution mutants of these residues reduced affinities (2- to 345-fold) for both agonistic and antagonistic compounds. Our data indicate that determining the inhibitory activity of antagonists is a potentially fruitful alternative to design specific two-component system inhibitors for the development of new drugs to inhibit processes regulated by two-component systems.

histidine kinases | isothermal titration calorimetry | *Pseudomonas* | two-component systems | aromatic hydrocarbons

The most widely distributed type of transcriptional control in prokaryotic microorganisms exposed to environmental cues is the two-component regulatory system (TCS) (1). In fact, genes encoding TCSs are present in almost all bacteria and typically represent ≤1% of their genomes (1, 2). TCSs are also present, although to a lesser extent, in archaea and eukaryotes such as fungi, slime molds, and plants (3, 4). These systems are often made up of a histidine protein kinase (HPK) and a response regulator (RR). The recognition of physical or chemical signals at the input domain of HPKs typically initiates modulation of its autophosphorylation activity. The phosphate of the HPK is transferred to the RR, triggering alterations in the functional properties of its output domain and eventually leading to the stimulation of transcription. The HPK and their RR together comprise a large and diverse group. Their diversity is particularly pronounced in the input domain of HPKs and the output domain of RR, which were shown to belong to many different protein families (1). This variety ensures that HPKs recognize many different signals and RRs are involved in the regulation of different cellular processes (5–7).

Although many TCSs have been studied so far, the primary environmental signals recognized by HPKs remain unknown for most TCSs (8). This lack of information is often a consequence of difficulties in expressing and purifying sensor kinases, which are membrane-bound in most cases (8). Furthermore, in recent years, an increasing number of TCSs have been able to recognize and respond to structurally different agonists. This finding is exemplified by PhoQ, which was initially reported to recognize bivalent cations (9), although recent studies showed that PhoQ also binds cationic antimicrobial peptides (10, 11) by the same binding site for both types of agonists (12). Signal-recognition HPKs are generally assumed to trigger a regulatory response. However, in the light of data showing that some HPKs recognize a wide range of chemical signals, it is of interest to establish whether the binding of a ligand at the sensor domain of an HPK is enough to trigger this kind of response.

We have attempted to shed light on these issues by investigating the TodS HPK in *Pseudomonas putida*, which forms a TCS with the TodT RR (13, 14). This TCS controls the expression of the toluene dioxygenase (TOD) pathway (15) responsible for the metabolism of toluene into Krebs cycle intermediates (16). The catabolic genes of the TOD pathway form an operon that is transcribed from the P_{todX} promotor (13–16).

The architecture of the 108-kDa HPK TodS is atypical and complex. TodS has two supradomains, each containing a periodic circadian-Ah receptor single-minded protein (PAS) sensor domain and a histidine kinase domain (Fig. 1), which are separated by an RR receiver domain. TodS lacks transmembrane regions and is thus likely to be located in the cytosol (8, 13). The N-terminal PAS domain of TodS binds toluene with high affinity ($K_D \approx 700$ nM) (14). This binding increases the basal autophosphorylation rate of TodS, leading to transphosphorylation of TodT and transcription stimulation from P_{todX} (14). TodS seems to belong to a subfamily of HPKs involved in the control of catabolic pathways for the degradation of solvents. For example, TmoS (82% identity with TodS) controls toluene degradation by the T4MO pathway in *Pseudomonas mendocina* (17), TutC (49% identity) regulates the anaerobic degradation of toluene in *Thaurea* sp. strain T1 (18), and StyS (41% identity) in *Pseudomonas* sp. strain Y2 is involved in styrene degradation (19).

In the present study, we used isothermal titration calorimetry (ITC) to measure the thermodynamic parameters for the binding of a wide range of different compounds to purified TodS. We then related these data to the capacity of these compounds to induce gene expression *in vivo* and to their ability to stimulate TodS autophosphorylation activity *in vitro*. Almost all of the mono- and bicyclic aromatics we analyzed bound to TodS. However, only some of these molecules induced gene expression *in vivo*, and this ability

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Abbreviations: HPK, histidine protein kinase; ITC, isothermal titration calorimetry; PAS, periodic circadian-Ah receptor single-minded protein; RR, response regulator; TCS, two-component system; TMB, trimethylbenzene; TOD, toluene dioxygenase.

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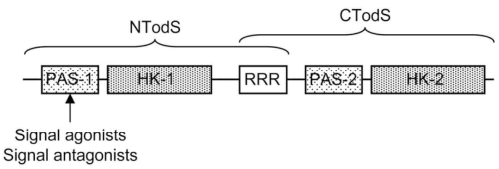

Fig. 1. Domain organization of TodS. The NTodS and CTodS recombinant proteins are indicated. Agonists and antagonists bind to the PAS-1 domain. PAS, PAS-type sensor domain; HK, histidine kinase domain; RRR, response regulator receiver domain.

was related to their capacity to increase TodS autophosphorylation *in vitro*.

Results

Comparison of the *in Vitro* Ligand Affinities of TodS and the Capacity of the Compounds to Induce Gene Expression *in Vivo*. Purified TodS was subjected to microcalorimetric titration with different compounds to determine the effector profile *in vitro*. In parallel, the potential of these compounds to stimulate gene expression *in vivo* was determined by measuring the β-gal activity with a P_{todX}::*lacZ* fusion. The *in vitro* binding parameters and β-gal measurements are listed in Table 1.

ITC experiments revealed that TodS bound benzene with an affinity as high as that of toluene (Table 1 and Fig. 2*A*), but not cyclohexane (Fig. 2*A*) and aliphatic compounds such as 1-hexanol. We then investigated the influence of single substitutions on the aromatic ring [supporting information (SI) Fig. 6]. Styrene was found to be recognized by TodS with the highest affinity (K_D = 580 ± 70 nM) among benzene derivatives carrying an aliphatic substitution. Ethyl, propyl, and butyl substitutions were recognized with lower affinity in comparison to benzene by a factor of 4, 23, and 110, respectively (Table 1). Ethylbenzene was found to be a weak inducer *in vivo*, and propyl- and butylbenzene did not induce expression from P_{todX} *in vivo* (Table 1). Nitro-, chloro-, and fluorobenzene bound to TodS with affinities in the low micromolar range and were found to be potent inducers of expression from P_{todX} (Table 1). Benzamide and benzoate were not bound by TodS, which is consistent with their failure to induce gene expression *in vivo*.

Taking into consideration that toluene is an efficient inducer *in vivo*, the next set of experiments was aimed at evaluating the impact of toluene substitutions on the binding parameters. Initial experiments were carried out with the three xylene isomers (methyl-substituted toluene). Surprisingly, all three xylenes bound to TodS with similar affinities (Fig. 2*B* and Table 1), but only *m*- and *p*-xylene were powerful inducers *in vivo*. In contrast, *o*-xylene failed to induce gene expression (Table 1).

To verify whether the data recorded for the three xylenes represented a general pattern in toluene derivatives, TodS was titrated with the three chlorotoluenes. Like xylenes, all three chlorotoluenes were bound by TodS, but only *m*- and *p*-chlorotoluene showed *in vivo* activity, whereas *o*-chlorotoluene was inactive *in vivo* (Table 1). To further verify these findings, we investigated the interaction of the three toluidines (amino tolu-

Table 1. *In vitro* thermodynamic parameters for the binding of different hydrocarbons to TodS and their capacity to induce expression from P_{todX} *in vivo*

Compound	Binding parameters to TodS *in vitro*		β-Gal expression, Miller units
	K_D, μM	Δ*H*, kcal/mol	
Benzene and singly substituted benzene derivatives			
Benzene	0.76 ± 0.1	−11.0 ± 0.2	79 ± 3*
Toluene	0.69 ± 0.1	−5.5 ± 0.1	333 ± 55*
Ethylbenzene	3.1 ± 0.1	−3.6 ± 0.1	15 ± 2*
Propylbenzene	18 ± 2	−2.7 ± 0.4	2 ± 1*
Butylbenzene	81 ± 4	−5.4 ± 0.1	2 ± 1*
Styrene	0.58 ± 0.1	−12.4 ± 0.3	129 ± 42*
Nitrobenzene	6.6 ± 0.1	−7.3 ± 0.9	141 ± 20*
Chlorobenzene	1.2 ± 0.1	−9.9 ± 0.4	92 ± 5*
Fluorobenzene	1.2 ± 0.1	−5.3 ± 0.1	111 ± 22
Disubstituted benzene derivatives			
o-Xylene	0.58 ± 0.1	−9.4 ± 0.1	2 ± 1*
m-Xylene	1.2 ± 0.1	−9.0 ± 0.1	34 ± 2*
p-Xylene	0.76 ± 0.1	−10.1 ± 0.1	88 ± 18*
o-Chlorotoluene	0.73 ± 0.1	−6.7 ± 0.2	2 ± 1†
m-Chlorotoluene	8.3 ± 0.1	−11.0 ± 2	186 ± 12*
p-Chlorotoluene	0.29 ± 0.1	−8.9 ± 0.1	146 ± 32*
o-Toluidine	3.2 ± 0.2	−28.7 ± 2.5	17 ± 4*
m-Toluidine	8.6 ± 0.7	−7.3 ± 0.8	52 ± 16*
p-Toluidine	11 ± 1	−20.7 ± 3	63 ± 14*
Catechol	16 ± 4	−1.6 ± 0.6	13 ± 2
TMB isomers			
1,2,3-TMB	0.58 ± 0.1	−4.2 ± 0.1	0*
1,2,4-TMB	1.9 ± 0.2	−2.6 ± 0.1	0*
1,3,5-TMB	133 ± 20	−3.2 ± 1.6	0*
Biaromatics			
1-naphthol	2.1 ± 0.1	−12.4 ± 0,3	0
2,3-dihydroxynaphthalene	7.5 ± 0.3	−15.1 ± 0.3	0

*Data were initially reported in ref. 13.
†No binding to TodS or transcription stimulation was observed for 1-hexanol, cyclohexane, benzamide, benzoate, anthracene, or naringenine.

MICROBIOLOGY

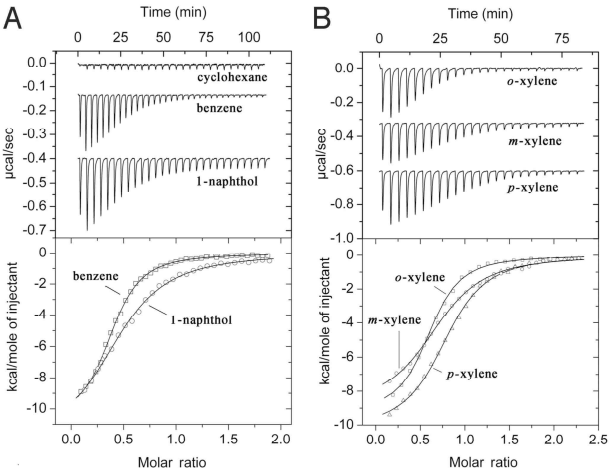

Fig. 2. Microcalorimetric titration of TodS with different hydrocarbons. (*A*) (*Upper*) Heat changes for the titration of 12 to 13.2 μM TodS with 4.8-μl aliquots of 750 μM cyclohexane and 1.6-μl aliquots of 750 μM benzene and 500 μM 1-naphthol. (*Lower*) Integrated and corrected peak areas for the titration with benzene and 1-naphthol. (*B*) Heat changes (*Upper*) and integrated peak areas (*Lower*) for the titration of 10–12 μM TodS with 1.6-μl aliquots of the three xylenes. For clarity, raw titration data have been shifted arbitrarily on the *y* axis. Derived thermodynamic data are given in Table 1.

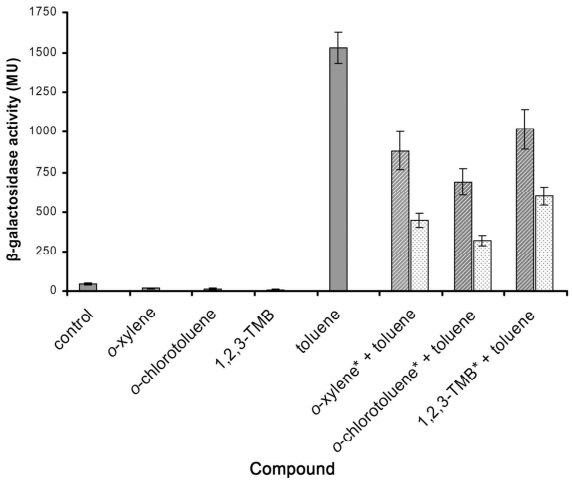

Fig. 3. Inhibition of toluene-mediated induction of P_{todX} by *o*-xylene, *o*-chlorotoluene, and 1,2,3-TMB. Eleven 25-ml cultures of *P. putida* DOT-T1E harboring pMIR66 (containing *todST*) and pMIR77 (containing a P_{todX}::*lacZ* fusion) were grown in LB to a turbidity of 0.2 at 660 nm. Then, six cultures were exposed to *o*-xylene, *o*-chlorotoluene, or 1,2,3-TMB (asterisk) at 0.3 mM (hatched bars) or 1.5 mM (dotted bars). When the cultures reached a turbidity of 0.5, buffer (control culture), *o*-xylene, *o*-chlorotoluene, or 1,2,3-TMB (all at a final concentration of 0.3 mM) was added to four cultures without addition, and 0.3 mM toluene was added to the seven remaining cultures. The β-gal activity was measured 2 h later. Data are the means and corresponding standard errors derived from at least three independent assays, each done in triplicate.

enes). Again, *o*-toluidine was found to be a significantly weaker inducer *in vivo* than the other two isomers (Table 1), although it bound to TodS more tightly than *m*- or *p*-toluidine. Our interpretation of the combined data for xylenes, chlorotoluenes, and toluidines is that *o*-substitutions either fully abolished (xylene, chlorotoluene) or reduced (toluidine) the *in vivo* response without exerting a significant impact on binding affinity. This apparent lack of correlation between the affinity measured *in vitro* and expression studies *in vivo* was further confirmed by the fact that the second-best inducer *in vivo*, *m*-chlorotoluene, was recognized by TodS with modest *in vitro* affinity (K_D = 8.3 ± 0.1 μM).

Because of the unexpected results for *ortho*-substituted compounds, we explored the influence of polysubstituted benzene derivatives on the binding parameters. The three trimethylbenzene (TMB) isomers bound to TodS but did not induce expression *in vivo*, which supports the notion that binding *in vitro* does not automatically translate into induction by a compound *in vivo*.

Because only monoaromatic compounds have been reported thus far to induce the TOD pathway (14), we carried out experiments to define the upper size limit of TodS ligands. The biaromatic compounds 1-naphthol (Fig. 2*A*) and 2,3-dihydroxynaphthalene bound to TodS with K_D values of 2.1 ± 0.1 and 7.5 ± 0.3 μM (Table 1), respectively, but were equally inactive *in vivo*. No binding of the polyaromatic hydrocarbon anthracene to TodS was observed.

In short, the compounds under study can be classified into three groups. The first group is made up of compounds such as cyclohexane or benzoate, which do not bind to TodS *in vitro* and do not activate gene expression *in vivo*. The second group, referred to here as agonists, consists of compounds that bind to TodS and induce expression from P_{todX}. The third group, called antagonists, includes chemicals that bind to TodS *in vitro* but exhibit no *in vivo* activity.

Agonists and Antagonists Bind to the Same PAS Domain. We then studied the mode of action of antagonists, among which *o*-xylene, *o*-chlorotoluene, and 1,2,3-TMB were chosen as representatives because they were recognized by TodS with similar affinities to toluene and in the range of 580–730 nM (Table 1). TodS is predicted to contain two sensor domains of the PAS type (20). One domain is located at the N-terminal end of TodS (amino acids 31–168 according to Pfam) (21), whereas the second domain is found at the C-terminal half (amino acids 613–725). We recently showed that toluene only binds to the N-terminal PAS sensor domain (14), a finding that raised the possibility that agonists and antagonists bind to different sensor domains, causing the differences observed *in vivo*.

