The IIANtr (PtsN) Protein of Pseudomonas putida Mediates the C Source Inhibition of the $\sigma^{54}$-dependent Pu Promoter of the TOL Plasmid*

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The gene cluster adjacent to the sequence of rpoN (encoding sigma factor $\sigma^{54}$) of Pseudomonas putida has been studied with respect to the C source regulation of the Pu promoter of the upper TOL (toluene catabolism) operon. The region includes four open reading frames (ORFs), two of which (named ptsN and ptsO genes) encode proteins similar to components of the phosphoenolpyruvatesugar phosphotransferase system. Each of the four genes was disrupted with a nonpolar insertion, and the effects in the inhibition caused by glucose on Pu activity were investigated with a lacZ reporter system. Although cells lacking ORF102, ORF284, and ptsO did not display any evident phenotype under the conditions tested, the loss of ptsN, which encodes the IIANtr protein, made Pu unresponsive to repression by glucose. The ptsN mutant had rates of glucose/gluconate consumption identical to those of the wild type, thus ruling out indirect effects mediated by the transport of the carbohydrate. A site-directed ptsN mutant in which the conserved phospho-acceptor site His$^{68}$ of IIANtr was replaced by an aspartic acid residue made Pu blind to the presence or absence of glucose, thus supporting the notion that phosphorylation of IIANtr mediates the C source inhibition of the promoter. These data substantiate the existence of a molecular pathway for co-regulation of some $\sigma^{54}$ promoters in which IIANtr is a key protein intermediate.

Pseudomonas putida cells harboring the catabolic TOL plasmid pWW0 degrade toluene, m-xylene, and p-xylene through a pathway encoded by two separate operons (1). The upper operon encodes enzymes for the oxidation of the methyl group of toluene, whereas the lower operon determines activities for the fission of the aromatic ring leading to pyruvate and acetaldehyde (2). The upper operon is transcribed from the $\sigma^{54}$-dependent promoter Pu, which is activated at a distance by the pWW0-borne regulator called XylR, of the NtrC family of proteins, in response to pathway substrates (1). Like other enhancer binding proteins, XylR has the modular structure common to this type of activator (3). XylR variants devoid of its N-terminal module (called the A domain), activate transcription from Pu (4, 5) in the absence of inducers. Besides XylR and the form of RNA polymerase containing $\sigma^{54}$ ($\sigma^{54}$-RNAP), Pu activation also requires the integration host factor, which both favors a DNA geometry optimal for the interplay of the different factors (6, 7) and assists the binding of the RNA polymerase (8).

The comparison of Pu promoter activity in vitro and in vivo has revealed a paradox that determines the regulation of this promoter under real environmental conditions (9). Although Pu is active in vitro by just mixing purified and preactivated XylR (i.e. deleted of its N-terminal domain) with $\sigma^{54}$-RNAP and integration host factor (5), promoter activity in vivo is down-regulated by various growth conditions (10–16). In particular, the presence in the medium of carbon sources like glucose, gluconate, and $\alpha$-ketogluconate inhibit Pu activity, whereas other compounds (citrate and fructose) do not seem to have any influence (11).

The molecular basis of this phenomenon is unknown. In enteric bacteria, such as Escherichia coli, glucose repression is mediated mainly by the cAMP receptor protein (17) and the PTS$^3$ (18). The latter is a complex and very branched whole of phosphotransfer proteins that mediate the intake of certain carbohydrates (which vary widely among species) through a mechanism involving the simultaneous phosphorylation of the sugar and its transport (18). The availability of adequate carbohydrates in the external medium that act as a drain of high energy phosphate determines the accumulation or the depletion of phosphorylated protein intermediates that have the ability to interact with and modify the activity of many other cell proteins. The PTS proteins have been traditionally classified as enzymes type I, HPr, and enzymes type II, the latter frequently involving a multiprotein array (18). As opposed to the situation in E. coli, cAMP appears to play no role in carbon repression in pseudomonads (19). In fact, the known cAMP receptor protein analogue in Pseudomonas aeruginosa (called Vfr) is fully alien to carbon regulation (20, 21). On the other hand glucose, which inhibits Pu activity (11), is not transported in Pseudomonas through the PTS (22), thus making unlikely an involvement of the housekeeping PTS intermediates in the effect. Finally fructose, which is transported through the PTS system in Pseudomonas (23) does not inhibit Pu activity. The mechanism should therefore be different of those known for other systems subjected to catabolite control.

