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Characterization of the gene cluster involved in allantoate catabolism and its transcriptional regulation by the RpiR-type repressor HpxU in *Klebsiella pneumoniae*

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Summary. Bacteria, fungi, and plants have metabolic pathways for the utilization of nitrogen present in purine bases. In *Klebsiella pneumoniae*, the genes responsible for the assimilation of purine ring nitrogen are distributed in three separated clusters. We characterized the gene cluster involved in the metabolism of allantoate (genes KPN_01761 to KPN_01771). The functional assignments of HpxK, as an allantoate amidohydrolase, and of HpxU, as a regulator involved in the control of allantoate metabolism, were assessed experimentally. Gene *hpxU* encodes a repressor of the RpiR family that mediates the regulation of this system by allantoate. In this study, the binding of HpxU to the *hpxF* promoter and to the *hpxU-hpxW* intergenic region containing the divergent promoter for these genes was evidenced by electrophoretic mobility shift assays. Allantoate released the HpxU repressor from its target operators whereas other purine intermediate metabolites, such as allantoin and oxamate, failed to induce complex dissociation. Sequence alignment of the four HpxU identified operators identified TGAA-N_8-TTCA as the consensus motif recognized by the HpxU repressor. [**Int Microbiol** 2013; 16(3):165-176]

Keywords: *Klebsiella pneumoniae* · allantoate metabolism · allantoate amidohydrolase · purine catabolism · RpiR-type repressor

Introduction

Bacteria, fungi, and plants have metabolic pathways for the utilization of nitrogen present in pyrimidine and purine bases [40,41]. When ammonia is limiting, many microorganisms obtain nitrogen from these organic compounds. Deamination of the purine bases adenine and guanine yields one molecule of ammonia, and hypoxanthine and xanthine, respectively. The catabolic pathway for hypoxanthine and xanthine assimilation occurs in two stages. In the first, both compounds are oxidized to uric acid, which is then converted to allantoate via allantoin by two sequential ring-opening steps. In the second, allantoate is degraded to carbon dioxide and ammonia through alternative metabolic routes depending on the organism [10,35,40,41].

In enterobacteria, purine catabolism has been well characterized in *Escherichia coli* and some species of *Klebsiella*. In the presence of oxygen, the adenine catabolic pathway is incomplete in *E. coli* [10,32,44] whereas *Klebsiella* can assimilate all adenine nitrogens under these conditions [28,33].

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In *K. oxytoca* M5a1, a cluster of 23 genes responsible for the utilization of purines as sole nitrogen source was identified [28]. Functional assignments of some genes of this cluster were based on growth, complementation tests, and sequence similarity. Comparison with the *K. pneumoniae* MGH78578 genome revealed that, at least in this strain, these genes are organized in three separate clusters [28], two of which have been studied in detail in *K. pneumoniae* strain KC2653 [15,33]. The first cluster (KPN_01661 to KPN_01666), involved in the oxidation of hypoxanthine to allantoin, is formed by seven genes organized in four transcriptional units, *hpxDE*, *hpxR*, *hpxO*, and *hpxPQT* [33]. Expression of this system is activated by nitrogen limitation and by the presence of specific substrates, with *hpxDE* and *hpxPQT* controlled by both signals. The induction of *hpxPQT* requires uric acid formation, whereas the expression of *hpxDE* is induced by hypoxanthine through the HpxR regulatory protein encoded by *hpxR* [33].

The second cluster (KPN_01787 to KPN_01791) is organized in three transcriptional units, *hpxSAB*, *hpxC*, and *guaD*. It contains genes involved in the metabolism of allantoin to allantoate and in guanine deamination. Gene *hpxS* encodes a regulatory protein that mediates regulation of the *hpxSAB* operon by allantoin, although full induction of *hpxSAB* by allantoin requires both HpxS and the nitrogen assimilation control protein (NAC), which regulates a subset of genes that are dependent on RNA polymerase bearing σ^{70} for their transcription [2]. The expression of *guaD* is mainly regulated by nitrogen availability through the action of NtrC [15].