To test this possibility, we conducted sequential ITC experiments. In an initial series of experiments, TodS was saturated with toluene, and this complex was titrated with *o*-xylene, *o*-chlorotoluene, or 1,2,3-TMB. Binding of the second ligand would suggest that the binding sites of both ligands did not overlap. If the second ligand failed to bind to the TodS–toluene complex, binding sites would either be the same or overlap. In all cases, none of the three antagonists bound to the TodS–toluene complex (data not shown). When these experiments were repeated in reverse order (i.e., the titration of TodS complexed to *o*-xylene, *o*-chlorotoluene, or 1,2,3-TMB with toluene), the same results were obtained (data not shown).

To corroborate these findings, the NTodS and CTodS recombinant fragments (Fig. 1) were titrated with the three antagonists. No binding of *o*-xylene, *o*-chlorotoluene, or 1,2,3-TMB to CTodS was observed, whereas all three antagonists bound to NTodS with a 2- to 7-fold lower affinity compared with the binding to full TodS (SI Fig. 7 and SI Table 3). This reduction in affinity was similar to that observed for toluene (14).

Antagonists Inhibit Toluene-Mediated Up-Regulation of Gene Expression *in Vivo*. ITC data suggested that agonists and antagonists most likely compete for the same binding site *in vitro*. Gene expression studies with P_{todX}::*lacZ* and the *todST* genes in pMIR66 were carried out to determine whether this competition was observed *in vivo* (Fig. 3). In parallel experiments, the β-gal activity in cultures induced with toluene was compared with cultures to which *o*-xylene, *o*-chlorotoluene, or 1,2,3-TMB was added before toluene (Fig. 3). When antagonists and toluene were added at equimolar concentrations, gene expression was reduced by approximately half (Fig.

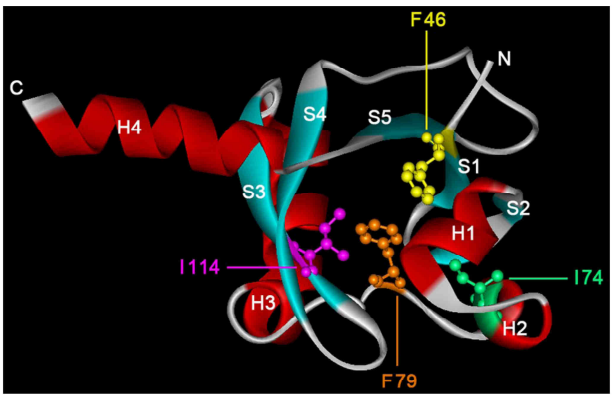

Fig. 4. The 3D model of the N-terminal signal sensor domain of TodS. Secondary structure elements are indicated by S (strand) and H (helix). The amino acids in the proposed effector-binding site, which were replaced with alanine residues, are shown in ball-and-stick mode. This model contains amino acids 43–164 of TodS.

3). When a 5-fold molar excess of antagonist was used, gene expression was reduced by a factor of ≈4 (Fig. 3). When antagonists recognized by TodS with low affinity were used, higher concentrations of these compounds (≈10-fold) were necessary to observe similar inhibition effects. Therefore, these results are consistent with competition between toluene and each of the antagonists for binding to TodS.

Identification of the Agonist/Antagonist Binding Site of TodS. To identify the amino acids involved in ligand binding at the N-terminal PAS domain of TodS, we generated a 3D model of this domain. With the help of the DALI algorithm (22), we aligned this model to the structure of the effector-binding domain of the HPK CitA (Protein Data Bank ID code 1POZ), which has been solved in complex with its agonist citrate (23). The model showed a hydrophobic cavity at a position analogous to the citrate-binding site of CitA, which we hypothesized to be the ligand-binding pocket of TodS. Four amino acids located in this pocket (F46, I74, F79, and I114) were postulated to be involved in effector binding (Fig. 4). All four amino acids were conserved in an alignment of the HPKs TodS, TmoS (17), StyS (19), and TutC (18). These three regulators responded to similar agonists such as toluene (TodS, TmoS, and TutC) and styrene (StyS) (14, 17–19). To investigate the potential role of these residues in effector binding, we prepared and characterized the corresponding alanine replacement mutants. All four purified mutants were subjected to ITC assays with agonists (toluene and benzene), as well as with the *o*-xylene and *o*-chlorotoluene antagonists (Table 2). The affinity of mutants F46A, I74A, and I114A for agonists and antagonists was found to be reduced 2- to 12-fold compared with the wild-type protein (Table 2), which supports the hypothesis that agonists and antagonists probably bind to the same site at TodS. The titration of mutant F79A with a 4- to 5-mM ligand solution only gave rise to small heat changes for binding, suggesting that this mutant had lost the ability to bind agonist and antagonist molecules. We then tested the mutants *in vivo* by replacing the wild-type *todS* allele in pMIR66 with the mutant variants and measuring induction from P_{todX}::*lacZ* as β-gal. As expected, TodSF79A did not stimulate transcription *in vivo* with any of the tested agonists and antagonists. Also, as expected, none of the other three TodS mutant variants responded to *o*-xylene and *o*-chlorotoluene, although they did induce transcription with toluene and benzene (data not shown).

Antagonist Compounds Do Not Stimulate TodS Autophosphorylation *in Vitro*. It often has been found that one of the critical steps in the mechanism of TCS's action is the autophosphorylation of

Table 2. Thermodynamic parameters for the titration of TodS mutants with agonists and antagonists

Mutants* and compounds	K_D, μM	Δ*H*, kcal/mol	K_D^{mut}/K_D^{wt}
F46A			
Benzene	10.0 ± 2	−8.20 ± 2.2	12
Toluene	3.03 ± 0.2	−14.6 ± 0.7	4.3
o-Chlorotoluene	3.48 ± 0.3	−13.5 ± 1	4.5
o-Xylene	2.46 ± 0.1	−5.66 ± 0.1	4.2
I74A			
Benzene	5.81 ± 1.3	−2.52 ± 0.8	7.7
Toluene	8.62 ± 1.0	−5.49 ± 1.3	12
o-Chlorotoluene	2.29 ± 0.7	−2.02 ± 0.5	3.1
o-Xylene	3.20 ± 0.6	−3.52 ± 0.8	12
F79A			
Benzene	>200†	—	>263
Toluene	>200†	—	>290
o-Chlorotoluene	>200†	—	>274
o-Xylene	>200†	—	>345
I114A			
Benzene	2.32 ± 0.2	−5.14 ± 0.3	3.0
Toluene	1.63 ± 0.3	−9.75 ± 1.2	2.4
o-Chlorotoluene	2.33 ± 0.1	−5.14 ± 0.3	3.2
o-Xylene	1.27 ± 0.2	−20.0 ± 9.3	2.2

K_D^{mut}/K_D^{wt} shows the ratio of the determined K_D to the corresponding value obtained for the wild-type (wt) protein.

*The location of the amino acids in the 3D model of the N-terminal PAS domain of TodS is shown in Fig. 4.

†A titration of this mutant with a series of 12-μl injections of 4–5 mM ligand solution gave rise to minor heats of binding, which did not permit data analysis. The K_D is estimated to be >200 μM.

HPK in response to ligands and the subsequent transphosphorylation of the RR. To shed further light on antagonists *in vitro*, we first performed autophosphorylation studies with TodS by using [γ-^{32}P]ATP in the absence and presence of various concentrations of agonists and antagonists (SI Fig. 8). After electrophoresis, [^{32}P]TodS was quantified densitometrically and plotted against time. From the linear curve fit of the data, we calculated the apparent rates of phosphorylation. In the presence of *o*-xylene, 1,2,3-TMB, or *o*-chlorotoluene, autophosphorylation rates were similar to the basal rate (0.8–1.4 ± 0.2) (Fig. 5). In contrast, the autophosphorylation rate in the presence of toluene was found to be 7.3 ± 0.1 times faster than in the absence of toluene. Other agonists such as fluorobenzene and ethylbenezene were also found to stimulate the rate of autophosphorylation between 3- and 5-fold (data not shown). This finding indicates that failure of the antagonists to induce gene expression *in vivo* correlates with their inability to promote autophosphorylation *in vitro*. We also tested autophosphorylation activity in mutant strain F79A in response to toluene. TodSF79A was found to have a relative basal autophosphorylation rate that did not increase in response to toluene (SI Fig. 9). We reasoned that antagonists could inhibit autophosphorylation if present in mixtures containing toluene. Similar assays to the ones described earlier were conducted with equimolecular concentrations of toluene and *o*-xylene. Under these conditions, the autophosphorylation rate was about half of that with toluene alone (data not shown).

Discussion

Both the Recognition of Agonists by TodS and Intra-TodS Signal Transmission Determine Whether a Compound Stimulates Expression from P_{todX}. This study was designed to answer the following question: Is ligand binding to HPKs enough to set up the regulatory cascade in response to environmental cues? To answer this question, we used the TCS TodS/TodT involved in

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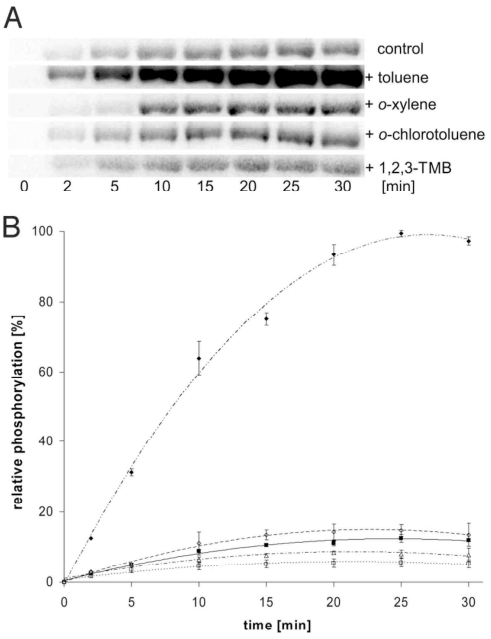

Fig. 5. Modulation of TodS autophosphorylation activity *in vitro*. TodS autophosphorylation activity with [^{32}P]ATP was measured in the absence of signal (control) and in the presence of 100 μM toluene, *o*-xylene, 1,2,3-TMB, or *o*-chlorotoluene. (*A*) SDS/PAGE of TodS in the absence and presence of different ligands. (*B*) Densitometric analysis of the data in *A*. Data are the means of three independent assays. Linear fit of the points at 2.5 and 10 min was used to calculate relative rates of autophosphorylation. Filled square, control; open triangle, *o*-chlorotoluene; open square, trimethylbenzene; open diamond, *o*-xylene; filled diamond, toluene.

toluene metabolism, and we analyzed recognition by TodS of molecules with different structural features. TodS only bound compounds containing at least one aromatic ring, although the presence of a benzene ring did not guarantee binding, as evidenced by the lack of recognition of benzamide and benzoate by TodS. Among the compounds recognized by TodS, we distinguished agonist and antagonist molecules, and three lines of evidence are consistent with the notion that agonists and antagonists share the same binding site: (*i*) agonists and antagonists compete for binding at TodS *in vitro* and *in vivo*, (*ii*) agonists and antagonists bind to NTodS but not to CTodS, and (*iii*) amino acid substitutions at the N-terminal PAS-binding domain of TodS reduce binding of agonists and antagonists in a similar fashion. Although all molecules appear to bind to the same site, the binding of agonists is likely to generate a signal that is transmitted through conformational changes to the kinase domain of the HKP, which in turn stimulates autophosphorylation activity. In contrast, the binding of antagonists seems to keep TodS in an inactive conformational state and thus has no significant effect on TodS autophosphorylation (Fig. 5).

Of the 26 mono- and biaromatic compounds analyzed here, 24 were found to bind to TodS *in vitro* (SI Fig. 6). Surprisingly, only 14 of these 24 compounds were able to increase gene expression *in vivo*. It is generally assumed that binding of ligands at the sensor domain of a histidine kinase is the dominant prerequisite for the regulatory activity of a TCS. However, the situation in TodS is different because the capacity of a molecule to stimulate gene expression from P_{todX} is not primarily determined by molecular recognition of the chemical by TodS, but rather by its ability to trigger the phosphorylation cascade.

Our findings with regard to the TodS sensor kinase system show clear parallels with the repressor TtgV (24, 25), a one-component regulator system that, like TodS, exerts its action in response to mono- and biaromatic compounds. We have shown that both types of compounds activate TtgV-mediated transcription with different efficiencies (26), and it was concluded that the effect of mono- and bicyclic compounds on TtgV intramolecular signal transmission is different from one to the other. It remains to be established whether such differences in the mechanism of intramolecular signal transmission are a general feature of regulatory proteins with a broad effector profile.

Identification of Amino Acids Involved in Signal Binding. A 3D model of the N-terminal sensor domain was generated and aligned to the structure of the sensor domain of CitA. A hydrophobic pocket in an analogous position to the citrate-binding site of CitA was hypothesized to be the effector-binding site of TodS. We generated alanine replacement mutants of F46, I74, I114, and F79 located in this pocket. Three of these mutants (F46A, I74A, and I114A) had a 2- to 12-fold lower affinity for agonists and antagonists, whereas the affinity of the F79A protein was reduced by a factor of >250 with the tested ligands (Table 2). These data suggest that the proposed hydrophobic cavity is the common binding pocket for agonists and antagonists in TodS. The aromatic side chain of F79 in TodS appears to play a central role in the recognition of a broad series of ligands, and we propose effector recognition to be dominated by pi-pi stacking interactions of effectors and protein residues, a common feature in the molecular recognition of aromatic ligands by proteins (27, 28).

Is High-Affinity Binding of Agonists a Typical Feature of Cytosolic Sensor Kinases? Ulrich *et al.* (1) analyzed the presence of one- and two-component regulatory systems in complete bacterial genomes and concluded that entirely cytosolic one-component systems are older, in evolutionary terms, than TCSs. The physiological reason leading to the emergence of TCSs, which frequently have an HPK with a periplasmic sensor domain, was their capacity to regulate cellular processes in response to extracytosolic signals. This capacity is of particular importance for soil bacteria such as *Pseudomonas*, which live in a rapidly changing environment. A subfamily of HPK has been identified that is entirely located in the cytosol and recognizes effectors (8). The physiological role of these cytosolic HPKs however, is unknown. The absence of transmembrane regions (13) and high solubility in the absence of detergents (14) are evidence that TodS belongs to this subfamily. Available data on the interaction between agonists and HPKs were obtained primarily with kinases containing a periplasmic sensor domain. The affinity of HPKs for their cognate agonists was found to be in the micromolar range, as exemplified by CitA, which has a K_D of 5.5 μM for citrate (29), and NarX and PhoQ, which have apparent affinities of $\approx$35 μM for nitrate and $\approx$300 μM for Mg^{2+} ions, respectively (30, 31). Here we show that TodS binds agonists with much higher affinities than those just mentioned. It remains to be established whether high-affinity ligand binding is a typical feature of cytosolic HPK. This information might help to solve the enigma surrounding the factors that led to the evolution of cytosolic HPKs.

***Ortho*-Substitutions of Toluene: Converting an Agonist Molecule into an Antagonist.** *In vivo* gene-expression studies showed toluene to be one of the most efficient agonists. The substitution of toluene at the *ortho* position by a methyl, chloro, or amino group either abolished or reduced the inducing capacity without producing a significant impact on affinity (Table 1). Thus, we have identified a structural modification that converts a powerful agonist into an antagonist. These antagonists behave as competitive inhibitors of the regulator activity mediated by agonists. This finding is relevant to the development of inhibitors of TCS such as PhoP/Q of *S. typhimurium* (32, 33), which were shown to be important virulence factors (6). A large number of inhibitors of different

TCSs have been developed *in vitro* (34). However, when tested for their antimicrobial potential *in vivo*, most of these compounds were not selective for signal transduction pathways, but exerted their effects through multiple mechanisms of action (35), making them unsuitable for any clinical application. Most of these inhibitors were developed by random screening, and most of them were shown to bind to the kinase domain, which might explain their restricted selectivity (36). In this study, we demonstrate that an HPK can be inhibited by structural analogues that behave as antagonists. Targeting the effector-binding domain of an HPK by specifically exploring the inhibitory activity of antagonists could thus be a productive line of research for the more rational development of TCS inhibitors.