An early clue on additional regulatory mechanisms for $\sigma^{54}$-dependent promoters in vivo was revealed by the sequencing of the DNA region around the gene encoding the sigma factor (rpoN) in Klebsiella pneumoniae (24) and E. coli (25). In both species rpoN is followed by four open reading frames later

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named ORF95, ptsN, ORF284, and ptsO (see Fig. 1A). Homologues of most of these genes have also been found adjacent to rpoN in various other Gram-negative species (26–30), including P. aeruginosa (31) and P. putida (32). Two of the most conserved ORFs, ptsN and ptsO, encode proteins with a considerable sequence similarity with known intermediates of the PTS sugar transport system. In particular, ptsN appears to encode a IIA-type enzyme, whereas ptsO seems to be a variant HPt protein (25). The corresponding proteins have been designated as IIA{sup N}r and NPr, respectively, on the basis of their hypothetical involvement in N metabolism (25). This was initially suggested by the observation that disruption of the ptsN gene of K. pneumoniae (originally called ORF152) or the adjacent rpoN increased to various degrees the activity of the σ{sup 54}-dependent promoter PstNl (24), whereas the loss of ptsO gene reduced it significantly (33). On the contrary, mutations in the equivalent ptsN (ORF2) gene P. aeruginosa did not affect the activity of the σ{sup 54}-dependent promoters for pilae and flagellin genes (31). Furthermore, some (but not all) σ{sup 54}-dependent promoters tested of Caulobacter and Rhizobium become less active upon the loss of ptsN (29). This protein may also be involved in more general metabolic activities, because the E. coli ptsN mutant displayed certain incompatibilities between C and N sources, typically glucose and alanine (25). In addition, this mutation also suppressed a temperature-sensitive allele of the gene era, which encodes an essential GTPase of unknown function (25).

Taken together, the observations above suggest that ptsN and the accompanying genes of the cluster may play a role not only in expression of σ{sup 54}-dependent promoters but also in their potential connection to N and C metabolism (34). Because such a connection with the central metabolism could account for the C source regulation observed in promoters such as Pu (11), we set out to explore the issue systematically in a well defined genetic assay system. The data presented in this work show that the ptsN gene of P. putida, formerly called ORF154 (32), participates in the inhibition of the Pu promoter of the TOL plasmid by glucose and that such inhibition involves a phosphorylation site of the ptsN-encoded IIA-type enzyme.

