The nitrogen-related branch of the phosphoenolpyruvate:carbohydrate phosphotransferase system (PTS) of *Pseudomonas putida* includes the *ptsN* gene encoding the EIINtr (PtsN) enzyme. Although the implication of this protein in a variety of cellular functions has been observed in diverse bacteria, the physiological signals that bring about phosphorylation/dephosphorylation of the PtsN protein are not understood. This work documents the phosphorylation status of the EIINtr enzyme of *P. putida* at various growth stages in distinct media. Culture conditions were chosen to include fructose (the uptake of which is controlled by the PTS) or glucose (a non-PTS sugar in *P. putida*) in minimal medium with casamino acids, ammonia, or nitrate as alternative nitrogen sources. To quantify the relative ratio of PtsN/PtsN$^*$ in live cells, we resorted to the in situ electrophoresis of whole bacteria expressing an E-epitope-tagged EIINtr followed by the fractionation of the thereby released native proteome in a non-denaturing gel. Although the PtsN species phosphorylated in amino acid His$^{68}$ was detected under virtually all growth scenarios, the relative levels of the non-phosphorylated form varied dramatically depending on the growth phase and the nutrients available in the medium. The share of phosphorylated PtsN increased along growth in a fashion apparently independent of any trafficking of sugars. The large variations of non-phosphorylated PtsN in different growth conditions, in contrast to the systematic excess of the phosphorylated PtsN form, suggested that the P-free PtsN is the predominant signaling species of the protein.

The phosphoenolpyruvate:carbohydrate phosphotransferase systems (PTS) are among the most widespread protein phosphorylation setups in both Gram-positive and Gram-negative bacteria. The PTS systems generally participate in the uptake of sugars into the cells by means of a cascade of high energy phosphate transfer processes (1, 2). As shown schematically in Fig. 1A, the archetypal PTS system consists of enzyme I (EI), HPr, and enzyme II (EII). Due to their ability to interact with other proteins in the cytoplasm, the set of phosphorylated and non-phosphorylated PTS proteins (more often HPr and EIIA) fulfills regulatory functions as diverse as catabolite repression, chemotaxis, the regulation of cyclic AMP synthesis, or nitrogen assimilation (2–4). Apart from these PTS components involved in sugar transport, many prokaryotes also have PTS branches that are not involved in carbohydrate traffic. This is because their EII component fails to have the permease partners (the so-called EIIB and EIIC domains) needed for sugar intake. However, such proteins still participate in regulation of some processes in a fashion dependent on their phosphorylation state. The PtsN protein of *Pseudomonas putida* (Fig. 1B) is one example of this case (5, 6). This polypeptide is orthologous to the so-called IIA$^{Ntr}$ protein of *Escherichia coli*. This product is hypothesized to play a role in N-metabolism because of the clustering of the *ptsN* gene with rpoN, which encodes the alternative, nitrogen-related sigma factor $\sigma^{Nt}$ (3). Sequence comparison with other IIA$^{Ntr}$ proteins revealed that PtsN can most likely be phosphorylated on the His$^{68}$ residue (6). Indeed, the IIA$^{Ntr}$ protein of *E. coli* can be phosphorylated in *vitro* both by HPr and by the homologous protein NPr (3), although this has never been demonstrated to occur in *vivo*.

The question of visualizing the phosphorylation state of PtsN in *vivo* is of essence for understanding the physiological control of biodegradative gene expression in *P. putida* and other soil bacteria. We (6) and others (8) have previously identified the *ptsN* gene as one of the elements that mediates the C-source repression of *m*-xylene catabolism determined by the pWW0 plasmid of *P. putida* mt-2. The principal *m*-xylene-responsive $\sigma^{Nt}$-dependent promotor of this system (called *Pu*) is regulated not only by the presence of pathway substrates but also subject to different physiological inputs that cause its down-regulation in *vivo* (6, 9–13). Specifically, glucose and other carbohydrates of the Entner-Doudoroff pathway repress transcription from *Pu* (14) through a mechanism that involves the phosphorylation of the His$^{68}$ residue of PtsN. The course of the phospho-transfer in the process or the origin and fate of the high energy phosphate that passes through PtsN in *vivo* is, however, difficult to assess. This is because phospho-histidines are labile in any of the procedures that allow detection of other phospho-amino acids (15, 16). To overcome this difficulty, we have resorted to the release of much of the intact proteome of live *P. putida* cells directly into a native polyacrylamide gel by means of the *in situ* electrophoresis of whole bacteria. With this experimental setup...
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in hand, we were able to examine the phosphorylation state of PtsN of *P. putida* through various growth stages and distinct nutrient conditions. These included media with fructose (a PTS sugar in *P. putida* (18)) or glucose (a non-PTS sugar (18)), along with casamino acids, ammonia, or nitrate as N-sources. The data presented below revealed that the phosphorylated form of PtsN systematically accumulates along growth regardless of the nutrient conditions. These included media with fructose (a PTS sugar) or 5 mM NaNO\textsubscript{3}. For induction of PtsN, cells were grown in nitro-