In summary, we demonstrate that agonists and antagonists bind to the same domain of TodS with similar affinities. The binding of agonist molecules stimulates the autophosphorylation activity of TodS, whereas antagonists do not modulate this activity. The binding of antagonists inhibits agonist-mediated transcriptional activation, which is relevant to the development of more specific inhibitors of TCS. X-ray crystallographic studies of TodS in the presence of different ligands are currently in progress to establish the molecular basis for the differential action of agonists and antagonists.

Materials and Methods

Generation of a 3D Model of the N-Terminal PAS Domain of TodS. A homology model of the N-terminal PAS domain of TodS (amino acids 31–168) was generated with the aid of 3D-JIGSAW software (37) by using the structure of FixL of *Rhizobium meliloti* (38) (residues 122–251; Protein Data Bank ID code 1D06) as a template. The model was validated by calculating the solvation profile with SolvX software (38), which gave a satisfactory score of −17.5. The DALI algorithm (22) was used to align this model with the structure of the sensor domain of CitA (23).

Site-Directed Mutagenesis. Plasmids encoding TodS mutants F46A, I74A, F79A, and I114A were prepared with the QuikChange site-directed mutagenesis kit according to the manufacturer's instructions (Stratagene, La Jolla, CA), with pTodS or pMIR66 (14, 17) as a template. For each mutant, two 30-mer complementary oligonucleotides were designed that contained the desired mismatch in the center. The pTodS derivatives were used for protein expression. The entire *todS* coding region and the flanking regions of the resulting plasmids were verified by DNA sequencing.

ITC. TodS and its mutant variants, NTodS and CTodS, were purified as described (14). ITC assays were performed with freshly purified protein by using a VP-microcalorimeter (Microcal, Amherst, MA) (39). Protein was dialyzed into ITC buffer [50 mM Tris·HCl/200 mM KCl/2 mM $MgCl_2$/2 mM DTT/0.1 mM EDTA (pH 7.5)]. Typically, the TodS concentration was in the range of 12–15 μM. Ligand solutions were prepared as described (14). Typically, TodS was titrated with 1.6-μl aliquots of ligand solution. If no binding heats were detected, the experiment was repeated with larger injection volumes ($\leq$12 μl). The mean enthalpies measured from the injection of agonists/antagonists into the buffer were subtracted from raw titration data before data analysis with ORIGIN software.

Expression of P_{todX} *in Vivo*. The β-gal measurements reported in Table 1 were obtained as described previously (14). The measurements reported in Fig. 3 were obtained with *P. putida* DOT-T1E bearing pMIR66 (17) (containing *todST*) or its mutant derivatives and pMIR77 (containing a P_{todX}::*lacZ* fusion). Cultures were grown on LB supplemented with 100 μg/ml gentamycin and 10 μg/ml tetracycline in the absence and presence of agonists and antagonists. The β-gal activity was determined in permeabilized whole cells as described (25).

***In Vitro* Autophosphorylation Assay.** Assays were carried out as described (14), except that 300 pmol of TodS and 4 μCi of [^{32}P]ATP were used.

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Capítulo 3: Mecanismo de fosfotransferencia TodS-TodT

The sensor kinase TodS operates by a phosphorelay mechanism involving two histidine autokinase domains

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El operon *tod* en *Pseudomonas putida* codifica para los genes del metabolismo del tolueno y su expresión es regulada a partir del promotor P_{todX} mediante el sistema de dos componentes TodS/TodT. La sensor-quinasa TodS alberga dos modulos funcionales con un dominio sensor y un dominio autoquinasa cada uno, separados por un dominio receptor interno. Mediante análisis de dominios recombinantes individuales y mutagénesis dirigida de los sitios conservados y susceptibles de participar en la transferencia de grupos fosfato a TodT (TodS: H190, D500, H760; TodT: D57) se identificó el modo de transferencia del fosfato a través de ensayos de transfosforilación *in vitro*. Ambos dominios histidín-quinasa tienen actividades basales de autofosforilación similares pero el tolueno únicamente estimula la autofosforilación del dominio histidín-quinasa N-terminal. El mutante H760A en el extremo C-terminal anula la transfosforilación de TodT, por lo que el dominio histidín-quinasa C-terminal es esencial en la transferencia de grupos fosfato de TodS a TodT. La idea de que TodT solamente se una a uno de los dos dominios la sustenta el hecho de que TodT se une a TodS con una estequiometría 1:1 y únicamente en presencia de ATP. La mutación H190A impide la estimulación de TodS por tolueno y la mutación D500A impide la transmisión del estimulo percibido en la histidín-quinasa N-terminal por tolueno hacia TodT. En ensayos *in vivo* estos exhiben reducida expresión de P_{todX}. Por tanto los aminoácidos H190, D500 y H760 son esenciales en la transmisión de señal hacia TodT. Esta es la primera evidencia de un sistema *phosphorelay* que opera a través de dos dominios quinasa y que no contempla un dominio HPT.

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The sensor kinase TodS operates by a phosphorelay mechanism involving two autokinase domains

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The two-component system TodS/TodT of *Pseudomonas putida* regulates the expression of the toluene dioxygenase (*tod*) operon which is under control of promoter P*todX*. The TOD pathway permits the metabolization of toluene, benzene and ethylbenzene. The sensor kinase TodS has a complex domain arrangement containing two functional modules, each harboring a sensor- and an autokinase domain and separated by a receiver domain. Based on site-directed mutagenesis of phosphoaccepting H190, D500 and H760 and *in vitro* transphosphorylation experiments with recombinant TodS fragments, we show that TodS uses a multiple-step phosphorelay mechanism to activate TodT. Both autokinase domains of TodS were shown to possess similar basal activities, but toluene binding stimulates exclusively phosphorylation of H190, which is followed by phosphotransfer to D500 and subsequently to H760 prior to phosphorylation of TodT D57. Mutation of H190, D500 and H760A prevented upregulation of toluene-mediated stimulation of TodT transphosphorylation *in vitro* and reduced *in vivo* expression of P*todX* to the basal level. Calorimetric studies support that TodT binds to the C-terminal kinase module with a *K*D of ~200 nM and 1:1 stoichiometry. This is the first report of a multiple-step phosphorelay mechanism of a sensor kinase which involves two autokinase domains.

The adaptation of bacteria to changes in environmental signals is often mediated at the transcriptional level by two-component systems (TCS), which contain as basic components a sensor kinase (SK) and a response regulator. SKs typically recognize signal molecules, which leads to an alteration of their phosphorylation state which modulates in turn transphosphorylation of the corresponding cognate response regulator. The ratio of phosphorylated to nonphosphorylated response regulator directly influences promoter activity (1, 2).

Based on domain arrangement and function, TCSs are grouped into two major families. One family, the so-called prototypal TCSs, is characterized by the fact that SKs and response regulators each contain a single phosphorylatable site and phosphotransfer occurs directly from the SK to the response regulator. The other family comprises the multiple-step phosphorelay systems (3), which in contrast to prototypal TCSs contain multiple phosphorylation sites. These systems involve initial phosphorylation at the autokinase domain, followed by phosphotransfer to one or several receiver domains and subsequently to the HPT (histidine containing phosphotransfer) domain prior to the phosphorylation of the response regulator (Fig. 1A). The HPT domain is able to accept phosphate from a receiver domain but lacks autokinase activity (4). Based on the location of the HPT domain three different types of multiple-step phosphorelays can be distinguished: (i) systems where the HPT domain is part of the sensor kinase which is well documented for tripartite hybrid SKs (Fig. 1) such as ArcB (5), BarA (6), EvgS, BvgS (7) and TorS (8), (ii) systems where the HPT domain is an individual single-domain protein as exemplified for the KinA/Spo0F/Spo0B/Spo0A system in *Bacillus subtilis* (9) and (iii) systems where the HPT domain is part of an individual but multidomain protein such as in the RcsC-YojN-RcsB system (10). The presence of the HPT domain appears to be an absolute requirement for a multiple-step phosphorelay mechanism since no such system was described which did not contain an HPT domain (11).

The domain organization of TodS from *P. putida* DOT-T1E is different from that of the tripartite SKs (Fig. 1). TodS has two modules each containing a PAS-type sensor domain and an autokinase domain. Due to this particular domain arrangement, members of this family have been termed "double SKs."

TodS forms, together with TodT, a TCS that regulates expression of the *tod* (toluene dioxygenase) operon in *P. putida* for toluene metabolism (12-16). Other members of the double SK family are also involved in the regulation of degradation pathways such as StyS (styrene degradation in *P. fluorescence* and *Pseudomonas sp.* Y2, 17, 18), TutC (toluene degradation in *Thauera aromatica*, 19) or TmoS (toluene degradation in *P. mendocina*, 14). Furthermore, genome sequencing projects have revealed that TodS-like proteins are found in other hydrocarbon-degrading bacteria such as *Dechloromonas aromatica* (20) and *Methylibium petroleiphilum* (21). The reason for this apparent functional link between the domain arrangement of TodS-type SKs and their involvement in the regulation of hydrocarbon degradation is unknown. A further TodS-like protein is found in the eukaryotic soil organism *Dictyostelium discoideum.*

The 108-kDa TodS protein is among the largest SKs ever described. We developed a protocol for the purification of the full-length protein and showed that it exhibits a broad effector specificity, recognizing a wide variety of mono- and biaromatic compounds (15, 16). We have also shown that effector molecules bind to the N-terminal PAS domain, causing an increase in the amount of phosphorylated TodS, which in turn stimulates transphosphorylation of TodT (15, 16). TodT binds to three different sites at the P_{todX} promoter in a cooperative and hierarchical manner. TodT-P in conjunction with IHF was found to stimulate promoter activity (22, 23). However, the functional reason for the complex architecture of TodS remains unknown and is the focus of this work. Two different modes of activation can be envisaged: either each of the modules containing a PAS and a kinase domain are independent units responding to different signals and serving potentially different regulators, or alternatively, all three phosphorylation sites are functionally linked by intramolecular communication. Using a set of site-directed mutants as well as different TodS fragments, we were able to establish that TodS uses also a multiple-step phosphorelay mechanism involving amino acids H190->D500->H760 followed by phosphorylation of D57 at TodT.

Results

TodS contains two functionally active autokinase domains. TodS was predicted to contain two autokinase domains (Fig. 1) and initial experiments were aimed at verifying whether both domains posses autophosphorylation activity. To this end, alanine substitution mutants of the histidine residues predicted to be the phosphoacceptors in each autokinase domain, namely H190 and H760, were generated by site-specific mutagenesis and autophosphorylation assays carried out. Equal amounts of TodSH190A and TodSH760A were incubated with [γ-^{32}P-ATP] for 20 min, and samples were analyzed by SDS-PAGE. Fig. 2A shows that a band of similar intensity was detected for each mutant. These results were corroborated by autophosphorylation assays of the TodS fragments NTodS-long and CTodS-long (Fig. 1B), each of which contain a single autokinase domain and a receiver domain.

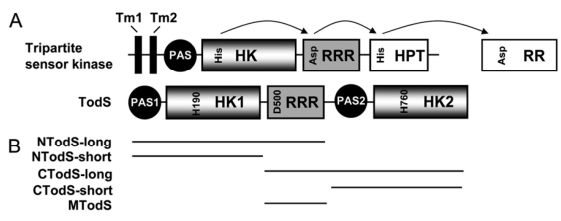

Fig. 1. *Schematic drawing of the domain arrangement of tripartite sensor kinases and TodS (A)* Tm, transmembrane region; PAS, PAS-type sensor domain; HK, histidine kinase domain; RRR, response regulator receiver domain; HPT, histidine-containing phosphotransfer domain. The domain arrangement for tripartite sensor kinases is taken from AcrB (5). The phosphorelay is indicated. TodS domains were predicted by SMART (42). *Summary of truncated TodS versions used in this study (B)* The limits of recombinant fragments were determined according to the domain annotation by SMART and secondary structure prediction of TodS. Fragment limits were placed in regions for

which a turn or coil secondary structure was predicted. All fragments were produced as His-tag fusion proteins and purified as detailed for TodS in (15). For further information see Table 1 and Materials and Methods.

As expected, both proteins were phosphorylated (Fig. 2B) although the amount of phosphorylated CTodS-long was less than NTodS-long. These results show that TodS possesses two functionally active kinase domains with similar basal activity.

Table 1. Bacterial strains and plasmids used.

Strains/Plasmids	Characteristics	Refs.
E. coli BL21(DE3)	F$^-$, *ompI*, *hsdS*$_B$ ($r_B^- m_B^-$)	(44)
P. putida DOT-T1E		
	phototroph, Tol$^+$ (*tod* pathway)	(45)
P. putida DOT-T1E*todST*	DOT-T1E, *todST*::Km, Tol$^-$, + pMIR66, Gm^R	(14)
pMIR77	Tc^R, P$_{todX}$::'*lac*Z inserted in pMP220	(14)
pMIR66	Gm^R, *todST* genes in pBBR1MCS-5	(14)
pTodS	pET28b containing *todS*	(15)
pNTodS	pET28b *todS* bps 1-1752 (aa 1-584)	(15)
pCTodS	pET28b *todS* bps1356-2934 (aa 452-978)	(15)
pHK1	pET28b *todS* bps 49-1347 (aa 16-449)	This work
pRRR	pET28b *todS* bps 1348-1755 (aa 450-585)	This work
pHK2	pET28b *todS* bps 1756-2928 (aa 586-976)	This work
pAB1	pTodS containing H190A	This work
pAB2	pTodS containing D500A	This work
pAB3	pTodS containing H760A	This work
pAB4	pMIR66 containing H190A	This work
pAB5	pMIR66 containing D500A	This work
pAB6	pMIR66 containing H760A	This work
pJLTodT	pET28b containing *todT*	(15)
pTodTD57A	pJLTodT containing TodTD57A	(15)

Gm^R, Km^R, Tc^R resistance to gentamicin, kanamycin and tetracycline, respectively. Tol$^+$/Tol$^-$ indicates that the strain grows or fails to grow on toluene, respectively.

Toluene binding to the N-terminal PAS domain increases phosphorylation of its neighboring autokinase domain. We have shown previously that effector molecules such as toluene bind exclusively to the N-terminal PAS domain of TodS (Fig. 1, 15, 16). Since this SK has two functional autokinase domains, effector binding could stimulate either one of the domains or potentially both. To address this question autophosphorylation assays of mutants TodSH190A and TodSH760A were repeated in the presence of toluene. No alteration in the phosphorylation state as compared to the absence of toluene was observed for mutant TodSH190A, whereas a significant increase in phosphorylation in response to toluene was noted for mutant TodSH760A (Fig. 2A). Since phosphorylation of the latter mutant is solely due to the action of the N-terminal kinase domain, our data indicate that toluene increased the phosphorylation level of only the latter kinase domain.

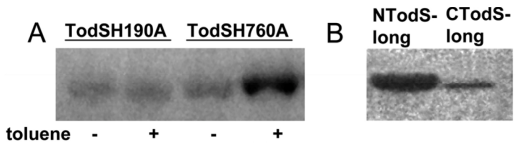

Fig. 2. *Autophosphorylation of site-directed mutants and fragments of TodS.* A) Protein at 20 μM was incubated with 200 μM ATP (containing 4μCi [γ-^{32}P-ATP]) for 20 min prior to analysis by SDS-PAGE. Autophosphorylation of TodSH190A and TodSH760A in the presence and absence of 100 μM toluene. Both mutants have similar basal activities but toluene stimulates only TodSH760A. B) Autophosphorylation of NTodS-long and CTodS-long in the absence of toluene.