**EXPERIMENTAL PROCEDURES**

**Strains, Plasmids, Media, and General Methods—**P. putida MAD2 (4) was used in all cases to examine Pu activity. This strain is a derivative of P. putida KT2442 bearing a chromosomal Pu-loac fusion along with the xylR allele named xylRAA, all assembled within a tellurite (Tel) mini-transposon vector. The loss of the N-terminal A domain of the protein makes XylR constitutively active in the absence of aromatic inducers (4). Bacteria were grown in either rich LB medium or in synthetic mineral M9 medium (35) supplemented with 0.2% casamino acids (CAA) to equal growth rates and to avoid effects related to the stringent response (36). To test carbon inhibition of promoter activity, the M9-CAA medium was amended with glucose, gluconate, or fructose at a final concentration of 10 mM. Overnight cultures of P. putida MAD2 or its derivatives were diluted to an A{sub 600} of approximately 0.05 in fresh medium and grown at 30 °C until the end of the exponential phase (A{sub 600} of approximately 1.0). Samples were collected 30 min after this point, and promoter activity was measured by assaying the accumulation of β-galactosidase in cells permeabilized with chloroform and SDS as described by Miller (37). Each enzymatic measurement was repeated at least twice, and deviations were less than 15%. The concentration of glucose and gluconate in culture supernatants were determined as described by Schleissner et al. (38). When required, the culture medium was supplemented with streptomycin (5 mM; 50 μg/ml), kanamycin (Km; 50 μg/ml), ampicillin (150 μg/ml), and potassium tellurite (Sm; 50 mM). Plasmids were generally maintained in E. coli DH5α and E. coli CC18 strains, although those containing conditional R6K replication origins were hosted in E. coli strain CC18Apir (39). DNA was manipulated following standard protocols (35). For constructing pJM154 (a ptsN− broad host range plasmid), a 0.78-kb PstI fragment from pARG5.1 (spanning the corresponding chromosomal region of P. putida; Ref. 40) was cloned in pNot18. This vector is a variant of pUC18 in which the entire σ{sup 54}-lacs segment and the Plac promoter of the plasmid are flanked by NotI sites (41). The result of this cloning step was plasmid pNot18−154, in which the promoterless ptsN sequence is transcribed through the Plac promoter of the σ{sup 54}-lacs segment. The corresponding 1.2-kb NotI fragment was cloned in pARG5.1 and cloned in the single NotI site of pPFS9, a mobilizable derivative of the broad host range plasmid R6K replicon bearing a Sm resistance gene (41). When required, the resulting construction (pJM154) was mobilized into P. putida recipients through triparental matings with helper strain E. coli HB101 (RK2031) as described by de Lorenzo and Timmis (39). For the xylR promoter chain reaction, separate colonies of the transformants were resuspended in 10 μl of H2O and boiled for 5 min. 1 μl of this material was diluted 100-fold and directly subjected to 25 cycles (1 min at 92 °C, 1 min at 55 °C, and 2 min at 72 °C) of amplification with Taq polymerase in the presence of 1.5 mM MgCl2, 1 μM dNTPs, and 50 pmol of the primers indicated in each case. DNA sequencing (kindly performed by E. Diaz) was carried out by applying a primer walking strategy along the insert of plasmid pDORB2 (see below) with fluorescent dNTPs. The sequence of the rpoN region has been deposited in the EMBL nucleotide sequence data bank under the reference PPU007689.

**Construction of Insertion Mutants—**Stepwise nonpolar knockouts of the four genes adjacent to rpoN were first generated in plasmids by directed insertions of a promoterless Km resistance cassette devoid of transcriptional initiation signals. Such a cassette was generated by chain reaction amplification of the sequence within coordinates 1920 to 2732 of pACYC177 (42). The resulting 0.8-kb DNA fragment was then recovered as a BamHI insert in pUC19 giving rise to plasmid pUCKn. Insertions in each of the ORFs were achieved as follows. A 3.2-kb SalI-Scal fragment from plasmid pNTR1 (spanning rpoN and 1.5 kb downstream; Ref. 40) was cloned in vector pBlueScript KS− digested with Smal and SalI. To generate a Km insertion in ORF102, the resulting plasmid (pBSNtr) was digested with HindIII, and the overhanging ends were filled in with dNTPs using the Klenow fragment of DNA polymerase (35) and ligated to an equally filled in 0.8-kb segment of pUCKn bearing the promoterless kanamycin cassette mentioned above. The resulting plasmid (pBSN102) bears ORF102 truncated at its 46th codon. Similarly, disruption of ptsN required the digestion of pBSNtr with Smal and its ligation to the blunt-ended kanamycin cassette of pUCKn. In the resulting plasmid (pBSN154) the Km resistance phenotype became apparent only when two cassettes were inserted in tandem, disrupting ptsN at its 53th structural codon. To knockout ORF284, the 1.2-kb XmaI fragment from pARG5.1 was ligated to the 2.3-kb XmaI fragment of the pHP45I (43). The resulting plasmid pARG5.1 was digested with BglII and SalI and ligated to the 0.8-kb BamHI fragment of pUCKn, giving rise to pHP284Km. In this plasmid, the 853 nucleotides following the 3rd structural codon of ORF283 have been replaced by the Km− cassette. The 1.2-kb fragment resulting from partial digestion of pH284Km with XmaI was then used to replace the corresponding XmaI fragment in plasmid pDORF2. This is a pNot19 derivative containing a 3.4-kb EcoRI−KpnI fragment from pHP284Km (44), a 0.8-kb SalI fragment from pARG5.1, spanning pUCKn and the 1.5 kb further downstream. The exchange of XmaI segments between pH284Km and pDORF2 rendered plasmid pDORFK. Disruption of ptsO was engineered by first cloning the 0.8-kb SalI fragment of pARG5.1 into pNot18 (41) thereby originating plasmid pNot18-90. This one was digested with Smal and ligated to the blunt-ended 0.8-kb BamHI segment of pUCKn encoding the promoterless Km− cassette, thus yielding the plasmid pNot18-90Km, which encodes a NPr protein truncated at position 16 of its predicted amino acid sequence.