\textbf{Materials and Methods}

\textbf{Strains, Plasmid, and Growth Conditions—All Pseudomonas strains used in this work were derived from strain *P. putida* MAD2, a derivative of the reference strain *P. putida* KT2440 (19). The *P. putida* MAD2 variants bearing directed chromosomal insertions of gene \textit{ptsN}, with either a kanamycin (Km) resistance gene or the xylE marker, have been described before (6, 9). Plasmid pVLT\textit{ptsN} tag, encoding a variant of the \textit{ptsN} gene with a short E-tag segment (see “Results”). Km-resistant *P. putida* exconjugants were examined by PCR for integration of pVLT\textit{ptsN} into the chromosome by a single DNA crossover event between the homologous sequences. One clone fulfilling all criteria was then kept for further analysis. Unless indicated otherwise, cells were grown at 30 °C in either rich LB medium or synthetic minimal M9 medium (23) supplemented with 0.2% casamino acids and 5 µg/ml tetracycline or 50 µg/ml kanamycin, in the presence or absence of 0.2% glucose or fructose. To analyze the influence of the N-source, cells were grown in nitrogen-free M9 medium supplemented with either 10 mM NH\textsubscript{4}Cl or 2 mM NaNO\textsubscript{3}. For induction of \textit{ptsN} expression born by plasmids pVLPT\textit{ptsN} tag and pVLPT\textit{NHA_E}, isopropyl-1-thio-β,δ-galactopyranoside was added to the cultures to a final concentration of 1 mM.

**Sample Preparation and Processing**—To obtain samples for analysis in the native PAGE system, \textit{cells} were grown in the media indicated, amended where required with suitable antibiotics and isopropyl-1-thio-β,δ-galactopyranoside. The cells were then harvested by centrifugation (2 min, 14,000 rpm, 4 °C) at the time points indicated, and the pellets were resuspended in a non-denaturing loading buffer (10% glycerol, 40 mM glycine, 5 mM Tris, pH 8.9, and 0.005% w/v bromphenol blue) such that a cell mass equivalent of 1 ml of culture at an \textit{A}_\text{600} = 1.0 was adjusted to disperse in 200 µl of the loading buffer. 10 µl of such intact cell suspensions were directly loaded into the wells of the non-denaturing gel system described below. Where required, protein specimens were stored at −20 °C until use (never longer than 1 week).

**Native and Denaturing PAGE and Western Blotting**—The non-denaturing gel electrophoresis system employed throughout this work is explained elsewhere. In brief, the gels consisted of a 10% polyacrylamide (10% (v/v) acrylamide/bis-acrylamide solution (29:1) polymerized with 0.05% TEMED and 0.05% ammonium persulfate in 1× running buffer (200 mM glycine, 25 mM Tris, pH 8.9) and were assembled in a mini-Protein gel box (Bio-Rad). 10 µl of the protein samples prepared as described above and adjusted to contain equivalent cell numbers were loaded in each well, and electrophoresis was carried out with a fixed current of 12.5 mA/gel (8.5 × 6.5 cm, width × height) at 4 °C for 35 min. The proteins were then transferred onto a polyvinylidene difluoride membrane (Immobilon-P, Millipore) using a semidyne transfer apparatus (Bio-
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Rad) as described (24). The anti-E-tag monoclonal antibody-peroxidase conjugate (Amersham Biosciences) was applied for luminescent detection of the PtsN-E-tag fusion proteins with the procedure described (24). The SuperSignal West Femto maximum sensitivity substrate (Pierce Biotechnology) system was employed for detection of chromosomally encoded E-tagged ptsN, according to the supplier’s manual. The intensity of the PtsN bands was quantified in a molecular imager Versadoc 4000 (Bio-Rad) with the Quantity One software.