Evidence for an intramolecular phosphorelay at TodS. TodS is predicted to contain three phosphorylatable amino acids: H190 and H760 of the autokinase domains and D500 of the response regulator receiver domain (Fig. 1). It has been reported that recombinant domains of large proteins can associate in a native-like fashion without significant loss of protein activity when mixed *in vitro* (24). To provide proof for phosphotransfer between the different domains of TodS we generated proteins corresponding to the NTodS-short, MTodS and CTodS-short fragments (Fig. 1B), each of which contained a single phosphoaccepting residue (H190, D500 and H760, respectively). These 3 proteins with a single phosphoaccepting residue were subjected to autophosphorylation assays.

The data available to date on response regulator receiver domains indicate that this domain cannot be phosphorylated by ATP. To verify whether this also holds for TodS, MTodS (Fig. 1B) was subjected to phosphorylation assays with [γ-^{32}P] ATP, which unequivocally revealed that it was not phosphorylated by ATP (not shown). Satisfactory autophosphorylation was observed for NTodS-short, but CTodS-short was devoid of activity (not shown). Therefore, attempts were focussed on detecting transphosphorylation between NTodS-short and MTodS, containing H190 and D500, respectively. To this end NTodS-short was phosphorylated in the presence of toluene and [γ-^{32}P-ATP] and applied to a gel filtration column to separate the protein from ATP. A stoichiometric amount of MTodS was added to the resulting protein and samples were taken at different times for SDS-PAGE analysis. As shown in Fig. 3, the band corresponding to NTodS-short gradually disappeared whereas a band representing phosphorylated MTodS became visible. Maximum MTodS phosphorylation was seen after 10 min, before this protein gradually dephosphorylated. This strongly suggests the existence of intramolecular phosphotransfer in TodS.

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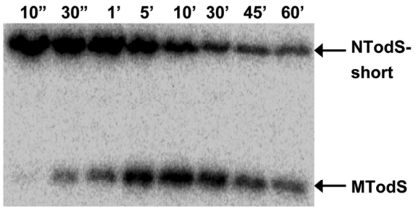

Fig.3. Transphosphorylation between TodS fragments. NTodS was phosphorylated with 200 μM ATP (containing 4μCi [γ-^{32}P-ATP]), and the solution was applied to NAP-5 gel filtration columns (GE Healthcare) to separate protein from ATP. An equimolar amount of MTodS was added to the protein fraction. At the time intervals indicated samples were removed for SDS-PAGE analysis.

TodT transphosphorylation occurs from the C-terminal kinase domain of TodS. Since TodS contains two kinase domains, transphosphorylation to TodT may occur from either of the domains or from both. To cast light on this issue transphosphorylation between various TodS fragments and TodT was studied (Fig. 4). To this end TodS polypeptides were phosphorylated which was followed by the addition of an equimolar amount of TodT. The first two lanes in Fig. 4 illustrate that NTodS-short and NTodS-long (Fig. 1) are phosphorylated by [γ-^{32}P] ATP. However, no transphosphorylation to TodT was detectable. Lane 3 shows that CTodS-long was phosphorylated, although to a lower extent than NTodS as already noted in Fig. 2. However, the addition of TodT to CTodS-long as shown in lane 4 of Fig. 4 resulted in a quantitative transfer of phosphate to TodT. The last lane, representing a mixture of NTodS-short, MTodS and TodT, was another control experiment which illustrated that NTodS-short maintained its capacity to transphosphorylate MTodS, but failed to phosphorylate TodT. In summary, these data are consistent with phosphotransfer from the C-terminal kinase domain towards TodT.

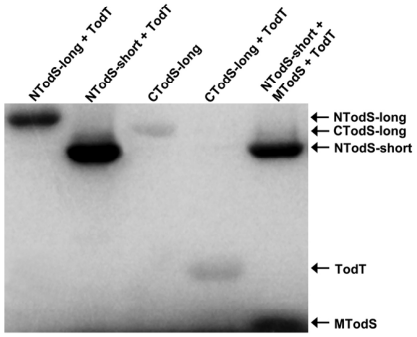

Fig. 4. *Transphosphorylation between TodS fragments and TodT.* TodS fragments NTodS-short, N-TodS-long and CTodS-long at 20 μM where incubated with 200 μM ATP containing 4 μCi [γ-^{32}P-ATP] for 20 min. An equimolar amount of MTodS was added to the sample in lane 5, and the corresponding amount of buffer to the remaining samples. After 10 min of incubation an equimolar amount of TodT was added to the samples in lane 1, 2, 4 and 5, and the corresponding amount of buffer was added to the sample in lane 3. Samples were taken after another 10 min of incubation.

Analysis of site-directed TodS mutants confirms the multiple-step phosphorelay mechanism and TodT transphosphorylation from H760. To provide further support for the existence of a His190→D500→His760 phosphorelay, transphosphorylation assays were conducted with TodT and the three TodS mutants in which the phosphoaccepting amino acid was replaced with alanine. Equal amounts of TodS, TodSH190A, TodSD500A and TodSH760A were incubated with [γ-^{32}P] ATP before TodT was added. Experiments were carried out in the presence of toluene which implies that activity of HK1 domain is stimulated whereas HK2 domain operates at basal activity (Fig. 2A). Transphosphorylation assays of the wild-type protein showed bands for both TodS and TodT (Fig. 5A). The band corresponding to TodT dominated, indicating that transphosphorylation was a relatively rapid process, which is in agreement with studies of other TCSs (25).

However, transphosphorylation assays of TodSH760A showed only a single strong band at the TodS level (Fig. 5A), whereas no band was seen for TodT. The absence of any transphosphorylation demonstrates that the phosphate departed from residue H760 in TodS towards TodT, which confirms data presented in Fig. 4. The strong band corresponding to TodSH760A confirms that toluene stimulated phosphorylation, but in the absence of transphosphorylation the phosphate remained at H190 and D500. Most interestingly, the amount of phosphorylated TodT seen in the transphosphorylation of TodSH190A and TodSD500A was very similar, probably because of the weak basal activity of the C-terminal kinase domain. Previous experiments have shown that toluene stimulated autophosphorylation of TodSD500A (not shown) but not of TodSH190A (Fig. 2). However, this stimulation was not reflected as an increase in phosphorylated TodT in transphosphorylation assays (Fig. 5B). These findings indicate that D500 was essential to transmit an increase in phophorylation of the N-terminal kinase domain to the C-terminal kinase domain. The notion that the His190→D500→H760 phosphorelay is interrupted by replacing D500 was supported by the fact that the amount of phosphorylated TodSD500A was larger than the phosphorylated wild-type TodS.

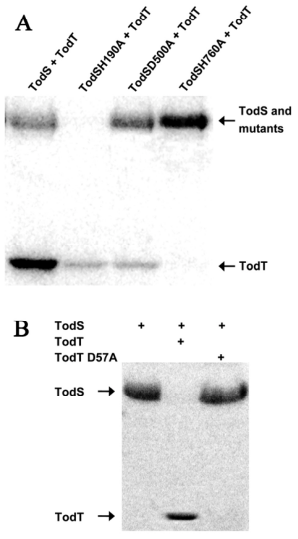

Fig. 5. *Transphosphorylation between wild-type and mutant forms of TodS and TodT.* A) Transphosphorylation between wild-type/mutant TodS with TodT. B) Transphosphorylation between TodS and wild-type/mutant TodT. Wild-type and mutant TodS at 20 μM were incubated with 200 μM ATP (containing 4μCi [γ-^{32}P-ATP]) and 100 μM toluene for 20 min prior to the addition of wild-type or mutant TodT. Samples were taken after 15 min.

TodT binds TodS with 1:1 stoichiometry: the presence of ATP is essential for interaction. We used isothermal titration calorimetry (ITC) (26) to study the interaction between TodS and TodT. ITC experiments of TodS with TodT at three different temperatures did not reveal binding heats, which suggested, unexpectedly, that the two proteins did not interact. Another series of ITC titrations of TodS with different nucleotides such as ATP, ADP and the non-hydrolyzable ATP analogue AMP-PNP revealed, surprisingly, that TodS bound only ATP. We then studied whether TodS interacted with TodT in the presence of 1 mM ATP, by adding a freshly prepared solution to both proteins prior to the experiment. The raw data from this experiment (Fig. 6) showed that initial peaks were broad at their base, which can be explained by the fact that initial binding heats were followed by heats from the kinetically slower process of TodS-TodT transphosphorylation. This distorted the enthalpic and entropic contribution, but did not impact on the binding constant or the stoichiometry of the interaction (26). After each injection the signal returned to the baseline, which enabled correct peak integration and curve fitting (Fig. 6, lower panel). Data analysis revealed that both proteins interacted with a stoichiometry of 1.06 ± 0.1 and a dissociation constant of 214 ± 10 nM. The control experiment with TodT/ATP and buffer/ATP yielded small, uniform peaks. When this experiment was repeated in the presence of 1 mM ADP, AMP-PNP or 100 μM toluene, no interactions was observed between TodS and TodT. Subsequently the interaction between NTodS-long and CTodS-long with TodT was studied in an analogous fashion, i.e. in presence of 1 mM ATP. CTodS-long was found to bind to TodT with an affinity of 65 ± 7 nM (not shown). No binding heats were observed for the titration of NTodS-long with TodT.

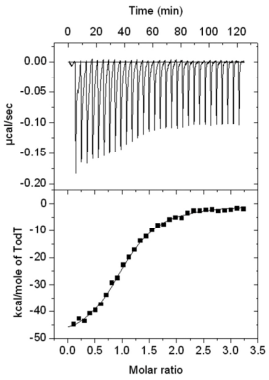

Fig. 6. *Isothermal titration calorimetry study of the interaction between TodS and TodT.* Upper panel: Raw titration data of 1.6 μM TodS with aliquots of 23 μM TodT in the presence of 1 mM ATP. For further experimental details see Materials and Methods and (26). Lower panel: Integrated and dilution-corrected peak areas from the raw data. The curves were fitted with the "one-binding-site" model of the MicroCal version of ORIGIN.

TodT D57 is the last step in the TodS/TodT phosphorylation cascade. The data presented so far suggested the existence of an intramolecular phosphorylation cascade at TodS, and that H760 of TodS is the amino acid which donates the phosphate to the response regulator. Sequence analysis of TodT predicted D57 as the phosphate-accepting residue. To verify this prediction transphosphorylation assays between TodS and TodT or TodTD57A were carried out (Fig. 5B). No transphosphorylation was detected for the TodTD57A mutant, which indicated that this amino acid is the last step in the H190-D500-H760-D57 phosphorelay in the TodS/TodT TCS.

In vivo proof for the deduced phosphorylation cascade in the TodS/TodT system. The effect of the amino acid substitutions H190A, D500A and H760A was evaluated *in vivo* by measuring expression from P_{todX} promoter fused to '*lacZ*. Plasmid pMIR77 bearing the P_{todX}::*lacZ* fusion was transformed in the knockout mutant *P. putida* DOT-T1E*todST*- bearing or not pMIR66 (*todST*) or plasmids pAB4, pAB5 and pAB6, which are pMIR66 derivative with a *todS* mutant allele that produces TodSH190A, TodSD500A and TodSH760A, respectively (Table 1). In cells with pMIR77 plus pMIR66, expression from P_{todX} was basal and low (25-40 MU) in the absence of toluene, and increased to 5000 ± 500 MU when toluene was added. However, in the three strains bearing pAB4, pAB5 and pAB6 encoding the above mentioned three TodS mutants, expression from P_{todX} was below 30 MU regardless of the presence of toluene. Therefore, the mutation of H190, D500 and H760 entirely abrogate toluene-mediated induction of P_{todX} activity *in vivo*. These data entirely support (a) that toluene stimulates phosphorylation of H190, (b) that D500 is essential for intra-TodS phosphotransfer and that there is no direct link between the two kinase domains and (c) that H760 is the site for transphosphorylation of TodT.

Discussion

TodS belongs to a new family of multiple-step phosphorelay sensor kinases. Two-component systems contain as basic components a SK and a response regulator. Data currently available indicate that response regulators involved in transcriptional control form a relatively well-conserved family of proteins. Galperin (27) showed that over 80% of DNA binding response regulators belong to either the NarL or OmpR families, which are

characterized by an N-terminal receiver domain and a C-terminal helix-turn-helix motif containing DNA binding domain. In contrast to the relative conservation of response regulators is the extreme functional and structural diversity of SKs (28), which can be membrane bound or not, have a large variety of different sensor domains in either the periplasm or cytosol, frequently show domain duplication, are in some cases the target of other regulatory proteins, and recognize signals in many different ways (28). To fully comprehend the physiological relevance of TCSs it appears indispensable to explore the functional and structural diversity of SKs.

TodS is clearly different from other SKs characterized to date because it contains two modules each of which has a sensor and a kinase domain. Based on our data we propose the functional model of TodS depicted in Fig. 7. Initial binding of toluene increases the phosphorylation state of the N-terminal autokinase domain. Phosphate is subsequently transferred to the internal receiver domain, and eventually to the C-terminal module before it is transferred to D57 of TodT, which is the primary regulator of the P_{todX} promoter.

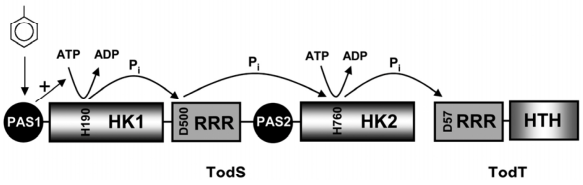

Fig. 7 Mechanistic model for the function of TodS. The binding of effectors, such as toluene, occurs at the N-terminal PAS sensor domain, which stimulates phosphorylation of its neighboring kinase domain. Phosphate is then passed on to D500 of the internal response regulator receiver domain and subsequently to H760 of the C-terminal kinase domain, which is the site for transphosphorylation of TodT. The C-terminal kinase domain has autokinase activity and it remains to be elucidated whether this is modulated by potential recognition of a signal by the PAS 2 sensor domain.

Effector binding stimulated phosphorylation of His190, whereas TodT phosphorylation occurred from His760—evidence of communication between the two kinase domains. Available data support the absence of a *direct* communication between the two kinase domains, and that D500 is essential for establishing this link. This statement is based on two lines of evidence. Firstly, *in vitro* autophosphorylation studies show that toluene stimulates activity of TodSD500A (increased phosphorylation at H190) but not that of the H190A mutant. However, the amount of phosphorylated TodT in transphosphorylation experiments withTodSD500A and TodSH190A is similar (Fig. 5B). Secondly, no toluene-mediated stimulation of transcription from P_{todX} was observed in *in vivo* gene expression studies of mutant D500A. At present we have not explored whether the phosphorelay occurs in the inverse direction (H760→D500→H190), which could be of functional and physiological importance.

All multiple-step phosphorelay TCSs characterized so far contain an HPT domain (11), and this domain appeared to be essential for this particular mode of action of a TCS. Here we demonstrate the existence of a multiple-step phosphorelay in TodS involving two autokinase domains and a receiver domain. The multiple-step phosphorelay is thus a mode of functioning which is not restricted to HPT domain-containing proteins. This is thus the first example of a multiple-step phosphorelay which does not involve an HPT domain. SKs predicted to contain two autokinase domains are present in numerous other bacteria such as *Legionella pneumophila*, *Bradyrhizobium* sp., *Stigmatella aurantica* and *Anabaena variabilis*. It is thus possible that these proteins also use a multiple-step phosphorelay mechanism.

Although tripartite SKs and TodS exhibit a different domain organization (Fig. 1), both operate using an intramolecular phosphorelay (29). What are the advantages of multiple-step phosphorelay systems as compared to prototypal SKs? There is evidence that additional steps in the intramolecular phosphorylation process can be subject to additional regulatory steps, thus providing a way to fine-tune the regulatory response by incorporating additional signals. An example of such an additional regulatory element is the SixA protein, which has phosphatase activity toward the HPT domain of ArcB (30). Furthermore, many response regulators can be phosphorylated by small phosphate donors such as acetyl phosphate, whose cellular concentration reflects the metabolic state of the cell (31). The phosphorylation of response regulators by low-molecular-weight compounds was shown to be of physiological relevance (31) and it remains to be established whether these compounds also act on SKs such as TodS, which contain internal response regulator receiver domains.