To transfer the various insertions into the chromosome of P. putida MAD2, the SalI-BamHI fragments of each of the pBSNtr derivatives bearing Km insertions in ORF102 (pBSN102) and ptsN (pBSN154) as well as the NotI fragments from pDORFK (ORF284::Km) and pNot18-90Km (ptsO::Km) were cloned in pKNG101 (44), a mobilizable suicide vector bearing the positive counter selection marker sacB. The resulting plasmids pKNG102, pKNG154, pKNG284, and pKNG90 were transferred to P. putida MAD2 through triparental matings with helper strain E. coli HB101 (RK2031) as described above. Homologous recombination of the mutated ORFs in the Pseudomonas chromosome was made by first selecting co-integrates of the suicide plasmids on LB plates containing Km and Sm (potassium tellurite; selection of the donor and helper strains). Plates with approximately 1000 exconjugant colonies were kept for 1 week at 4 °C, pooled in 1 ml of 1% NaCl and then replated in LB with Km and 5% sucrose for selection of a second recombination event. The colonies grown in such a medium were then scored for sensitivity to Sm. Those with a phenotype Sm− Km− were analyzed by polymerase chain reaction with oligonucleo-



tides spanning the first and last codon of each gene as follows. Disruption of ORF102 was verified with primers ORF102D (5'-ATGCCAGATCTTTCTGCAATTATCGAGGACG-3') and ORF102R (5'-GGGTATACATGTGGTTTGCAGGGC-3'). ORF154D (5'-ATGCCAGATTGTCTCTCAAG-3') and ORF154R (5'-ATGCAGAGCTTATGCTGAGGTGGGAGC-3'). Disruption of ORF284 was verified with primers ORF284D (5'-GACGGATCCGCTTGAGTCTGAAAT-3') and ORF284R (5'-ATGCAGGACATTGTCTCTCAAG-3').

Site-directed Mutagenesis of ptsN and Expression of the Mutant Alleles—The method developed by Kunkel et al. (45) was used to generate the H68A and H68D variants of ptsN. To this end, the SalI-BamHI fragment of PBSN102 described above was cloned in vector pG2C (46). Extension in vitro of the single-stranded, uracil-containing DNA from the resulting plasmid (pGC154) was primed independently with mutagenic oligonucleotides PtnSNH68A (5'-GGGTATACATGTGGTTTGCAGGGC-3') and PtnSNH68D (5'-ATGCAGAGCTTATGCTGAGGTGGGAGC-3') for H68A substitution and PtnSNH68D (5'-GGGTATACATGTGGTTTGCAGGGC-3') for H68D mutation (the changed nucleotides are shown in bold, and the triplets corresponding to the new amino acid residues are underlined). These changes introduced new SphI and BstEI restriction sites, respectively, to facilitate the screening of the mutants. The resulting plasmids pGC154-H68A and pGC154-H68D were used as templates for amplification with polymerase chain reaction of the new alleles with primers ORF154D and ORF154R, which incorporate new priming sites in the 5' ends of the resulting amplification products. This amplification step modified the N terminus of the mutant proteins from sites in ORF102 and ORF154 (32). To visualize the complete organization of the region, we sequenced 1.35 kb of the DNA flanking ORF102 and ORF154 (32). To visualize the complete organization of the region, we sequenced 1.35 kb of the DNA flanking ORF102 and ORF154 (32).

