**Mini-Review**

# From the phosphoenolpyruvate phosphotransferase system (PTS) to *selfish metabolism*: a story retraced in *Pseudomonas putida*

by

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**Running Title:** Regulation of *P. putida*'s physiology by the PTS

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**Abstract**

Although DNA is the ultimate repository of biological information, deployment of its instructions is constrained by the metabolic and physiological status of the cell. To this end, bacteria have evolved intricate devices that connect exogenous signals (e.g. nutrients, physicochemical conditions) with endogenous conditions (metabolic fluxes, biochemical networks) that coordinately influence expression or performance of a large number of cellular functions. The *phosphoenolpyruvate:carbohydrate-phosphotransferase system* (PTS) is a bacterial multi-protein phosphorylation chain which computes extracellular (e.g. sugars) and intracellular (e.g. phosphoenolpyruvate, nitrogen) signals and translates them into post-translational regulation of target activities through protein-protein interactions. The PTS of *Pseudomonas putida* KT2440 encompasses one complete sugar (fructose)-related system and the 3 enzymes that form the so-called nitrogen-related PTS ($PTS^{Ntr}$), which lacks connection to transport of substrates. These two PTS branches cross-talk to each other, as the product of the *fruB* gene (a polyprotein EI-HPr-EIIA) can phosphorylate PtsN ($EIIA^{Ntr}$) *in vivo*. This gives rise to a complex actuator device where diverse physiological inputs are ultimately translated into phosphorylation or not of PtsN ($EIIA^{Ntr}$) which, in turn, checks the activity of key metabolic and regulatory proteins. Such a control of bacterial physiology highlights the prominence of biochemical homeostasis over genetic ruling –and not *vice versa.*

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**Introduction**

The term *phosphoenolpyruvate:carbohydrate-phosphotransferase system* (PTS) deals with a diverse group of enzymes and protein modules that transfer high-energy phosphate (~P) originated in phosphoenolpyruvate (PEP) from one member of the chain to the next one in a stepwise fashion (Cases *et al.*, 2007). The PTS was first described in the mid 1960s by Kundig *et al.* (1964) in *Escherichia coli* as a carbohydrate transport system and since then it has been found in many different classes of bacteria (Postma *et al.*, 1993; Saier & Reizer, 1994). However, as time passed it became more evident that different types of PTSs do exist, varying substantially in enzyme composition and function, and that they are not restricted to mediation of carbohydrate transport (Deutscher *et al.*, 2006; Pflüger-Grau & Görke, 2010). A survey of the distribution of PTS domains in various genomes revealed that the main function of PTS proteins as devices for carbohydrate uptake upon phosphorylation is rather an exception found in *Enterobacteriaceae*, *Vibrionales* and *Firmicutes*, than a rule for all bacteria (Cases *et al.*, 2007). Instead there is evidence that such phosphorylation-mediated transport is just one evolutionary outcome originated from a core set of what appears to originally be carbon-sensing

phosphotransferases. These gravitated evolutionarily towards fulfilling a multitude of regulatory functions whereby the metabolic status of the cell, in particular the economy of carbon *vs.* nitrogen pools, is converted into physical actions on target proteins (Cases *et al.*, 2007). In this sense, the PTS of every bacterium acts as a complex biological sensor-actuator device that computes biochemical signals and transduces them into mechanical outputs (De Silva, 2007; Canton *et al.*, 2008). The best studied system is that of *Escherichia coli*, where ~ 60 genes encoding putative PTS proteins have been identified, although only a small fraction has been analyzed in detail (Tchieu *et al.*, 2001). This number exposes the complexity of the PTS in *E. coli* as compared to other bacteria where only very few PTS proteins and even just incomplete systems are found (Cases *et al.*, 2007).

Two general branches of the PTS are known in Gram-negative bacteria. The most widespread is the *canonical* PTS (or sugar-related PTS; Fig. 1a), which is responsible for the concomitant phosphorylation and transport of specific carbohydrates into the bacterial cell. These PTSs generally share the core EI and HPr phosphotransfer components but they diverge at the EII complexes, the proteins that bring about sugar import specificity (Barabote & Saier, 2005: Fig. 1a). The most ancient canonical PTS seems to be the one evolved to transport fructose, from which the other sugar-transporting devices have originated (Saier *et al.*, 2005). Detailed descriptions of the different classes of sugar-PTS can be found in Postma *et al.* (1993), Barabote & Saier (2005) and Saier *et al.* (2005). In addition to the canonical PTS bacteria often possess an abridged version i.e. the so-called nitrogen-related PTS ($PTS^{Ntr}$), which lacks EIIB and the membrane-spanning subunit EIIC necessary for carbohydrate transport. $PTS^{Ntr}$ is thus not involved in sugar intake but suggested to be involved in regulation of the carbon *vs.* nitrogen balance (Fig. 1b; Deutscher *et al.*, 2006; Görke & Stülke, 2008; Lengeler & Jahreis, 2009; Pflüger-Grau & Görke, 2010). The controversial designation *nitrogen-PTS* stems from the fact that two of its encoding genes, *ptsO* (NPr) and *ptsN* ($EIIA^{Ntr}$) map in the genomes of bacteria that carry them adjacent to *rpoN*, the gene encoding the $\sigma^{54}$ sigma factor of the RNA polymerase (Fig. 2; Reizer *et al.*, 1996; Comas *et al.*, 2008). These two proteins, NPr and $EIIA^{Ntr}$, form together with $EI^{Ntr}$ (encoded by the non-adjacent gene *ptsP*; Fig. 1b) an independent chain of PTS proteins that co-exists with various configurations of the sugar-PTS devices. Either PTS type is independently active, but they communicate under certain conditions by means of an active cross talk *via* phosphate group transfer (Pflüger & de Lorenzo, 2008; Zimmer *et al.*, 2008; see below). Both PTS branches have in common that the ultimate origin of ~P is PEP. This high-energy phosphate is then transferred through 3 major phosphotransferase complexes: the EI, HPr/NPr, and EII enzymes (Fig.1b). These complexes however differ between the two types in subunit composition or special features. $EI^{Ntr}$ usually carries an additional GAF domain on its N-terminal part (see below), a feature that is characteristic for the nitrogen-PTS and differentiates this EI protein from its counterpart in the sugar-PTS (Reizer *et al.*, 1996). $EI^{Ntr}$ sequences cluster