Another potential mode of fine-tuning of the transcriptional response might be associated with the C-terminal PAS domain of TodS (Fig. 7). The presence of a sensor domain neighboring the domain harboring the ultimate phosphorylation site is specific for the TodS family, and is not found in the tripartite SK family (Fig. 1). Another major difference is that the HK2 domain of TodS was shown to possess autokinase activity (Fig. 2) whereas HPT, the corresponding domain in ArcB, is devoid of this activity (4). This implies that the ultimate phosphoacceptor of TodS, H760, can receive phosphate from either ATP or D500. In this context it appears possible that the autophosphorylation activity of the C-terminal autokinase domain is modulated by an as yet unknown signal which is recognized by the C-terminal PAS sensor domain. For the TodS homologue StyS it was proposed that the C-terminal sensor domain might respond to the cell's redox potential (32). The existence and mechanism of action of a secondary signal which might modulate TodS activity remain to be documented.

Requirement of ATP for TodS/TodT interaction. There is no doubt that the TodS/TodT system represents a novel regulatory mechanism and presents specific features not found in other two-component systems. A feature which clearly distinguishes TodS/TodT from other systems concerns the molecular interaction between the two proteins. A large series of studies on other TCSs converged on the notion that both components interact *in vitro* with relatively modest affinity for a protein-protein interaction, as exemplified by FixL/FixJ (K_D=4 μM, 33), EnvZ/OmpR (1.2 μM, 34), CheA/CheY (0.9-1.3 μM, 35) and EvgS/EvgA (1.2 μM, 36). In all cases the presence of ATP was not required for molecular interaction. ITC experiments of TodS with TodT at three different temperatures did not reveal binding heats, a finding which strongly suggests that the two proteins do no interact. The same result was obtained in the presence of 100 μM toluene. However, when the experiments were repeated with both protein solutions containing 1 mM ATP, a sigmoid binding curve was observed (Fig. 6). We hypothesize that ATP binding at TodS induces conformational changes enabling interaction with TodT. The cellular ATP concentration is estimated to be between 3-5 mM implying TodS saturation and the potential physiological relevance of this finding awaits further study.

Specificity of two-component systems. A phenomenon which is still poorly understood concerns the structural basis of specificity between SKs and response regulators. Many organisms possess more than 20 TCSs (37), both components of which interact in a relatively specific manner. In cases where cross-talk between different TCSs is observed, it remains unclear whether this is artefactual or of physiological relevance (25). In their analysis of the 3D structure of the SpoB/SpoF complex, Zapf *et al* (38) concluded that the interaction between SK and response regulator is mediated by conserved amino acids—a factor that makes further elucidation of the specificity of interactions difficult.

Grebe & Stock (39) aligned all available sequences of TCSs for which proof of mutual interaction was available. SKs were found to cluster in 11 families, and response regulators in 6 different families. The authors were able to show that members of a given subfamily of SKs interact almost exclusively with one family of response regulators. Using the classification of Grebe & Stock (39) the N- and C-terminal kinase domains of TodS belong to classes HPK1a and HPK4, respectively, whereas TodT belongs to the RA4 subfamily. Both authors demonstrated that members of the HPK4 family interact almost exclusively with response regulators of the RA4 family. This confirms our data, which showed that TodT interacts with the C-terminal kinase domain of TodS, and underlines that minor differences in sequences determine the specificity of interactions between the two components.

SKs, which are primordial for bacterial signal transmission, are absent in the animal kingdom and rarely found in lower eukaryotes such as fungi and protozoa (40). Of the 11 classes of SKs mentioned above (39) one is found in lower eukaryotes. This led to the proposition that eukaryotes have acquired SK genes by lateral gene transfer from bacteria (40). In this study it is obvious that TodS operates through a sophisticated mechanism. Interestingly, a TodS-like protein is present in the eukaryote *Dictyostelium discoideum* (Q54SP4). Several functionally active SKs have been described in *Dictyostelium*, such as DokA (41) and DhkC (42). *Dictyostelim discoideum* and *P. putida* are soil organisms, which supports the possibility of lateral transfer of the *todS* gene between these organisms. This might place TodS at the border which separates the eukaryotic and prokaryotic kingdoms of life.

Materials and Methods

Bacterial strains and plasmids. The bacterial strains and plasmids used are listed in Table 1.

Recombinant DNA techniques. Site-directed mutations in the *todS* gene were introduced into plasmids pTodS and pMIR66 (Table 1) using the Quick Change Mutagenesis Kit (Stratagene) according to the manufacturer's instructions. Oligonucleotides used are listed in the supplementary material. For the generation of truncated TodS versions the corresponding gene fragments were amplified by PCR using pMIR66 as template. The resulting products were then cloned into pET28b using the *Nhe*I and *Bam*HI sites. All plasmids were verified by sequencing the insert and flanking regions.

Overexpression and purification of proteins. TodS, its site-directed mutants and fragments were expressed and purified as detailed for TodS in (15). TodT and TodTD57A were purified as described for TodT in (15).

Phosphorylation assays. Prior to analysis all proteins were dialyzed into 50 mM Tris-HCl, pH 7.5, 200 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 10% (v/v) glycerol, 2 mM DTT.
Autophosphorylation of TodS, its fragments or site-directed mutants was carried out with 200 μM ATP containing 4 μCi [γ-^{32}P]. In some cases 100 μl toluene was added. At different times samples were removed, mixed with 6×Laemmli buffer and analyzed by SDS-PAGE.
For transphosphorylation assays between TodS and TodT/TodTD57A, 20 μM TodS was incubated for 20 min with 200 μM ATP containing 4μCi [γ-^{32}P-ATP], then a slight molar excess of TodT was added. Reactions were stopped after 15 min.
For transphosphorylation experiments between fragments of TodS, NTodS- at 60 μM was phosphorylated as stated above. ATP was then separated from the protein by gel filtration on NAP-5 columns (GE Healthcare). Protein was then incubated with equimolar amounts of MTodS.
For transphosphorylation assays between TodS fragments and TodT, TodS polypeptides were phosphorylated as stated above. MTodS or an equivalent volume of buffer was then added. After 10 min an equimolar amount of TodT was added. Samples were taken after 10 min for SDS-PAGE analysis.

Isothermal Titration Calorimetry (ITC) - TodS and TodT were dialyzed into 40 mM Tris-HCl, pH 7.3, 200 mM KCl, 300 mM imidazole, 10 mM $MgCl_2$, 1 mM DTT, 10% (v/v) glycerol. Experiments were carried out at 25 ºC using a VP-microcalorimeter (MicroCal, Northampton, MA). TodS or fragments thereof at 1.6 μM were titrated with 23 μM TodT. Injection of 23 μM TodT into dialysis buffer resulted in small and uniform peaks typical of dilution heats. Data were analyzed with the MicroCal version of ORIGIN (Northampton, MA, USA).

β-Galactosidase assays. For these assays we used *P. putida* DOT-T1E *todST,* which is a mutant for *todST,* bearing pMIR77 (P_{todX}::*lacZ*) and pMIR66 or derivatives thereof (Table 1). LB medium was inoculated with a single colony from LB agar plates and cultured at 30°C overnight. These cultures were diluted 100-fold in the same medium supplemented or not with 1.5 mM of toluene, and cell growth was monitored over time. When the cultures reached an OD_{660} of 0.8 β-galactosidase activity was determined as described by Miller *et al* (43).

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V. Discusión General / Global discussion

Over the past decades, characterised by an exponentially increasing number of sequenced bacterial genome accompanied by a functional analyses of genes, the scientific community has obtained a clear vision on the complexity and versatility of bacterial metabolism. However, the sole description of the metabolic potential of bacteria has to be completed by studies of the associated regulatory processes to fully understand an organism. In the context of biodegradation it is therefore of primordial importance to determine under which experimental conditions a given metabolic machinery is operational.

Bacteria make use of periplasmic or cytosolic sensor/regulator systems to directly monitor environmental changes and to alter accordingly biological processes, which is in most of the cases the transcriptional process. A large part of these systems are two-component systems (TCS), to which cells dedicate near 1% of their genomic DNA (Ulrich *et al.* 2005, Ashby, 2004). Upon signal reception at the sensor domain of a SHK, which is located with high frequency in the periplasm, the autophosphorylation activity of the histidine kinase is modulated, which in turn impacts on the transphosphorylation activity towards its cognate response regulator (RR). Genome analyses reveal that there is a direct link between the extent of environmental changes and the number of TCS (Ashby, 2005). For instance soil bacteria, inhabiting a very variable environment, possess larger amounts of TCS than for instance enterobacteria or pathogenic bacteria that live in a more stable environment. The latter class of bacteria, for example, was shown to carry between none and about 15 TCSs, while soil bacteria have an average of about 50 TCSs. Especially versatile bacteria such as the species under study in this work, *Pseudomonas putida,* possess more than 90 TCS (Ashby, 2005).

The involvement of TCSs in the degradation of aromatic compounds was first described for strain F1 of *P. putida,* where toluene metabolism is regulated by the TodS/TodT TCS (Lau *et al*., 1997). Since the mid-90s an increasing number of degradation pathways of aromatic compounds have been shown to be regulated by TCSs (Ruíz *et al.*, 2004). This is exemplified by the regulation of pathways for styrene metabolism in *Pseudomonas* (Leoni *et al.*, 2003), but also in a *Rhodococcus* strain for biphenyl degradation (Pieper, 2005) and even in denitrifying bacteria such as *Aromatoleum* involved in anaerobic toluene metabolism (Heider, 2007). TodS/TodT homologues can also be found in other

strains like *Dechloromonas aromatica* (Coates *et al.*, 2001) or *Methylibium petroleiphilum* (Kane *et al.*, 2007) and it is likely that these homologues are also involved in aromatic compounds degradation, although their precise regulatory role has not been established. More interestingly, homologues are also present in the eukaryotic kingdom of life, such as in the slime molde *Dictyostelium discoideum*, which is also a soil organism. The fact, that these organisms share the same habitat supports the hypothesis of a horizontal gene-transfer event and not a convergent evolution of this type of TCS.

In *Pseudomonas*, the expression of most catabolic pathways of aromatic compounds is regulated by OCSs (Diaz and Prieto, 2000) which act as positive regulators of transcriptional activity in response to toluene, relevant toluene metabolites or other substrates, and in co-operation with various cofactors and global regulators (IHF, Crc, CyoB, RelA, PTS, Cases and de Lorenzo, 2005; Shingler, 2003; Aranda-Olmedo *et al.*, 2005). The TOD pathway on the other hand is regulated by the TCS TodS/TodT. TodS and homologous proteins like TmoS, TutC or StyS are so far the only members of the double sensor-histidine kinase family with an ascribed function. So far, this type of SHKs are known to be involved in the regulation of aromatic compounds metabolism and therefore suggest that the above mentioned systems regulate exclusively the degradation of monoaromatic compounds.

The proteins TodS and TodT under study were shown to specifically regulate the TOD pathway and to form a TCS where both proteins are essential in the activation process (Lau *et al.*, 1997, Mosqueda *et al.*, 1999). This doctoral thesis was undertaken to characterize TodS for which at the beginning of this work only a limited amount of experimental data were available. Emphasis was given to identifying the signals which were sensed by TodS and how signal transmission from TodS to TodT is accomplished. Getting insight into these issues is likely to further the understanding of the complex architecture of this multidomain protein.

Pseudomonas putida DOT-T1 and F1 are both able to use benzene, toluene and ethylbenzene as the sole carbon and energy source. These three compounds were expected to be specifically sensed by TodS. Suprisingly, *in vivo* around 20 different aromatic compounds were identified as inducers of the TOD pathway (Lacal *et al.*, 2006). ITC studies revealed that all compounds

which activate the pathway were found to bind to purified TodS. In addition, autophosphorylation studies showed that the binding of these compounds increased autophosphorylation *in vitro*. This apparent "energy-wasting" has been described also in other bacteria involved in mineralization of aromatic compounds and was called "gratuitous induction" (Martínez-Pérez *et al.*, 2007), a consequence of different substrate and inducer profiles, probably due to structural similarity of the sensed compounds. Although the TOD pathway specifically metabolizes only toluene, benzene and ethylbenzene to TCA cycle intermediates, the initial enzyme in this process, the toluene-2,3-dioxygenase has a broader substrate profile as shown in strain F1 (Boyd and Sheldrake, 1998; Gibson and Parales, 2000). Studies on the substrate specificity of the TOD pathway in strain F1 have revealed that *n*-propylbenzene, *n*-butylbenzene, isopropylbenzene and biphenyl can be biotransformed to only ring-fission dead-end products (Choi *et al.*, 2003; Cho *et al.*, 2000; Gibson *et al.*, 1968) but not futher metabolized by the TodF hydrolase, with a narrow substrate range (Furukawa *et al.*, 1993). Choi *et al.* (2003) showed in an adapted strain F1 able to grow on *n*-propylbenzene, *n*-butylbenzene, isopropylbenzene and biphenyl, that a hydrolase of another pathway in the same organism (CmtE, *cmt* pathway) able to degrade these dead-end products was recruited by the TOD pathway in a "metabolic bypass" to render metabolites that can than be further degraded to TCA intermediates by the TOD pathway. It would be interesting to provide data on whether how many of these non-substrate inducers are biotransformed in strain DOT-T1E. However, strain DOT-T1E is not able to grow on any of the mentioned alkylbenzenes and the dead-end products might be extruded and degraded by other organisms' enzymes. Further studies are necessary to clarify this issue. However, if a possible explanation of the broad inducer profile the promiscuity of the dioxygenase could be, communication between the structural and the regulatory genes would be necessary. Up to date only one example of such communication between pathway genes and their regulatory proteins exists (Martínez-Pérez *et al.*, 2007).

Functional activities of the SHK TodS

As the pathway substrates were hypothezised to be TodS effectors, initial studies were aimed in understanding where toluene binds to TodS and how effector binding to TodS is transformed into an effective response.

Fortunately TodS, predicted to be a cytosolic protein, could be purified as soluble recombinant full-length protein. *In vitro* studies of SHKs are normally hampered by their transmembrane character which makes it difficult to generate full-length active protein. TodS is therefore among the few SHKs which have been produced as full-length protein and might therefore serve as a model protein.

Using Isothermal Titration Calorimetry (ITC) we have shown that toluene binds with high affinity to TodS (K_D: 0.7μM). This affinity is significantly higher than that for other systems such as citrate binding to CitA (5.5μM), or affinities of about 35 μM and 300 μM for the interaction of NarX and PhoQ with their respective signals nitrate and Mg^{2+} (Gerhartz *et al.*, 2003; Lee *et al.*, 1999; Lesley and Waldburger, 2001). This high affinity of toluene binding to TodS might indicate that an effective response is already triggered at low toluene concentrations, which could be a evolutionary advantage as the compound induces its metabolization at relatively low cytosolic concentrations. As TodS has two putative ligand-binding PAS domains, we generated recombinant fragments NTodS and CTodS (Fig. 1, chapter 2, p. 47), and identified the N-terminal PAS domain as the effector binding site. Functionally, the PAS domain is a protein module generally associated with sensing of a dazzling variety of environmental signals. PAS domains like the N-terminal domain in TodS can directly sense small ligands or peptides like host antimicrobial cationic peptides in pathogenic bacteria (PhoQ sensor in *Salmonella typhimurium*, Bader *et al.*, 2005) producing changes in global gene expression in order to combat the host immune system. Detection of other signals such as voltage and nitrogen availability has been found not to require cofactors in the PAS domain, too (Kamberov *et al.*, 1995; Morais-Cabral *et al.*, 1998). Only a small subset of PAS domains are associated with cofactors, but this group includes some of the best-characterized members of the PAS family. Detection of signals such as diatomic gases, light, and redox potential is known to require cofactors such as

heme, FMN or parahydroxycinnamate, and FAD, respectively. Heme-groups can be found in PAS domains of oxygen-sensing proteins like FixL from *E. coli* (Gilles-González *et al.*, 1991; Gilles-González and González, 2004) and *Bradyrhizobium japonicum* (Hao *et al.*, 2002); chromophores like FMN in PAS domains of phototactic bacteria (Christie *et al.*, 1999); and FAD in the aerotaxis signal transducer Aer or the Arc redox switch of *E. coli* (Bibikov *et al.*, 2000; Georgelis *et al.*, 2001). The presence of two PAS domains in TodS implies that it is likely to be a dual sensor responding to different signals. Different clues about the second domains' signal come from two works: Lau *et al.* (1997) presumed an oxygen sensory domain. Because the *tod* pathway is oxygen dependent this could be a valuable hypothesis. However, spectral analysis of purified TodS did not show the characteristic absorption of the necessary heme-cofactor. Moreover, heme-groups were shown to be co-ordinately linked to the protein through the imidazole (ImH) side chain of a proximal conserved histidine, and the C-terminal PAS domain of TodS does not contain any histidine residues (Monson *et al.*, 1995; Hao *et al.*, 2002, Rodgers and Lukat-Rodgers, 2005). StyS is the TodS-homologue in the styrene degradation pathway and Rampioni *et al.* (2008) found the activity of StyS to be related to the redox potential and involved in carbon catabolite repression. In case of TodS this is a suitable possibility, TodS responding to two signals: toluene as the signal reflecting the mere need to activate the TOD pathway, and another signal integrating the metabolic status of the cell. We tested binding of different compounds involved in carbon catabolite repression and redox-sensing to the recombinant fragment CTodS. However, no binding was observed and therefore no answer to the question concerning the function of the C-terminal PAS domain could be found.