RESULTS

Organization of the rpoN Locus of P. putida and Its Encoded Products—The rpoN gene of P. putida was reported by Köhler et al. (48) to be followed by two ORFs that were called at that time ORF102 and ORF154 (32). To visualize the complete organization of the region, we sequenced 1.35 kb of the DNA further downstream. The presence of two additional genes (ORF284 and ptsO) completed the overall picture of the locus in this bacterium (Fig. 1B). Besides rpoN itself, the region includes a gene cluster located 78 base pairs downstream and consisting of four adjacent cistrons. Distances from ORF102 to ptsN or from ORF284 to ptsO are as small as 13 and 16 base pairs, respectively. The ptsN sequence overlaps by 1 base pair with that of ORF284. Such an organization resembles with relatively minor differences that found in E. coli (49) and K. pneumoniae (33) as shown in Fig. 1.

The protein encoded by ORF284 in P. putida is well conserved among bacterial species, but its sequence does not manifest any predictable function. The only hint of a role for the product borne by ORF284 of P. putida (49% identity and >60% similarity with the E. coli and K. pneumoniae homologues) is the presence of an 8-amino acid sequence that matches the phosphate binding loop of many ATP- and GTP-binding proteins (Ref. 25 and Fig. 1B). The P. putida ptsO gene was also very similar (34% identity and 58% similarity) to the ptsO gene of E. coli, which encodes the HPr-like enzyme termed NPr (25) and also contains the two sequence motifs that are conserved among phosphorylation sites of HPr-type phosphotransferases (Fig. 1B and Ref. 18).

Step-wise Disruption of the rpoN Gene Cluster: ptsN Mutants Lack Inhibition of Pu by Glucose—To have a reliable in vivo assay to examine the effect of the genes of the rpoN cluster in Pu activity, we employed strain P. putida MAD2 (4). This strain harbors all the regulatory elements that control expression of Pu assembled in a mini-Tn5 transposon inserted into the chromosome of P. putida. This includes a transcriptional Pu-lacZ fusion and a variant of the XylR activator named...
P. putida MAD2 bears a chromosomal insertion of a DNA segment encoding a constitutive allele of xylR (xylRΔA) and a Pu-locZ fusion, all assembled in a Tel' mini-Tn5 vector. *P. putida* MAD2 ptsN::Km is identical to *P. putida* MAD2 except for the nonpolar insertion of a Km cassette in the *ptsN* gene of the cluster adjacent to rpoN. Plasmid pJM154 encodes the wild type *ptsN* allele.

The *rpoN* Cluster of *Pseudomonas putida*

| C source added<sup>a</sup> | Strains | MAD2 | MAD2 ptsN::Km<sup>b</sup> | MAD2 ptsN::Km<sup>c</sup> (pJM154) |
|---------------------------|---------|------|-------------------------|-------------------------------|
| None                      | 4530 ± 460<sup>a</sup> | 4688 ± 604 | 3720 ± 564                |
| Glucose                   | 1456 ± 203 | 4650 ± 583 | 930 ± 126                |
| Glucuronate               | 1111 ± 146 | 5002 ± 670 | ND                      |
| Fructose                  | 3005 ± 631 | 9691 ± 702 | ND                      |

<sup>a</sup> *P. putida* MAD2 and its derivatives *P. putida* MAD2 ptsN::Km and *P. putida* MAD2 ptsN::Km (pJM154) were grown in M9-CAA medium supplemented with 10 mM of each of the C sources indicated.

<sup>b</sup> β-Galactosidase activity was determined in the cultures as explained under “Experimental Procedures.” The figures and the standard deviation of enzyme activities (expressed in Miller units) are the average of at least two independent experiments with samples taken in duplicate.

<sup>c</sup> ND, not determined.