together phylogenetically and they are distant from any other EI homologues, emphasizing their separation from the sugar-PTS components (Hu & Saier, 2002).

**The debated nitrogen connection of PTS$^{Ntr}$**

Designation of the second branch of the PTS as the nitrogen-PTS has not been devoid of controversy (Ninfa, 2011), as N-related phenotypes of PTS$^{Ntr}$ mutants of *E. coli* were later suspected of being artefacts (Reaves & Rabinowitz, 2011). Instead, the most clear functional role of this PTS branch in *E. coli* is the control of potassium uptake by direct interaction of the PtsN (EIIA$^{Ntr}$) protein with both the TrkA subunit of the Trk transporter complex, and the sensor kinase KdpD of the two-component system regulating the expression of a second transporter device, KdpFABC (Lee *et al.*, 2007; Lüttmann *et al.*, 2009). Since intracellular $K^+$ is thought to influence sigma factor competition by affecting the binding of $\sigma^{70}$ and $\sigma^S$ to core RNAP, it could be the case that many of the phenotypes attributed to EIIA$^{Ntr}$ could just reflect factor selectivity determined by $K^+$ levels (Lee *et al.*, 2010). Many reported nitrogen-related traits observed in *E. coli ptsN* mutants could therefore be misleading and not directly related to the PTS$^{Ntr}$ (Reaves & Rabinowitz, 2011). However, other facts have indicated otherwise. One key observation was that the N-terminal GAF domain of the EI$^{Ntr}$ is homologous to the *sensory* domain of the NifA protein of *Azotobacter vinelandii* (Reizer *et al.*, 1996), the $\sigma^{54}$-dependent transcriptional regulator of the nitrogen fixation genes. In turn, the GAF domain of NifA is known to bind $\alpha$-ketoglutarate (Martinez-Argudo *et al.*, 2004). Since this compound is the immediate precursor of glutamine from Krebs cycle components, this state of affairs prompted Dozot *et al.* (2010) to hypothesize a consistent model in which PTS$^{Ntr}$ could integrate C and N signals by means of the regulation of EI$^{Ntr}$ by $\alpha$-ketoglutarate. This somewhat inconspicuous chemical has turned out to be a major metabolic signal that orchestrates carbon catabolite repression in *E. coli* in respect to nitrogen and sulphur (You *et al.*, 2013). In this context, it came as a surprise that $\alpha$-ketoglutarate, which accumulates under nitrogen limitation, directly blocks glucose uptake by inhibiting the EI enzyme of the canonical PTS (Doucette *et al.*, 2011). Since such a default EI enzyme lacks the GAF domain of the EI$^{Ntr}$ counterpart, these data were used as an argument that the sugar-related PTS is able by itself to respond to N/C ratios and the nitrogen-branch of the system is in fact unrelated to N. Subsequent work, however, leaves this question still open. In particular, Lee *et al.* (2013) have suggested that $\alpha$-ketoglutarate and glutamine reciprocally regulate the autophosphorylation activity of EI$^{Ntr}$ (and thereby the phosphate flow through the PTS$^{Ntr}$) and that the GAF domain is necessary for such regulation. From this, it follows that sensing the $\alpha$-ketoglutarate/glutamine ratio by EI$^{Ntr}$ could in fact be the primary signal that triggers the phosphotransfer chain PEP → EI$^{Ntr}$ → NPr → EIIA$^{Ntr}$, thereby making a *bona fide* connection between the PTS$^{Ntr}$ and nitrogen availability (note that the canonical EI enzyme activity inhibited by $\alpha$-

ketoglutarate was not affected by glutamine; Doucette *et al.*, 2011). It is thus possible that one or more metabolic inputs signalling N and and/or the N/C ratio can be computed by a merged PTS, although many details on how this could be remain elusive at the time of writing this mini-review. It is also possible that the sensing and actuation mechanism through which physiological signals are converted by the PTS into regulatory actions may differ among bacteria (see below).