Subsequent experiments with TodS were focussed on the determination of autophosphorylation-, dephosphorylation- and transphosphorylation-activity towards the cognate RR with and without toluene. SHKs offer basically three modes to respond to signal input: 1) signal binding stimulates autophosphorylation 2) signal binding reduces phosphorylation and 3) signal binding apparently does not modulate autokinase activity and needs additional regulators. All three cases can be found in literature. Phosphorylation raises

upon signal perception in for example the PhoQ SHK of *Salmonella typhimurium* and is probably the most extended mode of response, leading to an increased affinity of the cognate RR PhoP to its target DNA-sequence (Bader *et al.*, 2005).

The second case, the reduction of phosphorylation upon signal sensing is found in the SHK CheA of *E. coli* involved in chemotaxis (Levit and Stock, 2002), where binding of an attractant inhibits CheA and consequently reduces phoshorylation of the RR CheY. In this case the RR directly interacts with the flagellar motor complex and not with a promoter sequence. Decreasing the level of phospho-CheY, the bacterium tends to continue swimming in favorable, attractant-rich direction. The third case is found in the RegB SHK controlling the redox switch between aerobic and anaerobic growth. RegB autophosphorylates at the same rate under both aerobic and anaerobic conditions confirming that an additional regulatory element is required to control/inhibit autophosphorylation. Activity of RegB was supposed to be regulated by its transmembrane domain increasing or lowering the dephosphorylation rate of the protein (Potter *et al.*, 2002).

The mechanism of action of TodS was shown to belong to the first group, as we could establish through *in vitro* phosphorylation studies that the binding of toluene in the presence of ATP to the N-terminal PAS domains of TodS was able to upregulate basal phosphorylation levels of TodS between 5-7 times. A toluene concentration at half the maximal autophosphorylation activity of 10 ± 2 μM was determined (Fig. 8, suppl. material, p. 79) The enhanced autophosphorylation rate of TodS in presence of the effector resulted in a higher transphosphorylation rate of TodT, confirming thus the *in vivo* data that suggested TodS-TodT to form a specific TCS, phosphorylated TodT acting as a positive regulator of transcription.

TodS has broad effector spectrum and binds both agonists and antagonists at the same site

As TodS was shown to bind toluene, the most potent inducer *in vivo*, purified TodS was subjected to further microcalorimetric titrations to determine the

effector profile *in vitro* and compare the *in vitro* binding affinities to the capacity of the compounds to induce gene expression *in vivo*. Five groups of structurally different compounds were defined and tested: benzene and single substituted benzene derivatives with an alkyl- or halogenated ring-substitution; disubstituted benzene derivatives in *ortho*-, *meta*- and *para*-position; the triple substituted trimethylbenzene (TMB) isomers; biaromatics and aliphatic hydrocarbons.

Our initial hypothesis was that all effectors which are active *in vivo* bind to TodS and that their binding affinities are probably positively related to the *in vivo* expression capacity. The results could not confirm this hypothesis as we found that, for instance, the compound with one of the highest affinities towards TodS, styrene (0.58 μM) induced *in vivo* transcription ~2.5 times less than toluene with lower binding affinity (Table 1, chapter 2, p. 47). This could probably be explained by the fact that not binding affinity but conformational changes produced upon binding affect directly their capacity to induce more or less effectively gene expression.

As we tested a broad range of effectors including those with *in vivo* induction capacity, we expected compounds which are inactive *in vivo* not to bind to TodS. This was the case for the non-aromatic compounds cyclohexane and 1-hexanol and also for the aromatic compounds benzamide and benzoate. Surprisingly, there were compounds which failed to induce expression from P_{todX} but bound with high affinity to TodS, like *p*-chlorotoluene, the compound with the highest overall binding affinity (0.28 μM). Therefore, we could classify the compounds studied into three classes: Those which do not bind to TodS *in vitro* and do not activate transcription *in vivo*; those ones, referred to as agonists, that bind to TodS and induce expression from P_{todX} and a third group including compounds that bind to TodS but which do not exhibit *in vivo* activity, referred to as antagonists. Therefore a main conclusion of this work was that not only the recognition of effectors is needed to trigger a regulatory response, but also the capacity of TodS to intramolecularily transmit the molecular stimulus created upon effector binding.

The next step was to identify whether both the agonists and antagonists share the same PAS effector binding site or whether, alternatively, each type binds to one of the both predicted PAS domains in TodS. We carried out ITC competition experiments to see whether TodS, previously saturated with an

agonist, was able to bind an antagonist (or *vice versa*) and tested the agonist toluene and the antagonists with similar binding affinities *o*-xylene, *o*-chlorotoluene and 1,2,3-TMB. In all cases the addition of a second effector did not produce any binding heats. To corroborate the hypothesis that both types of ligands bind to the same site, the ITC titrations were repeated with the NTodS and CTodS recombinant fragments of TodS (Fig. 7, and Table 3, suppl. material, p. 78/79) and both the agonists and antagonist were found to share the same binding site, the N-terminal PAS domain, the toluene binding site.

A total of 30 compounds were tested to establish an *in vitro* profile of the TodS effector binding properties. Aliphatic compounds were not bound by TodS and the aromatic benzene ring was found to be essential in signal recognition by TodS. A totall of 80% of the compounds tested were found to bind to TodS. However only 58% of these compounds were inducers of P_{todX} promotor activity *in vivo* (agonists). Within the monosubstituted benzene derivatives nitro-, halogen- and alkyl-substitutions up to -C_2 were found to be agonists. Longer sidechains reduced significantly the affinity and converted the effectors into antagonists. Within the disubstituted benzene derivatives, *ortho*-ring substitutions of chlorotoluenes and xylenes resulted in antagonistic behaviour whereas their *meta* and *para* counterparts were agonists. However, affinities of the three corresponding isomers were comparable, which underlines the notion that affinity does not determine the final regulatory response. Triple substituted compounds as the TMB isomers and biaromatic compounds were all found to be antagonists. In short, the broad effector profile encloses exclusively arenes (hydrocarbons that contain as structural unit the benzene ring) of one and two rings, agonists are monoaromatic compounds with a maximum of 2 benzene ring substitutions and short side-chains (≤ -C_2). Longer sidechains, *ortho*-substitutions, triply substituted benzene rings and biaromatics do not define antagonists but are indicative thereof (Fig. 6, suppl. material, p. 78). Compounds with two benzene rings were found to be the upper size limit of possible ligands as compounds with three benzene rings like naringenine or anthracene did not bind.

As the N-terminal PAS1 domain was identified as the agonist and antagonist binding site, a three-dimensional model of this domain was prepared using the 3D structure of the CitA sensor domain as template (Fig. 4, chapter 2, p. 49).

Alanine replacement mutants of hydrophobic residues exposed in the cavity of the model indicate a central role for the aromatic sidechain of F79, as affinities were reduced by a factor of ~350 for all tested agonists and antagonists. Autophosphorylation levels in the presence of agonists in the F79A mutant were reduced to basal levels (Fig. 9, suppl. material, p.79) Molecular recognition of aromatic compounds by proteins is normally conducted via pi-pi-stacking (Boehr *et al.*, 2002; Calero *et al.*, 2002) of aromatic sidechains and benzene derivatives tested in this work are proposed to directly interact with F79.

The subsequent aim was to identify the effect of agonist or antagonist binding on phosphorylation levels of TodS. This time the expected results could be confirmed and toluene, the agonist with the highest induction activity showed also the highest level of phosphoryaltion *in vitro.* In the presence of ethylbenzene, substrate and modest inducer *in vivo*, TodS showed only modest phosphorylation activity *in vitro*, too. This was also consistent for other inducers tested and a positive correlation between phosphorylation level *in vitro* and expression rate *in vivo* in the presence of agonists could be established. None of the antagonist was able to stimulate phosphorylation.

The final questions were thus whether the presence of both agonist and antagonist affect *in vivo* transcription activities if both type of effectors bind to the same site or, in other words whether antagonist binding reduces the stimulating effect of agonists.

To answer this question *In vivo* expression studies were carried out (Fig. 3, chapter 2, p. 48) where cultures induced with toluene were compared to cultures to which antagonists were added before toluene. These experiments showed that the higher the concentration and affinity of the antagonist, the lower the expression rate as compared to the culture only induced with toluene. We could conclude that antagonists compete for the same binding site also *in vivo* and inhibit toluene-mediated upregulation of autophosphorylation and therefore transcription activity. These data suggest a model in which agonist and antagonist binding involves different binding orientations within the ligand binding pocket, producing differential conformational switches and regulatory responses. Examples where the binding of antagonist to the agonist binding site have been observed before are FixL and AgrB. For FixL the kinetic, structural, and enzymatic studies to date point to a role of the ligand-induced changes in

the porphyrin shape, and particularly the positions of the heme propionates, in the regulatory conformational response (Gilles-González and González, 2004). AgrB is the sensor of the agr TCS which regulates quorum-sensing in *Staphylococcus aureus*. AgrB specifically binds an autoinducing peptide (AIP) and at least four specific groups of strains have been described, producing each group its own characteristic AIP. The authors (Lyon *et al.*, 2002) conclude that only AIPs of the own group are agonist for the SHKs AgrB. The AIPs produced by a bacterium belonging to a different group of bacteria has an antagonistic effect on AgrB, which is a consequence of binding to the PAS domain in a different orientation than the native AIP.

A general conclusion that can be drawn from this work is that both *in vivo* and *in vitro* approaches are necessary to fully understand the effector profile of a regulatory protein. *In vivo* approaches which measure transcriptional regulation allow differentiating inducing (agonists) from none-inducing compounds. However, none-inducing compounds might interact with the receptor and hamper the response of agonistic compounds. In this case, none-inducing compounds are called antagonists as they compete with the agonist for the same binding site, and can be identified by competitive *in vivo* studies as it was done in this thesis. However, *in vitro* binding studies are necessary to identify the molecular base for the inhibition observed *in vivo*. The identification of antagonist can be of utmost relevance in several research areas:

TCSs are extremely common in bacteria and play an important role in signal transduction for adapting to different stress factors. Since signal transduction in mammals occurs by a different mechanism, inhibition of SHKs is a potential target for antimicrobial agents against pathogenic bacteria (see review Matsushita and Janda, 2002). Inhibition of the gene expression regulated by TCSs was first achieved by Roychoudhury only 15 years ago (1993). Many sensor kinase inhibitors have been discovered since, mainly as a result of screening for kinase activity. However, it was shown that most of them bind to the kinase domain (Matsushita and Janda, 2002) and were shown to frequently to cause significant side effects in *in vivo* trials, an effect which is likely to be attributed to the inhibition of other ATP-utilizing proteins. An alternative to random screening for kinase activity inhibition and thus more specific approach could be the inhibition of SHKs with effector analogues – in cases were

effectors are known - focalized on TCS known to be involved in bacterial resistance like the KinA/SpoOF TCS of *Bacillus subtilis.*

Concerning the research area of this thesis, bioremediation, the data obtained on agonist/antagonist are of significant relevance. Petrol for example is a mixture of compounds which have agonistic and antagonistic effects on TodS activity. Therefore, data obtained are relevant for the biodegradation of aromatic compounds or for the development of biosensors. Antagonist binding could lower the sensibilty of such biosensors or mask the real concentration of contaminants. This depends on the real agonist/antagonist ratio, as we have seen that inhibition rises with antagonist concentration. Furthermore, as antagonists lower the expression rates of the toluene degradation pathways, this could be a handicap in applying microorganisms directly to fighting pollution with crude oil, slowing down the degradation process. However, this is a step forward in understanding how bacteria interact with an environment of different hydrocarbons and opens the question of how the antagonist-effect has to be considered.

New multi-step phosphorelay mechanism described for TodS

From the data discussed above, we know that all aromatic compounds tested, being agonist or antagonist, bind to the PAS1 domain and that the increase in the phoshorylation level of TodS as a consequence of agonist binding results in higher transphosphorylation to TodT. However, as the domain architecture of TodS is clearly different from other SHK characterized so far, with its two modules each containing a HK and a sensor domain, some questions remained open: Are both HK domains involved in the the modulation of the phosphorylation state in response to agonists? Are both domains functional? What is the role of the RRR domain? How do phosphoryl-groups reach the final receptor TodT? Which domain(s) of TodS does TodT interact with “to get the message”? Basically two scenarios were possible: i) TodT directly interacts with HK1, the domain which triggers autophosphorylation in response to agonists and the second sensor-kinase module (PAS2-HK2) responds to a different signal and serves a different regulator, the role of the RRR domain remaining open, or 2) other phosphorylatable residues of TodS are involved in

phosphotransmission towards TodT. In the latter case a putative second signal sensed by PAS2 could be integrated in the whole response.

To address these questions, two parallel experimental approaches were taken. Firstly, TodS fragments were prepared which contain only a single phophorylatable amino acid and, secondly, three full-length TodS mutants were prepared each having a phosphorylatable amino acid replaced by alanine (Busch *et al.*, 2009) The putative phosphorylation site of TodT (D57), the "end of the road" residue in the phosphorylation cascade, was also alanine-substituted (Lacal *et al.*, 2006). Phosphorylation and ITC assays conducted between recombinant fragments of TodS and TodT allowed to unravel the phosphorylation circuit between TodS and its cognate RR TodT as a multiple-step phosphorelay mechanism:

When an agonist is sensed, autophosphorylation of only the N-terminal HK domain is stimulated. Phosphorylgroups are then transferred from H190 to the internal RRR domain at D500, which bridges the communication between both HK domains. Signal transmission then departs exclusively form H760 to TodT, transphosphorylated in a 1:1 ratio at D57. S(H190→D500→H760)→T(D57) was found to be the only possible way of signal transmission from TodS to TodT, confirmed by in vivo P_{todX} expression studies with each of the substitution mutants. None of the single aminoacid exchange mutants was able to activate transcription in the presence of the best inducer toluene. The whole regulatory response is depicted in Fig. 7, chapter 3, p. 57.