XylRΔA, in which the N-terminal domain has been deleted to yield an effector-independent constitutive activator (4). In this genetic background, we inserted a promoterless Km cassette devoid of transcription terminators in each of the four cistrons of the rpoN cluster, namely ORF102, ptsN, ORF284, and ptsO (see “Experimental Procedures”). The effect of the C source added was examined in *P. putida* MAD2 and each of the derived mutant strains by growing them in mineral medium supplemented not only with the sugar under study but also with casamino acids to equal growth rates and to avoid effects related to the stringent response (36). As shown in Table I, the MAD2 strain faithfully reproduced the C source-dependent inhibition of the Pu promoter reported by Holtel et al. (11) but without the need to add an aromatic inducer to the medium, hence reducing the number of variables to be considered. The results of these assays indicated that although disruption of ORF102, ORF284, or ptsO did not have an apparent effect on the inhibition of Pu activity by glucose (not shown), the ptsN::Km mutant gave rise to a clear phenotype of apparent insensitivity to the added carbohydrate (Table I). The behavior of the ptsN::Km strain with glucose could be extended to other repressive carbon sources such as glucuronate, whereas Pu activity remained unaffected with fructose. That the release of Pu inhibition by glucose or glucuronate was due to the loss of ptsN and not to any other indirect effect caused by the Km insertion was verified by transforming *P. putida* MAD2 ptsN::Km with the ptsN<sup>+</sup> plasmid pJM154. As shown in Table I, the transformed strain reverted to the same phenotype of glucose inhibition of Pu activity as the wild type strain. These data suggested that ptsN was involved in the C source inhibition of Pu.

The Loss of ptsN Does Not Affect Glucose Transport or Metabolism—A trivial explanation to justify the phenotype of ptsN mutants regarding the inhibition of Pu by glucose could be that cells fail to transport and/or to metabolize this sugar. However, this is unlikely, because both the mutant and the wild type strain grew well in a minimal medium with glucose as the only carbohydrate source (not shown). However, to rule out that glucose transport in the ptsN mutant could be inhibited in the richer M9-CAA medium, we measured directly the concentration of glucose in the supernatants of cultures of ptsN<sup>+</sup> and ptsN<sup>−</sup> *P. putida* MAD2 cells along the growth curve. Because glucose is predominantly converted by *P. putida* cells to glucurate prior to its intake and subsequent metabolism (38), we also measured the extracellular levels of this carbohydrate. The results shown in Fig. 2 did not indicate any significant differences in either the growth rate or in consumption of glucose between the two strains. The sugar was, in fact, rapidly depleted from the medium, and the low levels of glucurate detected in the cultures suggested that glucose was normally used as a carbon source. The modest difference between the two strains regarding glucose consumption (Fig. 2) might simply reflect a small growth defect of the ptsN mutant.

A Genetic Approach to the Role of the His<sup>68</sup> Residue of IIA<sup>Ntr</sup> in C Source Control of Pu—The similarity of IIA<sup>Ntr</sup> with the PTS enzymes type II raised the possibility that the same phosphorylation switch that governs their activity (18) could also effect the action of the ptsN product on Pu. Fig. 3A shows that most members of the IIA<sup>Ntr</sup> family, with the one exception of IIA<sup>Ntr</sup> of *Hemophilus influenzae*, maintain almost perfectly the site of phosphorylation, including the conserved His residues that in various PTS enzymes type II is phosphorylated by the HPr<sup>−</sup>P (18). To examine whether the equivalent site of IIA<sup>Ntr</sup> located in position 68, played a role in the repression of Pu by glucose, we produced site-directed mutants of the protein in which His<sup>68</sup> was replaced by either an alanine or an aspartic acid residue. Because the H68A substitution prevents the site from being phosphorylated by a cognate kinase (50), the phenotype endowed by IIA<sup>Ntr</sup> H68A should correspond to either a total loss of function or that due to the protein locked in a nonphosphorylated state. On the other hand, the H68D substitution is also predicted to prevent phosphorylation while adding a negative charge at the former His residue, which may help the protein to maintain its native conformation and even mimic the phosphorylated state (50). The actual phenotypes raised by these mutants in connection with Pu activity are described separately below.