**What is PtsN (EIIA$^{Ntr}$) good for?**

PTS proteins exert their regulatory action by physical contact with their partners in a fashion that depends on their phosphorylation state and which results in either repression or activation of the corresponding biological function. A large variety of techniques have been applied for identifying such partners, typically pull-downs and 2-hybrid systems (Pflüger-Grau *et al.*, 2011; Karstens *et al.*, 2014). While both canonical HPr and EIIA/B proteins have been found to interact with a variety of regulatory, structural and enzymatic associates (Deutscher *et al.*, 2006), the repertoire of proteins known to be influenced by the PTS$^{Ntr}$ is thus far limited to those bound by PtsN (EIIA$^{Ntr}$). In most, if not all, cases the non-phosphorylated EIIA$^{Ntr}$ is the one species competent in protein-protein interaction. The best characterized interactions are those related to $K^+$ metabolism through binding to the low affinity potassium transporter TrkA (Lee *et al.*, 2007). In addition, EIIA$^{Ntr}$ regulates expression of the high-affinity K transporter KdpFABC by interacting with sensor kinase KdpD, which results in increased phosphorylation of the cognate response regulator KdpE (Lüttmann *et al.*, 2009). As mentioned above, changes in intracellular K concentration may result in different cell-wide transcriptomic regimes owing to redistribution of sigma factors in the population of RNAP molecules. This makes it difficult to ascertain whether the diverse effects of knocking-out *ptsN* in different bacteria are direct or indirect. It is also intriguing that EIIA$^{Ntr}$ modulates the activity of the histidine kinase PhoR which controls the phosphate starvation response of *Escherichia coli* (Lüttmann *et al.*, 2012). On the other hand, EIIA$^{Ntr}$ inhibits the binding of the response regulator SsrB to its target promoter of the SPI-2 pathogenicity island of *Salmonella* (Choi *et al.*, 2010). It is thus plausible that PTS$^{Ntr}$ modulates a number of two-component systems by either targeting the sensor kinase or the response regulator. Note that neither the mechanisms nor the sign of the effect of EIIA$^{Ntr}$ on such regulatory systems are necessarily conserved: while dephosphorylated PtsN stimulates the kinase activity of KdpD (Lüttmann *et al.*, 2009), it is the phosphatase activity that is inhibited in the sensor kinase PhoR (Lüttmann *et al.*, 2012). In other cases EIIA$^{Ntr}$ interacts with specific metabolic enzymes such as the pyruvate dehydrogenase complex (see below; Pflüger-Grau *et al.*, 2011). One additional target of EIIA$^{Ntr}$ turns out to be the SpoT1 enzyme of *Ralstonia eutropha*, a bifunctional ppGpp synthase/hydrolase that is a key enzyme of the stringent response in this bacterium. Interplay of

this enzyme with EIIA$^{Ntr}$ was not, however, observed in *E. coli* (Karstens *et al.*, 2014). It is also possible that virtually all ABC transporters of *Rhizobium leguminosarum* (not only KdpABC) are regulated by PTS$^{Ntr}$ in response to the cellular energy charge (Prell *et al.*, 2012) in order to save ATP for essential processes (including $K^+$ homeostasis). This indicates the plasticity and *stickiness* of the PTS for connecting dissimilar functions in different bacterial hosts.

**The minimalist PTS of *P. putida***

The Gram-negative soil and rhizosphere bacterium *Pseudomonas putida* was one of the first organisms in which a PTS, specifically one for fructose was described (Sawyer *et al.*, 1977). Identification of its molecular components had to wait until a draft sequence of the genome of *P. putida* KT2440 was available in the late 1990s. The sequence was finally completed and published by Nelson *et al.* (2002). An experimental match between the early biochemical characterization of the PTS$^{Fru}$ and the genomic data was not produced, however until much later (Velázquez *et al.*, 2007). This sugar-PTS includes the full set of components for fructose intake distributed in two co-expressed proteins, FruA and FruB (Fig. 2). Disruption of *fruB* leads to complete loss of the ability of *P. putida* to grow on fructose, thereby showing that the PTS$^{Fru}$ is essential for growth on this carbohydrate and that no other fructose transporters are present in this bacterium (Velázquez *et al.*, 2007). The multiphosphoryl transfer protein FruB carries an EIIA$^{Fru}$ domain (140 aa), a HPr domain (86 aa), and an EI domain (548 aa), whereas the sugar transporting component (FruA) bears a EIIB$^{Fru}$ domain (83 aa) and an EIIC$^{Fru}$ domain (340 aa). The genes *fruB* and *fruA* cluster together in the *fruR/fruBKA* operon (*fruK* encodes fructose-1-P kinase; Fig. 2), which is under the control of the *fruB* promoter. When cells are grown in a gluconeogenic regime (e.g. succinate as sole C source), expression of the *fruBKA* genes is strongly repressed by FruR, a regulator also known as the catabolite repressor/activator or the Cra protein (Chavarria *et al.*, 2011; Chavarria *et al.*, 2013). Such repression can be lifted only by endogenous accumulation of fructose-1-phosphate (F1P), which acts as an effector of Cra/FruR (Chavarria *et al.*, 2011). This is an important detail, as discussed below.

The fructose-PTS of *P. putida* is accompanied by a complete PTS$^{Ntr}$ formed by PtsP (EI$^{Ntr}$), PtsO (NPr) and PtsN (EIIA$^{Ntr}$), as shown in Fig. 2. While the *ptsO* and *ptsN* genes cluster together adjacent to the *rpoN* gene, they seem to belong to a different transcriptional unit (Kim *et al.*, 2013) that also includes the somewhat elusive genes *rpoX* and *yhbJ*. The RpoX protein, first thought to affect the activity or expression of *rpoN* (Nelson *et al.*, 2002), turns out in reality to promote the phenomenon known as *ribosome hibernation* (thereby the alternative name *hibernation promoting factor*, HPF), through which 70S ribosomes dimerize in stationary phase to form inactive

100S variants (Ueta *et al.*, 2008; Polikanov *et al.*, 2012). On the other hand, the protein YhbJ (also called RapZ) seems to be a RNA chaperone specific for some sRNAs. In *E. coli*, RapZ recruits the major endoribonuclease RNase E to degrade the sRNA called GlmZ, which activates translation of the *glmS* mRNA (encoding glucosamine-6-phosphate synthase; Kalamorz *et al.*, 2007; Gopel *et al.*, 2013) through RNA-RNA base-pairing. Other activities of the RapZ protein (which has homologues in bacteria lacking the $PTS^{Ntr}$ e.g. YvcJ of *B. subtillis;* Luciano *et al.*, 2009) might occur as well. The evolutionary pressure that has resulted in such different genes as *hpf* (*rpoX*) and *rapZ* being co-transcribed with *ptsO* and *ptsN* at a site immediately adjacent to *rpoN* in *P. putida* (Fig. 2) is quite a mystery. Yet, the synteny of the gene cluster in many other bacteria does suggest some type of coordination of the regulatory actions of each component of the group. In reality, all genes of this cluster are expressed (with some fluctuations; Yuste *et al.*, 2006; Kim *et al.*, 2013) under all growth conditions tested, the same being true for the stand-alone *ptsP* gene encoding the EI enzyme of the $Pts^{Ntr}$. It is thus likely that the performance of the system relies only on the signalling between pre-existing proteins rather than in the control of their expression.