RESULTS

PtsN Occurs in Vivo as Two Protein Species Differing by Phosphorylation of His68—The first step for examining the effect of various physiological conditions on the phosphorylation state of PtsN was to authenticate the protein forms that are released from P. putida cells upon in situ electrophoresis.3 To this end, we employed an epitope-containing variant of the PtsN protein fused to an E-tag sequence so that we could accurately follow its presence in various types of samples. As shown in Fig. 2, the PtsN protein freed from live P. putida ptsN::Km (pVLTptsN tag) cells grown in LB separated into two bands (lane 3) in the native gel system, possibly reflecting the phosphorylated and the non-phosphorylated forms. Unfortunately, neither are P ~ His bonds good substrates for commercially available phosphatases that act well on P ~ Ser and P ~ Tyr, nor do reliable procedures exist yet for analyzing P ~ His peptides by mass spectrometry. We thus chose a genetic procedure for recognizing phosphorylation of PtsN and for tracing it to a distinct position within the amino acid sequence of the protein. This consisted of examining the gel migration properties of a PtsN variant in which the conserved His68 residue, that is, the substrate of phosphorylation by cognate kinases, has been exchanged by an Ala and is thus locked in a non-phosphorylated form. P. putida cells bearing pVLTptsNHA_E were subject to the same treatment as before and examined in the non-denaturing system. The data of Fig. 2 show that such an H68A variant produces one band that migrates to the same position as the native system. The data of Fig. 2 show that such an H68A variant produces one band that migrates to the same position as the native system. The data of Fig. 2 show that such an H68A variant produces one band that migrates to the same position as the native system.

FIGURE 2. The PtsN H68A variant does not originate a faster-migrating protein form. P. putida ptsN::Km (pVLTptsN tag) and P. putida ptsN::xylE (pVLTptsNHA_E) were grown as indicated in M9-glucose or LB with suitable antibiotics, and the cells were collected in the exponential phase and subjected to the native PAGE system. The migration of the PtsN protein was then visualized by a Western blot procedure with an anti-E-tag antibody. Note the complete absence of the faster-migrating form of the protein in samples expressing the H68A form of PtsN (lanes 1 and 2). See “Results” for a comment on the abnormal migration of the sample in lane 2.

Due to the intrinsic production of PtsN at relatively low levels in vivo, the signals from the chromosomally encoded, E-tagged product in the Western blot were quite weak, almost at the detection limit of the procedure. A super-sensitive detection system and longer exposure times had to be used for identification of the bands (see “Materials and Methods”). To overcome this limitation, we resorted to expressing the same protein from plasmid pVLTptsN tag for both raising the level of intracellular protein and uncoupling expression from its native promoter. To ensure that this change still reflected the physiological scenario, we followed the evolution of PtsN in P. putida ptsN::Km (pVLTptsN tag) cells grown in LB under the same conditions of the chromosomally ptsN-tagged P. putida strain. The result (Fig. 3C, lower panel) reproduced dependably the behavior of the chromosomal ptsN-tagged strain (including the sharp decrease of PtsN signals at late stationary phase). Moreover, quantification of the products was facilitated by a higher expression of the proteins, and deviations between experiments decreased. All this endorsed P. putida ptsN::Km (pVLTptsN tag) as a strain of choice for examining the relative ratio of PtsN species in connection to the presence of diverse C-sources or N-sources in the medium.

PtsN ~ P Accumulates with Growth Regardless of the Culture Medium—As the phosphorylation state of PtsN is an indicator of the passage of high energy phosphate between the components of the abridged PTS system sketched in Fig. 1B, the pro-
FIGURE 3. Monitoring PtsN protein forms in P. putida cells along growth. A, E-tagging the genomic ptsN sequence by homologous recombination. The sketch summarizes the steps followed to deliver a C-terminal E-tag to the chromosomally encoded ptsN gene. This involved the mobilization of suicide plasmid pJP/ptsNΔΔN (which encodes a truncated, E-tagged ’ptsN sequence) and the formation of a co-integrate with the cognate chromosomal region (the coordinates of the enlarged genomic context are indicated). This leaves a single copy of the E-tagged ptsN expressed under the same promoter as the original ptsN gene. The reorganization of the resulting chromosomal region after co-integration was verified by PCR (not shown). B, growth of P. putida cells bearing the E-tagged ptsN gene. Bacteria were cultured at 30 °C in LB with Km added. Samples were collected at the times indicated with arrows. C, evolution of PtsN forms along growth. Intact cells were loaded in the native PAGE and Western blot analysis with an anti-E-tag antibody. The results with the strain bearing a monocopy (mono) E-tagged ptsN (upper panel) are compared with those of an equivalent experiment with P. putida ptsN::Km (pVLT/ptsN tag, multi) at the lower panel.