Phenotype of Mutant ptsNH68A—To assess the behavior conferred by the ptsNH68A allele we employed plasmid pMT/154 H68A, which expresses a IIA<sup>Ntr</sup> variant not amenable to phosphorylation. This plasmid, in which ptsNH68A can be induced with IPTG, was then introduced into wild type *P. putida* MAD2 as well as in its ptsN::Km derivative, so we could assess both the phenotype endowed by the mutant allele and its dominance or recessiveness versus the native protein. As a control, an equivalent plasmid encoding the wild type ptsN gene expressed through the same system was also introduced in identical strains. Each of the *P. putida* exconjugants was then grown in the presence or absence of glucose, and the accumulation of β-galactosidase was measured as before with or without addition of IPTG. The results of such an experiment are shown in Fig. 3B. The Western blot of each strain confirmed that plasmid...
The rpoN Cluster of Pseudomonas putida

DISCUSSION

We have analyzed the role of rpoN gene cluster in the physiological control of the transcription of an operon encoding the early steps for biodegradation of toluene in P. putida (pWW0). The 1.35-kb fragment of new DNA sequence downstream of the already known ORF102 and ptsN revealed the presence of two additional genes (ORF284 and ptsO), the whole of which was similar to those found in E. coli and K. pneumoniae adjacent to rpoN (Fig. 1). The most salient features of the gene cluster was the virtual identity of the ORF154 product to the E. coli protein named IIA^{Ntr} on the basis of its homology to EII-type proteins of the PTS system as well as the identity of ORF90 to the E. coli protein named NPr on the basis of its homology to housekeeping HPr phosphotransferases (25). These similarities are particularly true around the predicted phosphorylation sites of both proteins (Fig. 1B). That the array ORF102-ptsN-ORF284-ptsO forms a defined gene cluster is suggested by the fact that the only coding sequence found downstream of ptsO (similar to the guaA gene of Myxococcus xanthus) is expressed in an opposite orientation (Fig. 1B). The conservation of these genes and their genomic organization in E. coli, K. pneumoniae, and P. putida is in contrast with that found in other bacteria.

Note that pWW0 is unable to mediate any effect of glucose in media lacking a repressive carbon source. As shown in Fig. 3C, Pu-lacZ activity was inhibited to a degree dependent on addition of IPTG but could be further down-regulated when glucose was present in the medium. This can be explained by the presence of a wild type copy of the ptsN gene whose product could still respond to C source inhibition. Because the IIA^{Ntr}/H68D protein was produced at higher levels than the wild type product, even without IPTG, we tested whether the presence of an equivalent plasmid harboring the wild type allele had the same effect on Pu in media lacking a repressive carbon source. As shown in Fig. 3C, this was not the case. Overexpression of the ptsN gene made the promoter more sensitive to glucose but had no effect in the cultures not added with the sugar. This result rules out differences in intracellular concentrations of the wild type and the mutant ptsN products as the origin of the super repressed phenotype raised by ptsN^{H68D}.