The components of the *P. putida*'s PTS are shown in Fig. 2. As is the case in most known instances, PtsP consists of an EI domain fused to an N-terminal 137 aa GAF module. Also similarly to other bacteria, PtsO and PtsN are single-module enzymes, carrying a single NPr (83 aa) or $EIIA^{Ntr}$ (146 aa) domain, respectively (Fig. 2). Under metabolic conditions where the FruB protein is expressed, the $EIIA^{Fru}$-HPr-EI polyprotein can hand over high-energy phosphate from the $PTS^{Fru}$ to the $PTS^{Ntr}$ , thereby enabling a physiologically relevant cross-talk between the two systems *in vivo* (Pflüger & de Lorenzo, 2008; Fig. 3a). Since *fruB* expression is under the control of the FruR/Cra repressor that allows *fruBKA* transcription only when cells produce intracellular F1P (see above), such cross-talk is most evident when cells are grown in fructose (Chavarria *et al.*, 2013) and not detectable at all when consuming succinate or other carbon sources that enter the central metabolism below the upper glycolytic pathways. This means that in fructose-grown cells, the PtsN protein is phosphorylated by FruB regardless of any other ~P signal coming from PtsP. In fact, the only way to supress altogether PtsN phosphorylation is to mutate both *ptsP* and *fruB* (Pflüger & de Lorenzo, 2008; Chavarria *et al.*, 2013), as either of the two enzymes does the job equally well. Since non-phosphorylated $EIIA^{Ntr}$ protein is the form believed to generally interact with the functional targets of the $PTS^{Ntr}$ (see above) this state of affairs predicts that some phenotypes linked to a *ptsN* mutation should be manifested also when *P. putida* cells grow on fructose. This is because virtually the whole pool of the protein in these conditions is phosphorylated and thus unable to interplay with cognate companions. companions. In fact, when one compares core metabolic fluxes of a *ptsN* mutant of *P. putida* with a wild-type strain growing on fructose: the outcome of the two scenarios is quite similar (Chavarría *et*

*al.*, 2012). The practical consequence of the transfer of ~P from $PTS^{Fru}$ to $PTS^{Ntr}$ is that any signal that could originate in PtsP is altogether superseded by those coming from FruB in fructose-grown cells (or other conditions that allow expression of the *fruBKA* operon). To further examine this issue, a mathematical model describing the phosphorylation state of PtsN under different physiological states allowed determination of the phosphate flux distribution during growth on fructose (Kremling *et al.*, 2012). Interestingly, the model predicts that during growth on this sugar the great majority of phosphate molecules (~80%) are trafficked through the $PTS^{Ntr}$. This raises new questions: on one hand, metabolic signals can be entered into the $PTS^{Ntr}$ through either of the two EI enzymes available in *P. putida*. On the other hand, $EIIA^{Ntr}$~P may not be able to back-phosphorylate the $EIICB^{Fru}$ transporter, as suggested by the fact that a *fruB*$^{-}$ / *fruA*$^{+}$ strain fails to grow on fructose as sole carbon source (Velázquez *et al.*, 2007). Regardless of details, the model of Kremling *et al.* (2012) suggests that the PEP/pyruvate ratio, a gross proxy of carbon fluxes through the central metabolism (Hogema *et al.*, 1998) controls the extent of phosphorylation of PtsN.

**The metabolic links of the *P. putida*'s $PTS^{Ntr}$**

The tight connection between the biochemical status of the cell and phosphorylation state of PtsN is also reflected by the fact that the ratio $EIIA^{Ntr}$~P/ $EIIA^{Ntr}$ changes with the growth phase of *P. putida*. In the wild-type strain, PtsN becomes increasingly phosphorylated during growth, to the point that the P-free protein species disappears completely in stationary cells (Pflüger & de Lorenzo, 2007). PtsN phosphorylation is thus influenced not only by the immediate carbon source and N status (provided that *P. putida*'s PtsP behaves as in *E. coli*, see above), but also by the growth state of the cells. The N source also plays a role in PtsN phosphorylation: while cells grown with a rich nitrogen source show both PtsN species, a poor nitrogen source results in only the phosphorylated form being detectable (Pflüger & de Lorenzo, 2007). Since the non-phosphorylated PtsN species is the one able to contact (most often inhibiting) its targets, it seems that a general role of the protein is to post-translationally hinder their activities during C-rich and N-rich growth but unleash their action when cells face nutritional limitations. Consistent with this notion, we found that cells lacking PtsN have significantly higher carbon fluxes in the reactions of the pyruvate shunt, which could be traced to increased activity of the corresponding metabolic enzymes (Chavarría *et al.*, 2012).