Regarding the third gene cluster (KPN_01761 to KPN_01771) presumably involved in allantoate catabolism, there have been no studies dealing with its transcriptional regulation, and functional assignment has only been reported for gene KPN_01762 (*hpxJ*). This gene encodes a protein that belongs to the pyridoxal 5′-phosphate (PLP)-dependent aspartate aminotransferase superfamily, specifically, an aminotransferase that preferentially converts ureidoglycine plus an α-keto acid into oxalurate plus the corresponding amino acid [12]. The gene encoding this aminotransferase is also related to the allantoate amidohydrolase genes in several organisms [42,43]. For instance, a gene related to alanine-glyoxylate aminotransferase (*pucG*) has been reported in the purine degradation cluster of *Bacillus subtilis*. *Bacillus* species are known to have an efficient metabolic system for the use of oxidized purines. This activity seems to enable soil and gut bacteria to use animal-produced purine waste as a source of carbon and nitrogen [30].

In this study, we were able to functionally identify the *hpxK* gene product in *K. pneumoniae* strain KC2653 as an allantoate amidohydrolase. This gene belongs to the *hpxFGHIJK* operon (KPN_01766 to KPN_01761), which is located next to the regulatory gene *hpxU* (KPN_01767) and genes predicted to be involved in oxalurate metabolism *(*KPN_01768 -KPN_01771; *hpxWYXZ)*. Analysis of the transcriptional regulation of this cluster by HpxU is also presented.

Materials and methods

Bacterial strains and plasmids. The genotypes and sources of the bacterial strains, plasmids, and promoter fusions are given in Table 1. All *K. pneumoniae* strains were derived from strain W70 [23].

Growth conditions and preparation of cell extracts. Cultures were grown at 30 ºC with aeration in Luria broth (LB) [6] or in W4 minimal medium [38] supplemented with glucose at 0.4 % as the sole carbon source. For nitrogen-limiting conditions, freshly prepared glutamine was used at 0.04 %. Ammonium sulfate and glutamine, both at 0.2 %, were used to achieve nitrogen excess [4,5]. Allantoin, allantoate, ureidoglycolate, and oxamate were usually used at 0.05 %. When required, the following antibiotics were added to the indicated final concentrations: ampicillin (Ap), 100 μg/ml; kanamycin (Km), 50 μg/ml; streptomycin (Sm), 50 μg/ml; chloramphenicol (Cm), 20 μg/ml; rifampicin (Rf), 50 μg/ml; and tetracycline (Tet), 12.5 μg/ml. 5-Bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) and isopropyl-β-D-thiogalactoside (IPTG) were used at 30 and 10 μg/ml, respectively.

Cell extracts were obtained by sonic disruption of bacterial cells collected by centrifugation at the end of the exponential phase and resuspended in the appropriate buffer.

Enzyme activities. For β-galactosidase assays, the cultures were grown to an OD_{600} of 0.5. The cells were collected by centrifugation, washed in 1 % KCl, and suspended at a concentration that contained 1–1.5 mg of protein per ml [1]. β-Galactosidase activity was assayed in detergent-treated whole cells using *o*-nitrophenyl-β-D-galactopyranoside (ONPG) as the substrate and expressed as U/mg of cell protein [25]. One unit of β-galactosidase activity corresponds to the amount of enzyme that hydrolyzes 1 nmol of ONPG per min. The data reported are the averages of at least four separate experiments performed in triplicate.

Allantoate amidohydrolase activity was assayed spectrophotometrically as described by Muratsubaki et al. [26]. The reaction mixture consisted of 100 mM Tris–HCl buffer (pH 8.5), 0.1 mM $MnCl_2$, 0.3 mM NADPH, 0.25 mM α-ketoglutarate, 2.5 mM allantoate, and yeast glutamate dehydrogenase (1 kU/ml). The reaction was started by the addition of the enzyme and activity was assayed by monitoring the decrease in absorbance at 340 nm induced by NADPH oxidation. One unit of enzyme activity was defined as the amount of enzyme that transforms 1 μmol of ammonia per min. Protein concentration was quantified by the method of Lowry et al. [22], with bovine serum albumin as the standard.