FIG. 3. Phenotypes of the H68A and H68D ptsN variants regarding Pu activity. A. Alignment of the region of the known IIA^{Ntr} proteins containing the phosphorylation motif of the EIIA family of PTS proteins. The conserved His residue in position 68 was changed to Ala or to Asp, the latter introducing a negative charge. B. The resulting exconjugants were grown as before, and the levels of the ptsN gene and its variants in each of the strains and growth conditions was monitored with the blots probed with an anti-IIA^{Ntr} serum shown below the bar diagrams. Equal amounts of total cell protein were loaded in the lanes, so that the intensities of the bands highlighted in the blots are representative of the relative intracellular protein were loaded in the lanes, so that the intensities of the bands highlighted in the blots are representative of the relative intracellular protein instability or proteolytic degradation, thus suggesting that the IIA^{Ntr}-H68A mutant is produced as a full-size protein. The data of Fig. 3D indicated that the mutant protein failed to restore the phenotype of repression by glucose that is lost in the ptsN::Km mutant. In the same conditions, plasmid pJMT154, which encodes wild type IIA^{Ntr}, fully complemented the lack of ptsN in that respect. Finally, plasmid pJMT154-H68A did not have any effect in the inhibition of Pu by glucose displayed by the wild type P. putida MAD2 host, even if the H68A variant was overproduced upon addition of IPTG, thereby indicating that the H68A allele was a recessive mutation and that it is unable to mediate any effect of glucose in Pu.
For instance, *Caulobacter crescentus* has an additional ORF of unknown function between the *rpoN* gene and the *ORF102* homologue (which has in fact 208 amino acid residues in this microorganism; Ref. 29). A similar gene is present downstream of *rpoN2* of *Bradyrhizobium japonicum*. This organism harbors two different *rpoN* genes, and one of them, *rpoNL*, lacks these ORFs in its 3’ end (26). On the contrary, *H. influenzae*, which has no *rpoN* gene as it has been revealed by the complete sequencing of its chromosome, presents an abbreviated cluster, consisting only in the *ptsN* gene and the ORF284 homologue (28). A short version of the cluster is also found in *Acinetobacter calcoaceticus*, in which only the ORF102 homologue is present, followed by two unrelated genes (27).

When each of the four genes of the *rpoN* cluster of *P. putida* were knocked out with a Km cassette, none but the *ptsN* gene as it has been revealed by the complete sequencing of its chromosome, presents an abbreviated cluster, consisting only in the *ptsN* gene and the ORF284 homologue (28). A short version of the cluster is also found in *Acinetobacter calcoaceticus*, in which only the ORF102 homologue is present, followed by two unrelated genes (27).

This suggested that the loss of *lack* inhibition by either glucose or gluconate but did not influence significantly the consumption of these sugars (Table I and Fig. 2). This suggested that the loss of *ptsN* made Pu to lack inhibition by either glucose or gluconate metabolism but rather with the transduction pathway that translates the presence in the medium of a repressive carbon source into inhibition of the Pu promoter. The phenotypes endowed by the *ptsNH68A* and *ptsNH68D* alleles (Fig. 3) indicate that the phosphorylatable H68 residue of IAA\(^{Ntr}\) plays a key role in carbon inhibition of the upper TOL operon. Many proteins of the PTS system are known to interact directly with a variety of polypeptides of diverse functions not necessarily related in sequence or structure. Typical examples of this include the phosphorylation-dependent ability of IIA\(^{Glu}\) to interact with a number of per meses (51) or the formation of the CcpA-HPr repressor complex in Gram-positive bacteria (52). Similarly, the interplay of phosphorylated/nonphosphorylated forms of IIA\(^{Ntr}\) could cause the inhibition of Pu activity through the interaction of this protein with a thus far unknown target in the transcription machinery.

Although our data would fit well in a model in which the phosphorylated form of IIA\(^{Ntr}\) (imitated by the H68D mutant) would cause repression and the nonphosphorylated form (imitated by the H68A mutant) would prevent such a repression, the mechanism by which the His\(^{68}\) residue may be modified in *vivo* deserves further clarification. Whereas the IIA\(^{Ntr}\) proteins of *E. coli* and *K. pneumoniae* can be phosphorylated by the PTS enzyme HPr in *vivo* (25, 53), it is also possible that IIA\(^{Ntr}\) of *P. putida* follows in *vivo* a route alternative to the housekeeping PTS pathway. A potential good candidate for being the phospho-donor to IIA\(^{Ntr}\) could be NPr, the protein encoded by the *em5* promoter. Disruption of *ptsN* gene as it has been revealed by the complete sequencing of its chromosome, presents an abbreviated cluster, consisting only in the *ptsN* gene and the ORF284 homologue (28). A short version of the cluster is also found in *Acinetobacter calcoaceticus*, in which only the ORF102 homologue is present, followed by two unrelated genes (27).
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