Still, the most direct proof of interaction of the $PTS^{Ntr}$ with central metabolism of *P. putida* is the physical contact of PtsN with the E1 subunit of the pyruvate dehydrogenase (PDH) complex of this bacterium (Pflüger-Grau *et al.*, 2011). PDH controls *inter alia* the flux of carbon from catabolism of carbohydrates into the Krebs cycle and thus

operates as a divider of metabolic resources towards distinct physiological fates (Fig. 3b). The PDH complex is formed by multiple copies of 3 enzymatic subunits, the decarboxylase E1 (encoded by *aceE*), the acetyltransferase domain E2 (*aceF*) and the dihydrolipoamid dehydrogenase E3, (*lpdG*). These three enzymes together are responsible for the decarboxylation of pyruvate to acetyl-CoA. As this is an irreversible process, the PDH complex thereby serves as sort of a *gatekeeper* of the TCA cycle, as the amount of acetyl-CoA introduced into the cycle fuels its performance. From detailed analysis of PDH activities in cell extracts of various *ptsN* mutant backgrounds, it has been demonstrated that [i] the non-phosphorylated form of PtsN is the inhibitor of PDH activity and that [ii] this mechanism does not involve phosphate transfer to/from the PDH complex. Thus, the phosphorylation state of PtsN seems to be directly communicated to PDH, thereby influencing its activity, and thus modulating the amount of acetyl-CoA produced in the cell.

The link between PtsN and acetyl-CoA could account for why a *P. putida ptsN* mutant accumulated about 70% more polyhydroxyalcanoates (PHAs) from octanoates than the wild type, whereas *ptsO* or *ptsP* mutants were almost totally incapable of PHAs production (Velázquez *et al.*, 2007). At that time, we argued that the loss of PtsN is sensed by the PHA synthesis machinery as an indicator of carbon surplus with respect to other limiting nutrients. This could then be the signal that channels much of the available octanoate to the synthesis of PHAs. Conversely, the PHA polymerization machinery of cells lacking either PtsP or PtsO would detect a shortage of carbon, thereby directing octanoate to other destinations. In view of the more recent data concerning PDH (Pflüger-Grau *et al.*, 2011) it could well happen that the extra acetyl-CoA predicted to be produced in a *ptsN* mutant strain is either diverted directly into the accumulation of the PHA or serves as a signal for a more intense PHA synthesis. This is consistent with the fact that a *ptsO* mutant produces less PHAs: since the loss of NPr results in a greater concentration of non-phosphorylated PtsN, the PDH complex is inhibited and less acetyl-CoA is produced. The same reasoning can be applied for a *ptsP* mutant, which also fails to accumulate PHAs (Velázquez *et al.*, 2007). In summary, it seems that the $PTS^{Ntr}$ of *P. putida* has connections to both upper (e.g. glycolysis) and lower (PDH, Krebs cycle) central metabolic routes as well as to peripheral biochemical activities such as PHA synthesis.

A more complicated scenario is posed by the role of PtsN in mediating the carbon-source repression of *m*-xylene catabolism encoded by the pWW0 plasmid of *P. putida* mt-2 (Cases *et al.*, 1999; Cases *et al.*, 2001). While the phenomenon is clear in that *ptsN* mutants do not display the distinct repression by glucose of the $\sigma^{54}$-dependent and *m*-xylene responsive promoter *Pu* that drives transcription of the *xyl* genes for degradation of the aromatic substrate, the mechanism behind it is still uncertain. This is because not only PtsN but also other regulatory

components such as the RNA-binding Crc protein (Moreno *et al.*, 2010) and other unknown actors (Silva-Rocha & de Lorenzo, 2011) influence the expression of the *xyl* genes as well. This makes it difficult to distinguish direct from indirect effects while one wonders why a simple metabolic trait is subject to such an intricate regulation (Silva-Rocha *et al.*, 2013). Sorting out the interplay between PtsN, Crc, small RNAs and the omnipresent Hfq RNA-binding factor in the regulation of catabolic systems of *P. putida* is currently under investigation by different research groups.

**The *selfish metabolism* – or who rules here?**

The data discussed above verify that in *P. putida* [i] the phosphorylated form of PtsN is present in all growth stages, [ii] PtsN~P accumulates in the stationary phase, [iii] the non-phosphorylated PtsN appears under growth conditions not limited by C or N, [iv] the lack of PtsN leads to changes of the metabolic fluxes of central carbon metabolism and [v] PtsN decreases the activity of the PDH complex. The information encrypted in the phosphorylation ratio of PtsN/PtsN~P is then decoded through protein-protein interactions into instructions for the functioning of different metabolic modules. All these results point to a role of the $PTS^{Ntr}$ as a non-genetic, protein-based actuator of the physiological situation of the cell in response to endogenous and exogenous signals. The question is whether this is just one more element of the repertoire of devices that cells use to maintain their homeostasis or, in fact, that it unveils a different dimension of a key biological problem. Since the late 1970s the metaphor of the *selfish gene* as proposed by Dawkins (2006) has guided a view of evolution that takes for granted that the sole agenda of biological systems is to ensure self-perpetuation of the genes they contain. This notion is however growingly challenged by a more integrated view in which the highly interconnected network of genes, proteins and metabolites, with metabolism holding the leading role, becomes the actual driver of both short-term functioning and evolutionary adaptation on the longer run (de Lorenzo, 2014). While the flow of information in any live object is DNA→ RNA → proteins → metabolism, the *chain of command* is just the opposite: metabolism controls gene expression and often rules directly the functioning of RNAs and proteins. Metabolism seems to have in fact its own evolutionary drive towards both conquering of new chemical landscapes (de Lorenzo, 2014) and sorting out biochemical conflicts (de Lorenzo *et al.,* 2014). The rest of the cell machinery could well be submitted to this drive, an occurrence that we have termed *selfish metabolism* (as opposed to the *selfish DNA* metaphor).