DNA manipulation and site-directed mutagenesis. Bacterial genomic DNA was obtained using the Wizard Genomic DNA purification kit (Promega). Plasmid DNA was prepared using the Wizard Plus SV Midipreps DNA purification system (Promega). DNA manipulations were performed essentially as described by Sambrook and Russell [34]. DNA fragments were PCR-amplified using chromosomal DNA as template. When necessary, specific restriction sites were incorporated at the 5′-ends of the primers to facilitate cloning of the fragments into the appropriate vector. PCRs were performed with *pfu* DNA polymerase under standard conditions. DNA was se-

Table 1. Strains, fusions and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source/reference
K. pneumoniae strains		
KC2653	*hutC515* Δ*[bla]*-2 *dadA1str-6*	[21]
KC2738	*hutC515 ntrC2*::Tn*5*-131	[3]
JB101	KC2653 *hpxK*::*cat*	This study
E. coli strains		
DH5αF′	ϕ80d *lacZ*ΔM15 *recA*1 *endA*1 λ^- *gyrA*96 *thi-1 hsdR*17 (r_K^- m_K^+) *phoA supE*44 *relA*1 *deoR* Δ*(lacZYA-argF)* U169	Gibco BRL
XL1-Blue	*recA1 endA1 gyrA96 thi*-1 *hsdR17 supE44 relA1 lac* [F' *proAB lacI*^{q}ZΔM15 Tn*10*(Tc^r)]	Stratagene
S17 λ pir	Tp^r Sm^r *recA thi pro hsdR*$^-$ *M*$^+$RP4::2-Tc::Mu::Km Tn7 λ	Biomedal
EB6193	RP4-2 tet::Mu -1 Kan::Tn7 integrant *leu*-63::IS10 *recA*1 *creC510 hsdR17 endA1 zbf-5 uidA*(ΔMuI)*:pir+ thi* Sp^R/Sm^R	RA Bender
Fusions[a]		
ϕ(*hpxF-lacZ*)	*hpxF* (–180 to +140) fused to *lacZ*	This study
ϕ(*hpxU-lacZ*)	*hpxR* (–195 to +105) fused to *lacZ*	This study
ϕ(*hpxW-lacZ*)	The same fragment as for *hpxU* but cloned in the opposite direction	This study
Plasmids		
pGEMT	Ap^r; cloning vector for PCR products	Promega
pCAT19	Ap^r Tn*9*-CAT(Cm^r)	[14]
pUC18Not	Ap^r; identical to pUC18 but with NotI in the multiple-cloning site	Biomedal
pRS415	Ap^r; promoterless *lacZYA* reporter for operon fusions with replication origin of pBR322	[37]
pUTmini-Tn5 Tc	Ap^r oriR6K *mob*RP4 tnp* mini-Tn*5* Tc	Biomedal
pCB1583	Ap^r Km^r; promoterless *lacZ* reporter for integration of operon fusions on host genome with oriR6K replication origin; *rpsL*	RA Bander
pMAL-c2x	Ap^r; vector for cytoplasmic expression of MBP fusion proteins	N.E. BioLabs

[a]Nucleotide sequences are given in the 5′- to 3′- direction for the coding strand of each gene and numbered relative to the gene transcription initiation nucleotide at position +1.

quenced using an automated ABI377 DNA sequencer and fluorescent dye termination methods. The primers used in this work are available upon request.

To construct an HpxK-defective mutant, a fragment encompassing *hpxK* was PCR-amplified and cloned into pUC18Not. The resultant recombinant plasmid was then digested with *Psy*I (with a single restriction site in *hpxK*) and ligated with a Cm cassette amplified from plasmid pCAT19 [14]. The recombinant plasmid, pUC18-*hpxK*::*cat*, was then digested with *Not*I to obtain the *hpxK*::*cat* insert. This fragment was cloned into the *Not*I restriction site of pUTmini-Tn*5*Tc and introduced into *E. coli* S17-1(pir) by electroporation. To introduce the *hpxK*::*cat* mutation into the *K. pneumoniae* chromosome, conjugation was performed with a Rf-resistant derivative of strain KC2653 as a recipient. Transconjugants displaying the Rf^s Cm^r Tc^s phenotype were selected. Chromosomal insertion was confirmed by PCR.