But why involves the TodS mechanism the phosphorylation of three and not of only a single residues as is in sensor kinases of prototypal TCSs? Other multiple-step phosphorelay SHKs have been shown to respond to multiple signals in the regulatory response. The SHKs which initiates the sporulation process, KinA (Fig 2, p. 13) possesses three PAS domains and the most N-terminal domain (PAS-A) was found to bind ATP and catalyze exchange of phosphate between ATP and nucleoside diphosphates. However, ATP probably reflects the energy status of the cell and is not the primary signal (Stephenson and Hoch, 2001). PAS-B and PAS-C therefore could potentially bind the primary signal and where shown to be involved in homodimerisation, the active state of KinA (Wang *et al.*, 2001). More interestingly, the decision of *Bacillus subtilis* to sporulate is not only modulated by KinA domains, but by inhibitors of the SHK

KinA (i.e., KipI and Sda; Wang *et al.* 1997; Burkholder *et al.* 2001; Rowland *et al.* 2004), which altogether form part of an integrated final response, including the signals ATP, DNA-damage (Sda) or the presence of glucose (KipI).
In the ArcB/ArcA phosphorelay system the phosphatase SixA was found to affect the dephosphorylation rate of the HPT domain, responsible in transferring phosphoryl-groups to the cognate RR (Ogino *et al.*, 1998). So far, in phosphorelay systems where the phosphotransfer mechanism has been studied, i.e. ArcB/ArcA (Kwon *et al.*, 2000), EvgS/EvgA, BvgS/BvgA (Uhl and Miller, 1996; Perraud *et al.*, 1998, 2000), TorS/TorR (Jourlin *et al.*, 1997), and TodS/TodT (Busch *et al.,* 2009) only the most C-terminal domain was able to transphosphorylate the cognate RR. Therefore, all incoming signals must be channelled through this domain, indicative for an accurate response at the very first level of regulation, signal sensing, before the executing RR receives the signal to exert its regulatory role. It is likely that the complex mechanism of TodS, with several phosphorylation sites, offers additional steps for regulation (modulation of phosphotransfer), which represents the inclusion of additional signals in the final response.

In this thesis we bring along several new aspects which are a significant contribution to the field of TCS mediated signal transduction as generally there is scarce information on direct SHK-effector interaction. We describe in detail the effector binding profile *in vitro* and test the induction capacities of all binding compounds and can differentiate two types of effectors: agonists and antagonist. Furthermore, this is the first example of a double sensor histidine kinase described at its biochemical and molecular level. The molecular mechanism of phosphotransfer from signal recognition to activation of the RR TodT has been revealed as a multi-step phosphorelay mechanism, which involves two autokinase domains rather then an autokinase and a histidine-containing phosphotransfer domain as in all phosphorelay systems described so far.

VI. Conclusiones / Conclusions

- **TodS se puede purificar como proteína soluble en su conformación nativa y activa, por lo cual es probable que su localización sea el citosol.**
- **Al añadir tolueno la actividad de autofosforilación de TodS y de transfosforilación de TodS a TodT aumentan.**
- **La vida media de fosforilación de TodS es de 70 min y comparable a otras histidín-quinasas.**
- **Tolueno se une a TodS con una afinidad sub-micromolar.**
- **Tolueno se une al dominio PAS1 N-terminal de TodS.**
- **A TodS se une con alta afinidad un amplio rango de aromáticos mono- y bicíclicos.**
- **Los efectores se dividen en agonistas y antagonistas:**
    - **agonista: efectores que se unen a TodS y que activan la transcripción a partir de P_{todX}**
    - **antagonista: efectores que se unen a TodS y que no activan la transcripción a partir de P_{todX}**

  Conclusión: la transmisión intramolecular de la señal y no su reconocimiento molecular determina la activación de los genes *tod*.
- **Tanto agonistas como antagonistas se unen al dominio PAS1 N-terminal.**
- **Sustituyentes en posición *ortho* del anillo aromático derivados del tolueno reducen o impiden la respuesta *in vivo*.**
- **No hay correlación entre la afinidad de unión de efectores y el nivel de inducción de P_{todX}.**

- **Hay correlación positiva entre el nivel de estimulación de la autofosforilación de TodS y el nivel de transcripción a partir de P_{todX} *in vivo*.**
- **El aminoácido F79 en el dominio PAS1 es esencial para el reconocimiento de efectores.**
- **Tolueno únicamente estimula la actividad autoquinasa de el dominio histidín-quinasa N-terminal.**
- **TodS tiene dos dominios de auto-quinasa funcionalmente activos.**
- **TodS opera a través de un mecanismo "multistep-phosphorelay" que involucra los aminoácidos H190→D500→H760.**
- **En la fosfotransferencia de TodS a TodT están involucrados únicamente los aminoácidos H760 de TodS y D57 de TodT.**
- **El sistema "multistep phosphorelay" TodS/TodT es el primero descrito que no comprende un dominio HPT de fosfotransferencia y sí dos dominios histídin-quinasa.**

- **TodS can be purified as soluble, active full-length protein and is likely to be a cytosolic sensor kinase.**
- **The addition of toluene increases TodS autophosphorylation and transphosphorylation of TodT**
- **The half life of TodS phosphorylation is 70 min and comparable to other sensor kinases.**
- **TodS binds toluene with sub-micromolar affinity.**
- **Toluene binds at its N-terminal PAS1 domain of TodS.**
- **TodS binds a broad range of mono- and biaromatic compounds with high affinity.**
- **Effectors can be divided into agonists and antagonists:**
    - **agonist: effectors which bind to TodS and which activate transcription of P_{todX}**
    - **antagonist: effectors bind to TodS but which do not activate transcription of P_{todX}**

  Therefore: intramolecular signal transmission and not molecular recognition determines activation of *tod* genes.
- **Agonists and antagonists bind to the N-terminal PAS1 domain.**
- ***Ortho*-substitutions of toluene derivatives reduce or abolish in vivo responses.**
- **There is no correlation between binding affinity and the level of P_{todX} induction.**
- **There is correlation between stimulation of TodS autophosphorylation in vitro and P_{todX} transcription.**

- **Phenylalanine F79 is an essential amino acid in the recognition of effector molecules.**
- **Toluene stimulates autokinase activity of only the N-terminal histidine kinase domain.**
- **TodS has two functionally active autokinase domains.**
- **TodS operates by a multistep-phosphorelay mechanism involving amino acids H190→D500→H760.**
- **TodS-TodT phosphotransfer involves exclusively TodS H760 and TodT D57.**
- **The TodS/TodT multiple-step phosphorelay system is the first described which does not use a phosphotransfer (HPT) domain.**

VII. Material suplementario / Supplementary material

ad Chapter 2

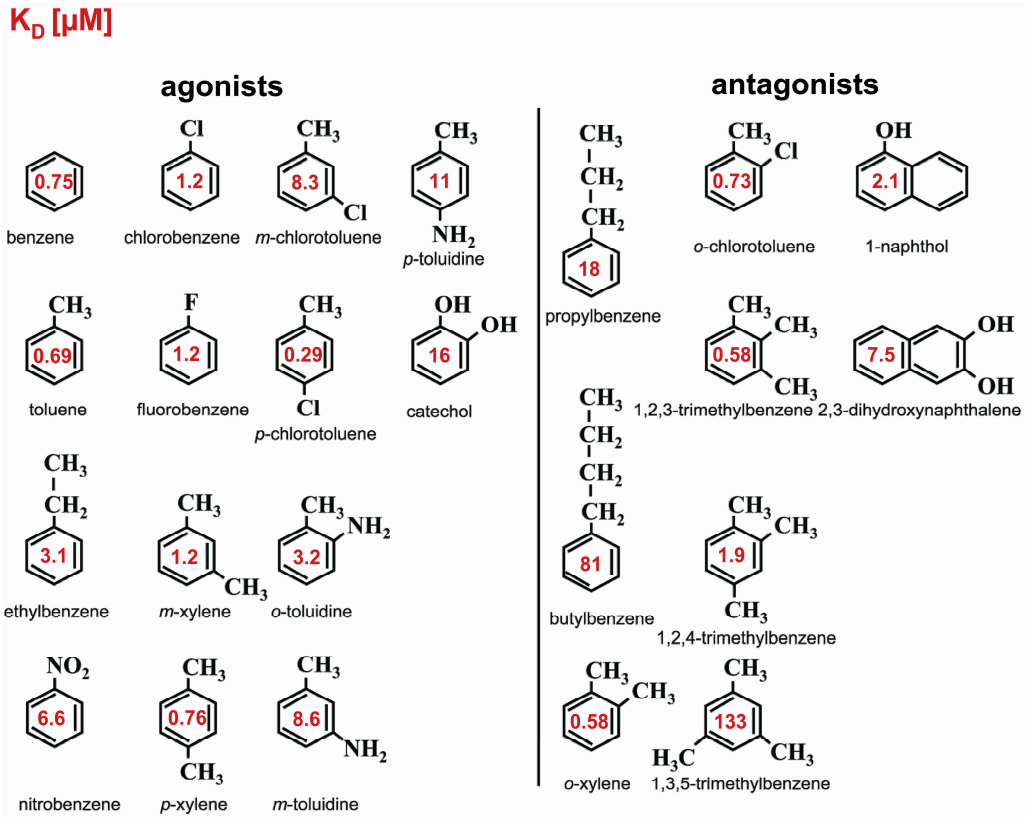

Fig. 6. Structures of signal agonists and antagonists analyzed.

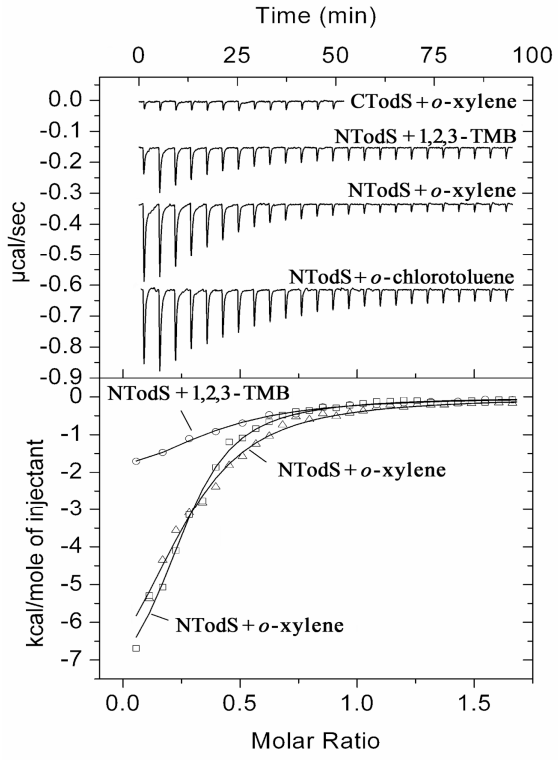

Fig. 7. Microcalorimetric titration of NTodS and CTodS with different hydrocarbons. (*Upper*) Titration of 21 mM CTodS with *o*-xylene and 20 mM NTodS with 1,2,3-TMB, *o*-xylene, and *o*-chlorotoluene. The ligand concentration was 1 mM in all cases. For clarity, raw titration data have been shifted arbitrarily on the*y* axis. (*Lower*) Integrated and corrected peak areas for the titration of NTodS with 1,2,3-TMB,*o*-xylene, and *o*-chlorotoluene. Derived thermodynamic data are given in Table 3.

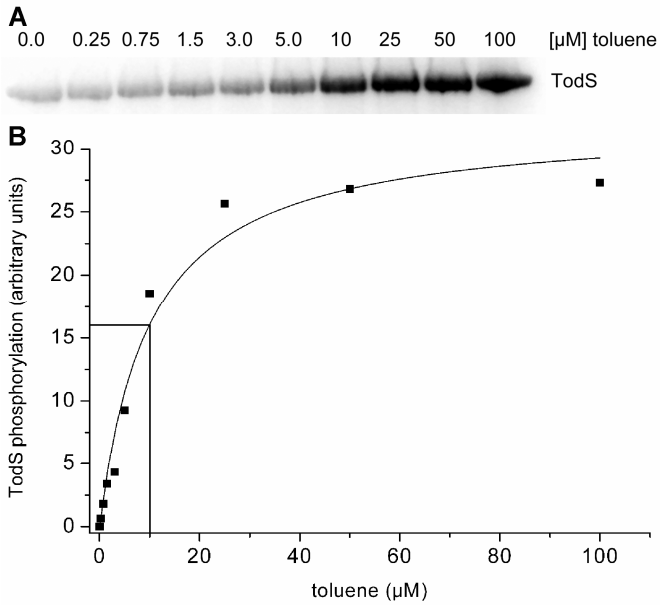

Fig. 8. Autophosphorylation of TodS in the absence and presence of different toluene concentrations. (*A*) TodS was incubated for 20 min with [^{32}P]ATP and toluene at the concentrations indicated before separation on SDS/PAGE gels. The first lane corresponds to the basal phosphorylation rate of TodS (absence of toluene). (*B*) Densitometric analysis of data shown in *A*. The value corresponding to the basal autophosphorylation (first lane in *A*) was subtracted from each of the remaining values. Data were then fitted with the Michaelis-Menten equation, and a toluene concentration at half the maximal autophosphorylation activity of 10 ± 2 µM was determined.

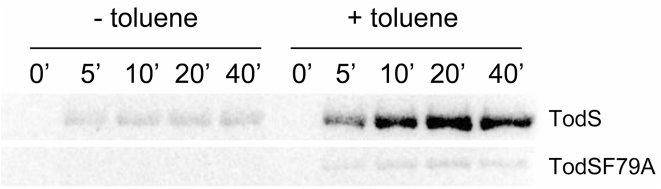

Fig. 9. Autophosphorylation of TodS protein and its F79A mutant. Experiments carried out in 50 mM Tris·HCl (pH 7.5)/200 mM KCl/2 mM $MgCl_2$/0.1 mM EDTA/2 mM DTT; 300 pmol TodS was incubated with 0.2 mM [g-^{32}P]ATP containing 4 µCi -^{32}P in the absence and presence of 100 mM toluene. At the indicated time points, 7.5-ml samples were obtained and analyzed by SDS/PAGE.

Compound	K_D,mM	Δ*H*, kcal/mol
Toluene	2.3 ± 0.1	-18.3 ± 0.2
o-Xylene	1.3 ± 0.1	-8.4 ± 0.3
o-Chlorotoluene	2.2 ± 0.1	-8.2 ± 0.3
1,2,3-TMB	4.1 ± 0.5	-2.9 ± 0.2

Table 3. Thermodynamic parameters for the binding of different hydrocarbons to the N-terminal fragment of TodS (NTodS) comprising amino acids 1-584. Parameters were derived from microcalorimetric titrations of NTodS with the compounds. Representative examples of these experiments are shown in Fig. 6.