FIGURE 4. Monitoring non-phosphorylated versus phosphorylated PtsN ratios in P. putida ptsN::Km (pVLT/ptsN tag). Cells were grown at 30 °C in the M9 medium indicated in each case amended with tetracycline to favor plasmid retention. Samples were collected at various times after cells had reached an A600 ~ 0.1, and intact cells were analyzed in the native PAGE and Western blot system with an anti-E-tag antibody. Note the presence of the PtsN ~ P species in all conditions and its prevalence in late stationary phase. CAA, casamino acid.

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The PTS system (components of which are encoded in almost every bacterial genome known (2)) has evolved to provide a mechanism by which the activities of distinct sets of proteins are regulated epigenetically. This occurs by means of significantly depending on the growth medium and the growth stage. For instance, Fig. 4 shows that there is a considerable amount of the non-phosphorylated PtsN in glucose-grown cells at a growth phase in which the same form has virtually disappeared from cells grown in the presence of fructose. In Fig. 5, the various ratios of PtsN/PtsN ~ P species are shown as a function of growth phase. Although all of them shared the predominance of the phosphorylated form at late growth stages, the disappearance of the non-phosphorylated species seems to occur earlier in the fructose-grown cells. A different case is posed by varying the N-source from ammonia (NH4+) to nitrate (NO3−) in the culture (Fig. 6). This anion cannot be used as a terminal electron acceptor by P. putida as this bacterium lacks the physiologioal change caused by NO3− is, therefore, the replacement of an easy N-source by a less direct counterpart. The result shown in Fig. 6 suggests that the phosphorylated form of PtsN is virtually the only product observed with NO3−, whereas both forms can be detected in NH4+-grown bacteria. That the PtsN/ PtsN ~ P ratios can be changed by growth phase, C-source, and N-source suggests that the phosphorylation state of this protein is more a reflection of the metabolic status of the cells than a component of any specific sugar-traffic system.

DISCUSSION

The PTS system (components of which are encoded in almost every bacterial genome known (2)) has evolved to provide a mechanism by which the activities of distinct sets of proteins are regulated epigenetically. This occurs by means of...
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![FIGURE 5. Evolution of the share of non-phosphorylated PtsN in \textit{P. putida} ptsN::Km (pVLTPtsN tag). Cells were grown in the media indicated, and samples were collected at the times indicated by arrows. The relative contents of the two PtsN forms were then determined in live cells as before. The bar diagrams represent the average of at least three separate experiments. Note the important presence of non-phosphorylated PtsN in cells grown in casamino acids (CAA) and glucose as compared with bacteria grown in fructose. In either case, the non-phosphorylated PtsN forms virtually disappear during the late stationary phase.](Image 373x26 to 400x38)

![FIGURE 6. Effect of nitrogen sources in the phosphorylation state of PtsN in \textit{P. putida} ptsN::Km (pVLTPtsN tag). Cells were grown at 30 °C in N-free M9 minimal medium and glucose, supplemented with either 10 mM ammonia (NH\textsubscript{4})\textsubscript{2} or 2 mM nitrate (NO\textsubscript{3}). Culture samples were collected at the late exponential (late exp, lanes 1 and 2, A\textsubscript{600} of 0.8 for NH\textsubscript{4}\textsuperscript{+} or 0.3 for NO\textsubscript{3}) and early stationary (early stat, lanes 3 and 4, A\textsubscript{600} of 1.5 for NH\textsubscript{4}\textsuperscript{+} or 0.7 for NO\textsubscript{3}) phase, and cells were subject to the standard analysis of the PtsN forms. Note that PtsN − P is the only species that appears in cells growing in NO\textsubscript{3} as N-source.](Image 61x459 to 289x734)