Apart from well-known regulatory duties in allosteric control of enzyme activities, intermediate metabolites and small molecules are increasingly being identified as trigger agents in transcriptional attenuation, regulating

riboswiches and ribozymes. Metabolites thus deploy their regulatory role not only by interacting with proteins but also with RNA e.g. determining translational activity. Genome, proteome and metabolome are not 3 distinct entities, separated from each other, but should be perceived as a highly interconnected whole (Fig. 4), in which none can exist and fulfil its duties without the control and feed-back of the other, but all subordinated to metabolism. These features account for the high efficiency and robustness of cellular physiology. In many cases, the loss of one gene can be compensated by the joint activity of the whole, leading only to minor deficiencies, many still not detectable with today's methods. However, changing the level of a core metabolite leads to drastic effects on metabolic fluxes, which in turn have an influence on transcriptional and translational activity of the whole cell (see de Lorenzo, 2014 for a thorough discussion). The interplay between these 3 levels, however, requires molecular mediators and we suggest that the PTSs are key go-betweens that make connections between genome, proteome, and metabolism. In *P. putida* we have shown that various physiological factors such as C source, N source, or growth rate are reflected in the phosphorylation state of PtsN which in turn modulates the activity of the PDH complex, thereby changing the levels of the core metabolite acetyl-CoA. In *E. coli* the $PTS^{Ntr}$ was recently shown to connect carbon metabolism to potassium homeostasis, as outlined above, and this same phenomenon in *P. putida* is currently under investigation in our laboratories. Nevertheless, metabolic changes both sensed and transmitted through the $PTS^{Ntr}$ are likely to have greater consequences than the mere resetting of the transcriptome and the proteome of individual cells in that they can also propagate into population behaviour e.g. swimming and formation of biofilms (Fig. 5). In sum, we argue that non-genetic regulation of cell homeostasis as endowed by the PTS will be key not only to understanding the functioning of extant cells but also to guiding the engineering of new traits of biotechnological interest in bacteria like *P. putida* (Nikel *et al.*, 2014).

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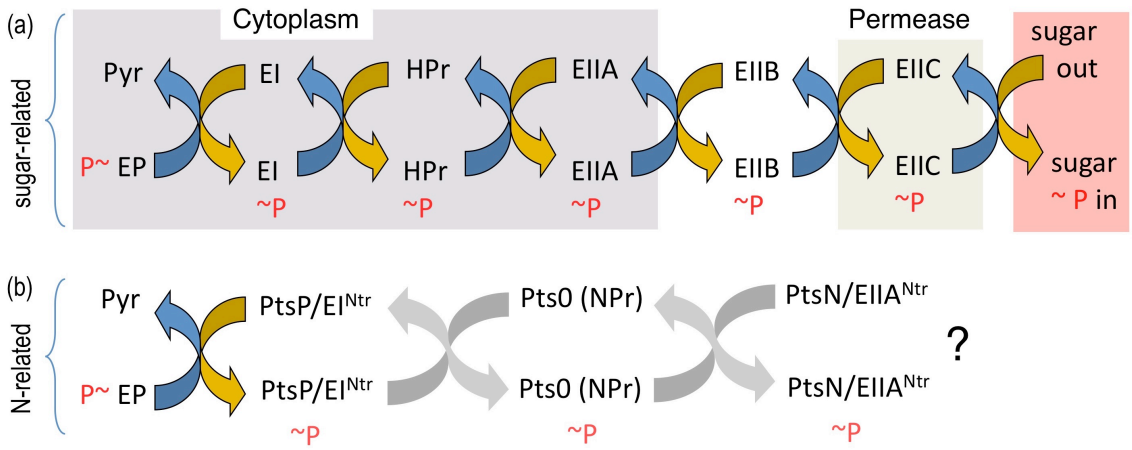
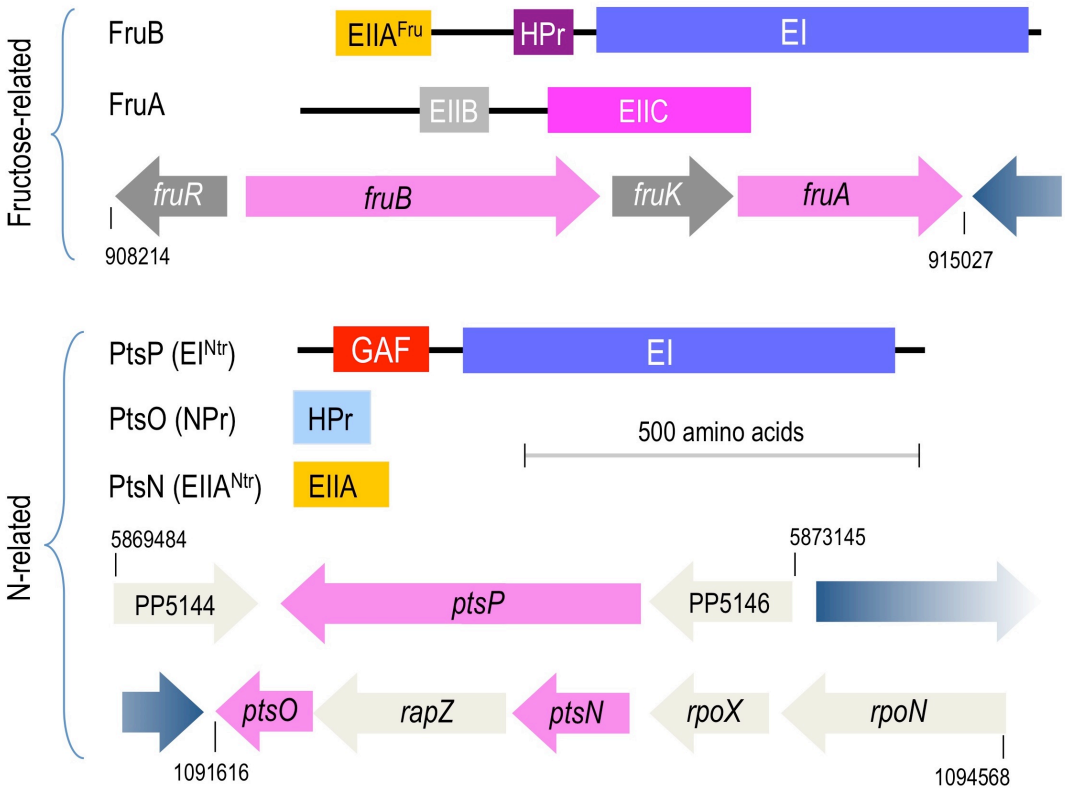
**FIGURES**