Sequences

TodS and TodT nucleotide and amino acid sequences

(corrected version of sequence data available on www)

```
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197 SLILGPLEAVMAAEAGRESPYWKQFEVIQRNAMTLLKQVNTLLDLAKMD
246 ARQMGLSYRRANLSQLTRTISSNFEGIAQQKSITFDTKLPVQMVAEVDC
295 EKYERIILNLLSNAFKFTPDGGLIRCCLSLSRPNYALVTVSDSGPGIPP
344 ALRKEIFERFHQLSQEGQQATRGTGLGLSIVKEFVELHRGTISVSDAPG
393 GGALFQVKLPLNAPEGAYVASNTAPRRDNPQVVDTDEYLLLAPNAENEA
442 EVLPFQSDQPRVLIVEDNPDMRGFIKDCLSSDYQVYVAPDGAKALELMS
491 NMPPDLLITDLMMPVMSGDMLVHQVRKKNELSHIPIMVLSAKSDAELRV
540 KLLSESVQDFLLKPFSAHELRARVSNLVSMKVAGDALRKELSDQGDDIA
589 ILTHRLIKSRHRLQQSNIALSASEARWKAVYENSAAGIVLTDPENRILN
638 ANPAFQRITGYGEKDLEGLSMEQLTPSDESPQIKQRLANLLQGGGAEYS
687 VERSYLCKNGSTIWANASVSLMPQRVGESPVILQIIDDITEKKQAQENL
736 NQLQQQLVYVSRSATMGEFAAYIAHEINQPLSAIMTNANAGTRWLGNEP
785 SNIPEAKEALARIIRDSDRAAEIIRMVRSFLKRQETVLKPIDLKALVTD
834 TSLILKAPSQNNSVNLDVVADDELPEIWGDGVQIQQLIINLAMNAIEAI
883 SQADCETRQLTLSFSGNDTGDALVISVKDTGPGISERQMAQLFNAFYTT
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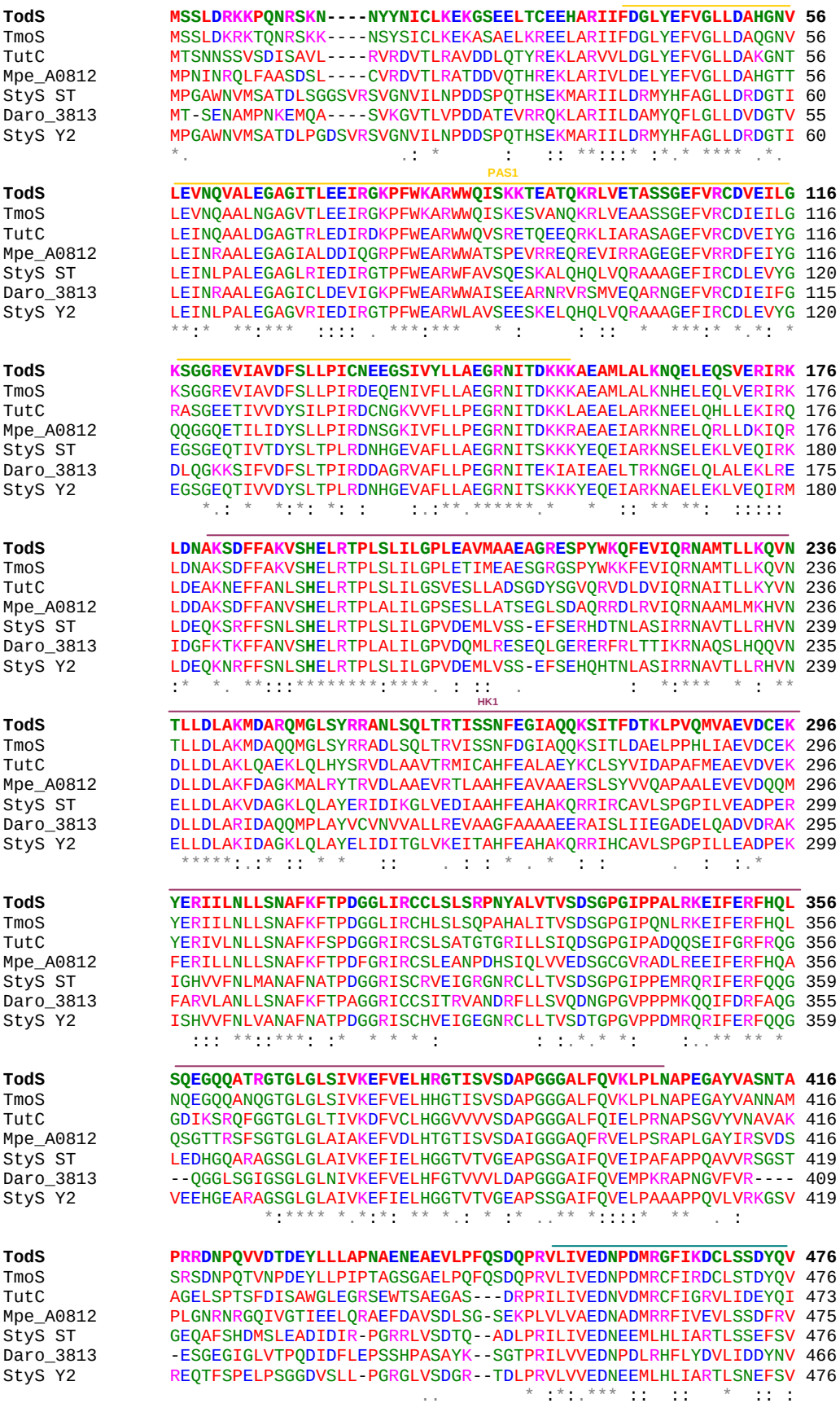
Multi-alignment of TodS



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TutC           SVAADGEQALELITSSPPDLVITDLMMPKVSGQLLVKEMRSRGDLANVPILVLSAKADDG 533
Mpe_A0812      VHAADGLQALTQARAQAPDAIITDLMMPKLGGDKLVSELRSTPELAHIPVLVLSAKADES 535
StyS ST        ECAGNGEQGLGLMLANPPDLVIRDLMMPGMSGEKLIRLMREEAQLTQIPVLVLSARADED 536
Daro_3813      TLAANGALALTSALEDPPDLVITDLMMPHFDGEQFVRELRTSGCFPNLPVLVLSARADDA 526
StyS Y2        ECASNGKQGFAYMLANPPDLVITDLMLPGMSGEKLIRRMREEGALTQIPVLVLSARADEE 536
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TodS           LRVKLLSESVQDFLLKPFSAHELRARVSNLVSMKVAGDALRKELSDQGDDIAILTHRLIK 596
TmoS           LRVKLLSESVQDYLLKPFSAHELRARVSNLISMKIAGDALRKELSDQSNDIALLTHRLIK 596
TutC           LRIKLLAESVQDYVVKPFSATELRARVRNLVTMKRARDALQRALDSQSDDLSQLTRQIID 593
Mpe_A0812      LRLKLLSDSVQDYIVKPFSSRELLVRVRNIVTMKLAREALQKELASQNEDLAQLTQQLIA 595
StyS ST        VRMTLLANMVQDYVTKPFFIPELLSRVRNLVMTRRARLALQDELKTHNADLVQLTRELIS 596
Daro_3813      QRETLLEELVQDYLTKPFSPQELRARVRNLVTVKRTVDILQKELNTQASDVGELTAGLVA 586
StyS Y2        LRMTLLATLVQDYVTKPFFIPELLSRVRNLVMTRRARLALQDELKTHNADFVQLARELIS 596
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TodS           SRHRLQQSNIALSASEARWKAVYENSAAGIVLTDPENRILNANPAFQRITGYGEKDLEGL 656
TmoS           SRHRLQQSNIALTASEARWKAVYENSAAGIVLTDTENRILNANPAFQRITGYTEKDLAQL 656
TutC           NRQELQRSHDALQESESRWRAVYENSAAGIVLTNLDGLILSANQAFQKMVGYAEDELRVI 653
Mpe_A0812      SKQGLQRSHDALKESERRWRAVYENTAVGVSLSDLQGNMHAANPALQEMLGYTESELIGL 655
StyS ST        GRQAIQRSLEAQQKSERRWRAIHENSAVGIAVVDLQWRFVNANPAFCRMLGYTQEDVLGY 656
Daro_3813      SRKSLQDSLVALQISERRWQGLYRNSAVGIALADREGRILKANPALQQMLGYSEAEIVGV 646
StyS Y2        GRRAIQRSLEAQQKSELRWRAIHENSAVGIAVVDLQWRFVNANPAFCRMLGYTQEELLGH 656
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                                          PAS2
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TmoS           SMEQLTPPNERTQMKQRLARLLQSGGAEYSVECSYLCKNGSTIWANASVSLMSPRVDEPQ 716
TutC           EISDLVPEHDREKIRSRVSNLISGRVDDYQVQRQCRRKDGRMMWANVRASLIPGLANQSP 713
Mpe_A0812      GNLMTDAEAGHEDRRLQLERLVNGSQVEMRQQRRYRHRNGMTILANVRESLIPGTSDLPP 715
StyS ST        SVLELTHPDDRNITDQRLHHLLDGRLQTYHHQKRFLHKDGHSLWTRSSVSVIPGSGDTPP 716
Daro_3813      SFIDISDESQRAMTLRNVHGLFDGSIDHYHVQKRYERRDGSFLWANVSASLIPAVDVEGP 706
StyS Y2          SVLEHTHPDDRNITDQRLHHLLDGRLRTYHHQKRFLHKDGHSLWTRSSVSVIPGSGDTPP 716
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TodS           VILQIIDDITEKKQAQENLNQLQQQLVYVSRSATMGEFAAYIAHEINQPLSAIMTNANAG 776
TmoS           VILQIIDDITEKKQAQETLNQLQQELVQVSRSATMGEFAAYIAHEINQPLSAIMTNANAG 776
TutC           MVVRIFDDITEKIQTEAELARAREKLTRVMRVTAMGELAASIAHELNQPLAAIVTNGHAS 773
Mpe_A0812      TLITVVEDITTQKRAEVELAQTKDALARVSRVTTMGELAASIAHEVNQPLTAVVVNGHAC 775
StyS ST        LMIGVVEDIDEQKRAEHELERARSELARVMRVTAMGELVASITHELNQPLAAMVANSHAC 776
Daro_3813      RLAVIVEDVSSRKEAESALAATQTELARVSRFTAMGELVASIAHEVNQPLSAIVTNSQAA 766
StyS Y2        LMIGVVEDIDAQKRAEHELERARSELARVMRVTAMGELVASITHELNQPLAAMVANSHAC 776
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TodS           TRWLGNEPSNIPEAKEALARIIRDSDRAAEIIRMVRSFLKRQETVLKPIDLKALVTDTSL 836
TmoS           TRWIGNEPPNIMEAKEALARIIRDSDRAADIIRMVRSFLKRQGPVLKPIDLKALVADTTL 836
TutC           LRWLGSEPCNLLEAVEAVRRIIHDANRASEIIKRIRGFLQRGEGRRSAVDIFQVVADVAA 833
Mpe_A0812      LRWLSTEPRNDLEVQDAIQRIVRDANRASEVIARIRGFLKRSKTDRTMVCMDNVVEDVIG 835
StyS ST        RRWLNSNPPNLKEGVASVEAVVRDSQRAAEVVLRLRMFMRRGEIQHEPLNLSGVVEEVLD 836
Daro_3813      LRWLARETPDYQEVVAALNRVNRDASLAGEVIARIRNFLSMGGMQRERLVVRPILENLLQ 826
StyS Y2        RRWLNSSPPNLKEGIASVEAVVRDSQRAAEVVLRLRMFMRRGETQHEPLNLSGVVEEVLG 836
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                                          HK2
TodS           ILKAPSQNNSVNLDVVADDELPEIWGDGVQIQQLIINLAMNAIEAISQADCETRQLTLSF 896
TmoS           ILKAPSQSNGVSLNVIAGDTLPAIMGDAVQIQQLVINLAMNSIEAMSQVGCETRQLALSF 896
TutC           IVSDMARSHCIDMRYQAVGQLSLVIADKVQLQQVILNLCINGIESIVGGNSERGELSITV 893
Mpe_A0812      LARDSLRSAGVQLIKHVDSDLPRVFADSVQLQQVILNLMMNGIEAMGSCATLERQLELRV 895
StyS ST        YVRESLVMQSISLETTLPTDLPVVLADRVQLQQVVLNLLLNAIEAIQARSPNVPRLKLRI 896
Daro_3813      MLQTMLQEADVEVDLRIAPGLPDLLADPVQLQQVLLNLVVNAVDAMREEKERARRLSISV 886
StyS Y2        YVRESLVMQGISLETTLPTDLPMVLADRVQLQQVVLNLVLNAIEAIQAASPSVPRLTLRI 896
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TmoS           SSNASNDALIICVKDTGPGIPEDQIGQLFNAFYTTKKEGLGMGLAICLTIAEVHNGKIWA 956
TutC           T-QSDKRFLTVSVHDSGPGLAPGEAENVFDAFYTSKVEGLGMGLAISRSIIEAHGGRLDV 952
Mpe_A0812      V-KHG-GDIDVSVSDSGTGLVTADFERIFEAFYTTKPDGMGMGLAICRSIVEAHGGRLWA 953
StyS ST        CRSPDNGDLQLEVEDNGCGVPALQAERIFEPFYTTKSQGMGMGLAICRTILEAHGGQLNL 956
Daro_3813      S-ADTAGSVLFSVSDTGPGIPPDKAAKIFDALFSTKSRGLGMGLAISRSIVENHGGRLRL 945
StyS Y2        CRSPDNGPLRLEVEDNGCGVPSSQTERIFEPFYTTKSHGMGMGLAICRTILEAHGGQLNL 956
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TmoS             ESP-----PAGGACFFVSIPVS--------- 973
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StyS ST          LPPSDSGSAASGSVFQVVLPTDQGALR---- 983
Daro_3813        VP-----EAAGGAHFVFNIPVQP-------- 963
StyS Y2          LPPSDNCSASGSVFQVILPTDQGTLL----- 982
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Secondary structure prediction of TodS

(GOR and SOPMA prediction methods)

GOR secondary structure prediction method version IV, J. Garnier, J.-F. Gibrat, B. Robson, Methods in Enzymology,R.F. Doolittle Ed., vol 266, 540-553, (1996)

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Sequence length :   978
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   3₁₀ helix       (Gg) :     0 is   0.00%
   Pi helix        (Ii) :     0 is   0.00%
   Beta bridge     (Bb) :     0 is   0.00%
   Extended strand (Ee) :   135 is  13.80%
   Beta turn       (Tt) :     0 is   0.00%
   Bend region     (Ss) :     0 is   0.00%
   Random coil     (Cc) :   380 is  38.85%
   Ambigous states (?)  :     0 is   0.00%
   Other states         :     0 is   0.00%
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SOPMA:Geourjon, C. & Deléage, G., SOPMA: Significant improvement in protein secondary structure prediction by consensus prediction from multiple alignments., Cabios (1995) 11, 681-684

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SOPMA :
   Alpha helix     (Hh) :   509 is  52.04%
   3₁₀ helix       (Gg) :     0 is   0.00%
   Pi helix        (Ii) :     0 is   0.00%
   Beta bridge     (Bb) :     0 is   0.00%
   Extended strand (Ee) :   138 is  14.11%
   Beta turn       (Tt) :    57 is   5.83%
   Bend region     (Ss) :     0 is   0.00%
   Random coil     (Cc) :   274 is  28.02%
   Ambigous states (?)  :     0 is   0.00%
   Other states         :     0 is   0.00%
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Resumen

La especie bacteriana *Pseudomonas putida* DOT-T1E es de gran utilidad en la lucha contra la contaminación con derivados del petróleo por su alta resistencia a estos compuestos aromáticos. Es capaz de usar tolueno, etilbenceno y benceno como única fuente de carbono a través de la ruta de degradación TOD, cuyos genes están codificados en el operón *tod*. Éste se transcribe a partir del promotor P_{todX} y su expresión está regulada por el sistema de dos componentes TodS/TodT. En esta Tesis Doctoral se describe el mecanismo molecular de respuesta a efectores de la histidín-quinasa sensora TodS y la transferencia de señal al regulador de respuesta TodT, el activador de la transcripción a partir de P_{todX}.

TodS tiene 5 dominios funcionales, dos dominios autoquinasa (HK) con su respectivos dominios sensores (PAS), separados por un dominio receptor interno. TodS se puede purificar en la fracción soluble y posee todas las actividades características de las sensor-quinasas: autofosforilación, defosforilación y transfosforilación hacia TodT. El dominio PAS N-terminal es capaz de unir un amplio rango de efectores mono- y bicíclicos con alta afinidad, los cuales se dividen en agonistas y antagonistas. Agonistas son capaces de estimular la autofosforilación de TodS que se traduce en un aumento de transfosforilación hacia TodT y por tanto en un aumento de la expresión de los genes de la ruta TOD a partir de P_{todX}. Los antagonistas se unen a TodS, pero no alteran el nivel de fosforilación basal de TodS y por tanto no activan la transcripción. Ambos dominios HK de TodS son funcionales, pero únicamente el dominio HK N-terminal es estimulado en su autofosforilación a partir de la unión de agonistas. TodS/TodT opera a través de un mecanismo denominado “multistep-phosphorelay”, novedoso por implicar dos dominios HK. Los grupos fosforilo son transferidos en TodS desde el dominio de respuesta a efectores N-terminal vía dominio regulador interno a el dominio HK C-terminal. A través del dominio HK C-terminal TodT es transfosforilado en su dominio receptor. Una vez fosforilado TodT, este adquiere su conformación activa y tiene lugar el inicio de la transcripción a partir de P_{todX} .