The intriguing side of what appears to be a separate branch of the PTS system (Fig. 1B) is that the protein encoded by \textit{ptsN} (EI\textsuperscript{Ntr}) lacks the membrane-associated permease moieties EIIB and EIIC that typically tie PTS proteins to sugar transport (4). An early hint about the functions of such proteins was derived from the genetic association of \textit{ptsN} (and the adjacent \textit{ptsO} gene) to \textit{rpoN} in several bacteria (3). \textit{rpoN} encodes a major sigma factor (\sigma\textsuperscript{N}) involved in many other functions (27–29) in nitrogen metabolism. The proposition thus was that these PTS genes could be related to sensing the N \textit{versus} C balance (\textit{i.e.} the excess of one nutrient source \textit{versus} the other), through a thus far unknown mechanism. One useful clue in this direction is the recent observation that the \textit{P. putida} EI\textsuperscript{Ntr}, NPr, and EI\textsubscript{II}N\textsuperscript{tr} proteins act in concert to control the intracellular accumulation of polyhydroxylalkanoates, typical products of carbon overflow in respect to other essential nutrients, \textit{e.g.} nitrogen (18).

The whole set of EI\textsuperscript{Ntr}, NPr, and EI\textsubscript{II}N\textsuperscript{tr} proteins of \textit{E. coli} have been purified and shown \textit{in vitro} to sustain a typical flow of high energy phosphate: phosphoenolpyruvate $\rightarrow$ El\textsuperscript{Ntr} $\rightarrow$ NPr $\rightarrow$ EI\textsubscript{II}N\textsuperscript{tr} (3). The three-dimensional structure of PtsN (EI\textsuperscript{Ntr}) has been determined (31, 32), and its interactions in solution with NPr have been studied (33). \textit{ptsN} mutants of \textit{E. coli} have been generated and subjected to a variety of phenotypic analyses. For instance, \textit{ptsN} mutants are extremely sensitive to leucine-containing peptides (LCPs (34)), a phenomenon that can be traced to the interaction of EI\textsuperscript{II}N\textsuperscript{tr} with the K$^+$ transporter TrkA (35). In addition, growth of the \textit{ptsN} mutant of \textit{E. coli} is inhibited by several sugars and tricarboxylic acid cycle intermediates in a medium containing an amino acid or nucleoside base as a combined source of nitrogen and carbon (3). Such an inhibition can be reversed by supplying ammonium salts, an indication that \textit{ptsN} is indeed related to N-metabolism. \textit{ptsN} mutants of \textit{P. putida} change the sensitivity of the m-xylene-responsive \sigma\textsuperscript{54} promoter \textit{Pu} to the presence of glucose in the medium (6, 8, 36–38) and alter the expression pattern of a large number of proteins of the cell proteome (36), \textit{ptsN} is also involved in the response of \textit{Pasteurella multocida} to iron starvation (39). Finally, inactivation of \textit{ptsN} in \textit{Rhizobium etli} reduces growth on medium containing succinate, lessens production of melanin, and inhibits expression of the N-fixation gene \textit{nifH} (40). Despite these multiple observations, the phosphorylation state of PtsN \textit{in vivo} or the physiological circumstances that alter such a state have not been examined before.

Perusal of the results shown in Figs. 3–6 clearly indicated that, regardless of the culture conditions: (i) the phosphorylated form of PtsN is present in all growth stages; (ii) PtsN − P accumulates in the later stationary phase; and (iii) the non-phosphorylated PtsN protein appears associated to rapid growth. Although the expression of the \textit{ptsN} gene from a heterologous promoter of a plasmid does unbalance the native stoichiometry of the system, we have shown that our experimental setup reasonably reflects the physiological scenario, probably because equilibrium is reached in the phosphorylation of all the proteins of the PTS system. Although we detect phosphorylated PtsN in virtually all growth conditions, the non-phosphorylated form seems to be subject to growth-dependent variations. If so, the protein form with more regulatory significance should be the...
non-phosphorylated PtsN species, the one that clearly varies with the carbon source at the onset of stationary phase. This notion is consistent with the appearance of a slower-migrating band in non-denaturing gel analysis of an extract expressing the H68A variant of PtsN (Fig. 2, lane 2), which may consist of complexes between PtsN and other proteins. Moreover, our results show that the disappearance of the non-phosphorylated PtsN does not occur simultaneously in all cases. Instead, the decline of the P-less species seems to happen earlier in the presence of fructose than with glucose (Fig. 5) and earlier in the presence of nitrate ($\text{NO}_3^-$) than with ammonia ($\text{NH}_4^+$, Fig. 6). The regulatory significance of these differences is still unclear and deserves further studies. However, they do suggest that it is the physiological conditions of the bacteria and not so much the traffic of specific sugars (as it happens with the canonical PTS) that dictates the relative phosphorylation states of the PtsN protein and possibly of the other enzymes of the N-related PTS of *P. putida*.

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