**Fig. 1.** Organization of the phosphotransfer chain in PTSs.



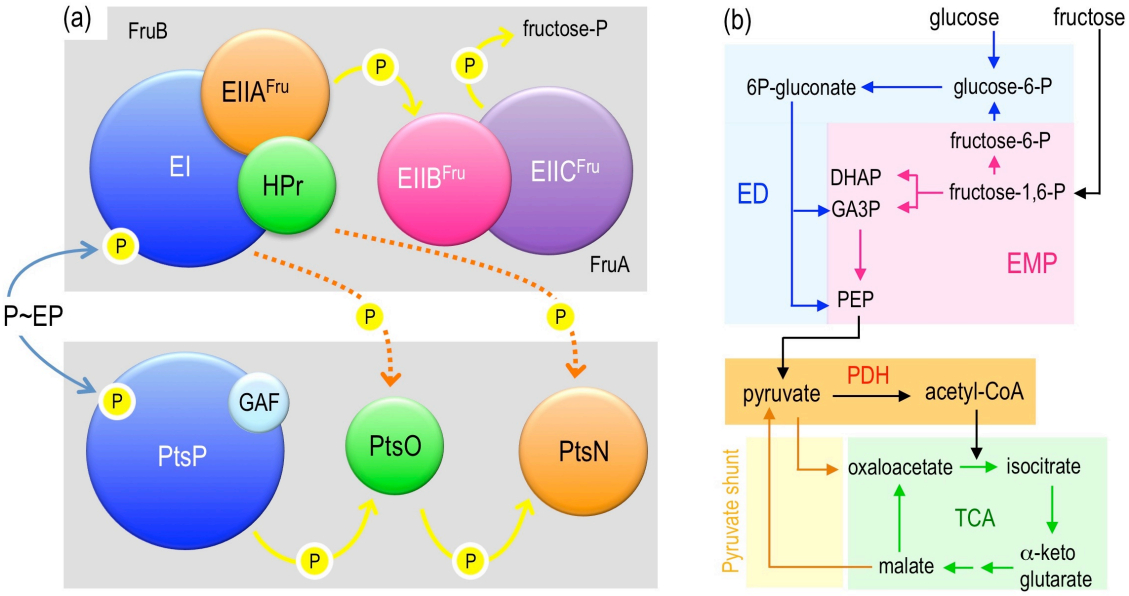

**(a)** The canonical, sugar-related PTS involves a flow of high energy phosphate (~P) that originates from phosphoenolpyruvate (PEP) and runs through the EI, HPr, and EII enzymes –which can be stand alone modules or domains associated to other proteins. EII enzymes are commonly composed of three domains EIIA, EIIB and EIIC, which may also appear as either fused or separated proteins. The membrane-bound component EIIC is a permease that couples phosphorylation of a specific sugar to its uptake (EIIC may or may not be fused to EIIB). This basic scheme has a large number of variations, including the fusion of PTS to non-PTS protein domains. **(b)** The nitrogen-related PTS includes three proteins with EI, HPr and EIIA motifs (named with synonyms EI/PtsP, NPr/PtsO, and EIIA/PtsN, respectively), which circulate ~P groups as shown in the scheme. The ultimate destination of the ~ P bound to the $EIIA^{Ntr}$ protein is unknown.

**Fig. 2.** The components of the PTS of *P. putida*.



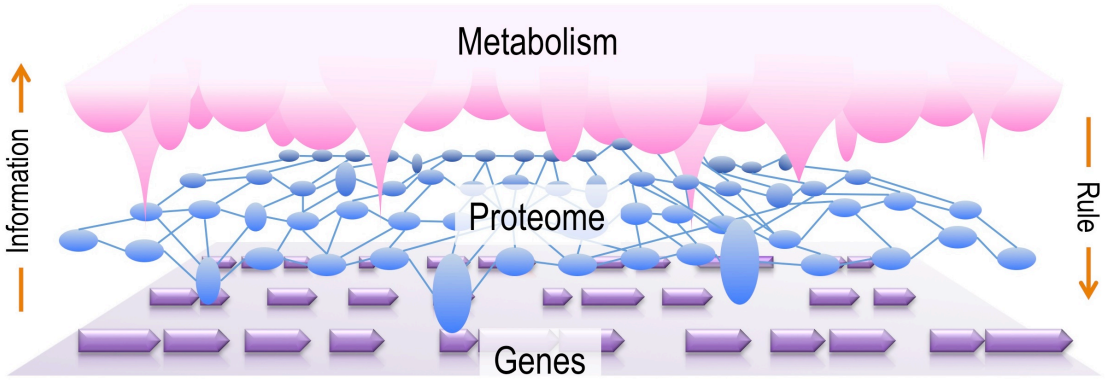

The distribution of EI, HPr, and EII domains in each PTS protein is indicated along with the context of the corresponding genes in the chromosome of *P. putida*. Along with *fruK* (fructose-1-P kinase), *fruB* and *fruA* form part of a cluster for uptake and metabolism of fructose. The organization of the two PTS genes found downstream of *rpoN* (*ptsN* and *ptsO*) is shown along with the other two genes that complete the transcriptional unit: *rpoX* (encoding the *ribosome hibernation promoring factor* HPF) and *yhbJ (rapZ)*, a sRNA chaperone. The *ptsP* gene stands alone in the *P. putida* chromosome, flanked downstream and upstream by orphan genes PP5144 and PP5146.

**Fig. 3.** Physiological activities of the *P. putida*'s PTS.



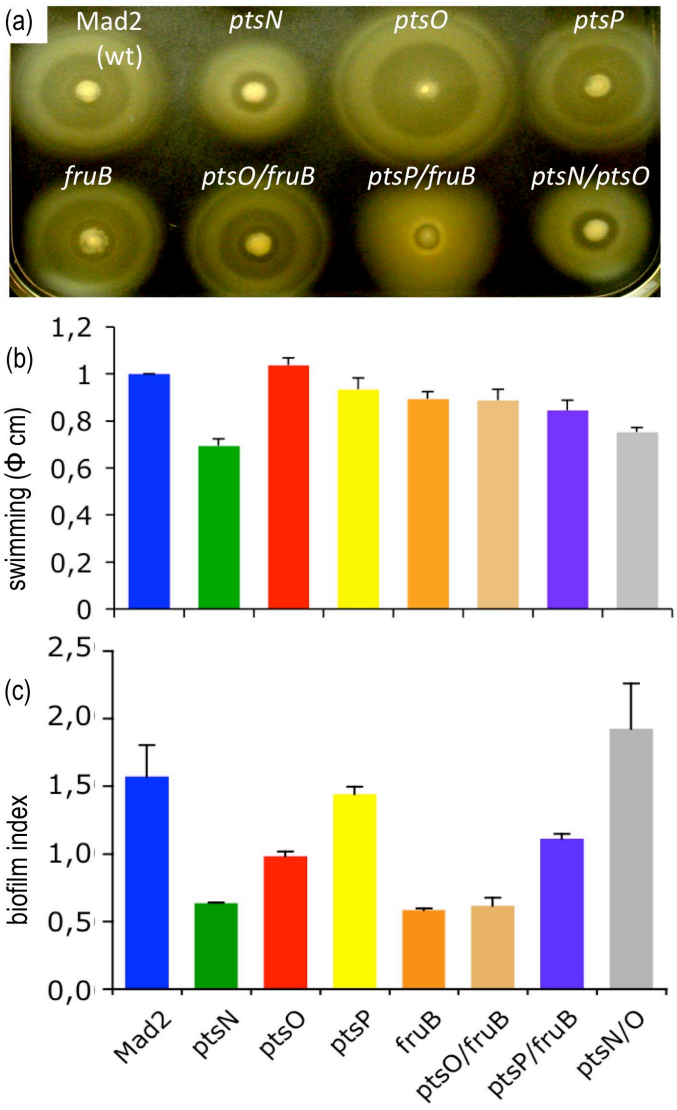

**(a)** Cross talk between the two branches of the *P. putida* PTS. The upper panel shows the standard ~P traffic from PEP to fructose through the various domains of the PTS$^{Fru}$ proteins FruA and FruB. The lower panel shows the same traffic through the PTS$^{Ntr}$ constituents. The yellow arrows indicate the standard phosphorylation steps, whereas the orange dashed arrows indicates phosphorylation cross talk under glycolytic regimes. **(b)** The pyruvate dehydrogenase complex (PDH) is the metabolic target for PtsN and links glycolysis to the Krebs cycle. The PDH complex is formed by multiple copies of 3 enzymatic subunits: the decarboxylase E1 (which is the actual target of PtsN) encoded by *aceE*, the acetyltransferase domain E2 (*aceF*) and the dihydrolipoamid dehydrogenase E3 (*lpdG*). These three enzymes together are responsible for the decarboxylation of pyruvate to acetyl-CoA (orange box). The PDH complex thereby links glycolysis –whether the Entner-Doudorodff (ED) or the Embden-Meyerhof-Parnas (EMP) branches, to the TCA cycle (green box), as the amount of acetyl-CoA introduced into the cycle fuels the activity of the TCA cycle.

**Fig. 4.** Metabolism, proteome, and genome form a tightly interconnected unit.




The metabolism, especially the small metabolites (represented in pink) communicates with the enzymatic network (represented in blue), which has an influence on gene regulation and transcription (genes are shown in purple), which in turn regulate the abundance of certain components of the protein network. Note that the direction of information transfer from DNA to metabolism is opposite to the ruling hierarchy of metabolism over genes (DNA).

**Fig. 5.** Metabolism determines multicellular behaviour.




**(a)** Swimming assays of *P. putida*'s PTS mutants. Each of the strains indicated was tested for flagellar swimming on low-agar LB medium. Note the different multi-cellular patterns, which could be related to the changes in metabolic trafficking caused by the mutations. **(b)** Quantification of relative swimming differences (halo diameters) among PTS mutants using the wild-type strain Mad2 as reference **(c)** Biofilm formation by the same mutants. Biofilm indexes were calculated as the ratio of crystal violet (CV) staining ($A_{595}$) to planktonic cell density ($OD_{600}$). Methods are described in detail in Martínez-García *et al.* (2014).

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