Mapping of the 5´-end of the transcripts. The 5′-end of the transcripts was determined by the rapid amplification of cDNA 5′-ends (5′-RACE) [34] using a commercial 5′-RACE kit (Roche Diagnostics). Total RNA was isolated from KC2653 cells grown aerobically to an OD_{600} between 0.5 and 1 in glucose minimal medium with Gln 0.04 % as nitrogen source (nitrogen-limiting conditions) using the Qiagen RNeasy total RNA kit and then treated with RNAse-free DNase (Ambion). The cDNAs were transcribed from RNA with specific *hpxU*, *hpxFGHIJK*, or *hpxWXYZ* antisense oligonucleotides. A homopolymeric(dA) tail was added (via terminal transferase) to the 3′-termini of the corresponding cDNAs. The reverse transcription products were amplified with nested gene-specific primers and an oligo(dT) anchor primer. The double-stranded cDNAs obtained were cloned into pGEMT vectors for sequencing.

Construction of *lacZ* transcriptional fusions. Transcriptional fusions were constructed by inserting the promoter fragments into plasmid pRS415 [37], which carries a cryptic *lacZ* operon and confers resistance to ampicillin. To construct the *hpxF-lacZ* fusion, a 320-bp fragment encompassing the *hpxF* 5′-region (positions –180 to +140) was PCR-amplified. The *hpxW-lacZ* fusion was constructed by PCR-amplifying a 300-bp fragment encompassing the *hpxU-hpxW* intergenic region (positions –195 to +105 with respect to the *hpxU* transcriptional start site). The same fragment was cloned in the opposite direction to obtain the *hpxU-lacZ* fusion. In all cases, primers bearing *Bam*HI or *Eco*RI sites at their 5′-ends were used to facilitate cloning

of the fragment into the *Eco*RI–*Bam*HI sites of plasmid pRS415. For all constructs, plasmid DNA was sequenced to ensure that the fragment was inserted in the correct orientation and that no mutations were introduced during the amplification.

To transfer the *lacZ* fusions into *K. pneumoniae* chromosome as a single copy, the recombinant plasmid was first digested with *Eco*RI and *Sac*I, and the fragment containing the promoter fusion was subcloned into plasmid pCB1583 [21]. In this λpir-dependent plasmid the *lacZ* gene is flanked by genes of the *K. pneumoniae* D-ribose operon, thus allowing integration of the cloned fusion by homologous recombination into the D-ribose operon of the recipient strain. After the transformation of strain EB6193, recombinant plasmids containing ϕ(*hpxF-lacZ*), ϕ(*hpxU-lacZ*), and ϕ(*hpxW-lacZ*) were selected as blue bacterial colonies on LB-Xgal-Km plates and then introduced into strain KC2653 by electroporation. After several rounds of selection in different growth media, stable recombinants were isolated as those displaying a Km^S Sm^R D-ribose negative phenotype.

Expression and purification of recombinant proteins. HpxU and HpxK were purified using the *malE* gene fusion system. For this purpose, the corresponding genes (*hpxU* and *hpxK*) were PCR-amplified and cloned into plasmid pMal-c2x. The overproduction of MalE-fused proteins was achieved in strain XL1-Blue incubated at 37 ºC for 16 h, in the presence of 0.3 mM IPTG. The fusion proteins were then affinity-purified chromatographed with amylose resin (New England BioLabs) as described previously [15]. The MalE-HpxK fusion protein was eluted with column buffer containing 10 mM maltose and then digested with factor Xa by incubation at room temperature for 12 h. However, MalE-HpxU folding hindered cleavage of the fusion protein in solution. Thus, instead, recombinant MalE-HpxU was used in gel shift experiments, since the MalE protein was unable to bind to the analyzed promoter regions (not shown). Purified proteins were analyzed by SDS–PAGE, performed according to the standard procedure [19], and then either used immediately or stored at –20 °C in 20 % glycerol.

DNA binding studies. The non-radioactive digoxigenin (DIG) gel shift kit for the 3′-end labeling of DNA fragments (Roche Applied Science, Indianapolis, IN) was used for protein-DNA binding assays. The fragments obtained by PCR were labeled with terminal transferase and digoxigenin-ddUTP according to the manufacturer's instructions. Labeled DNA fragments were incubated with purified HpxU in 10 mM Tris-HCl (pH 7.4), 100 mM KCl, 10 mM $MgCl_2$, 10 % glycerol, and 2 mM dithiothreitol in a total volume of 20 µl. All mixtures contained 500-fold molar excesses of poly(dI-dC) as non-specific competitor. HpxU binding mixtures were incubated at 37 ºC for

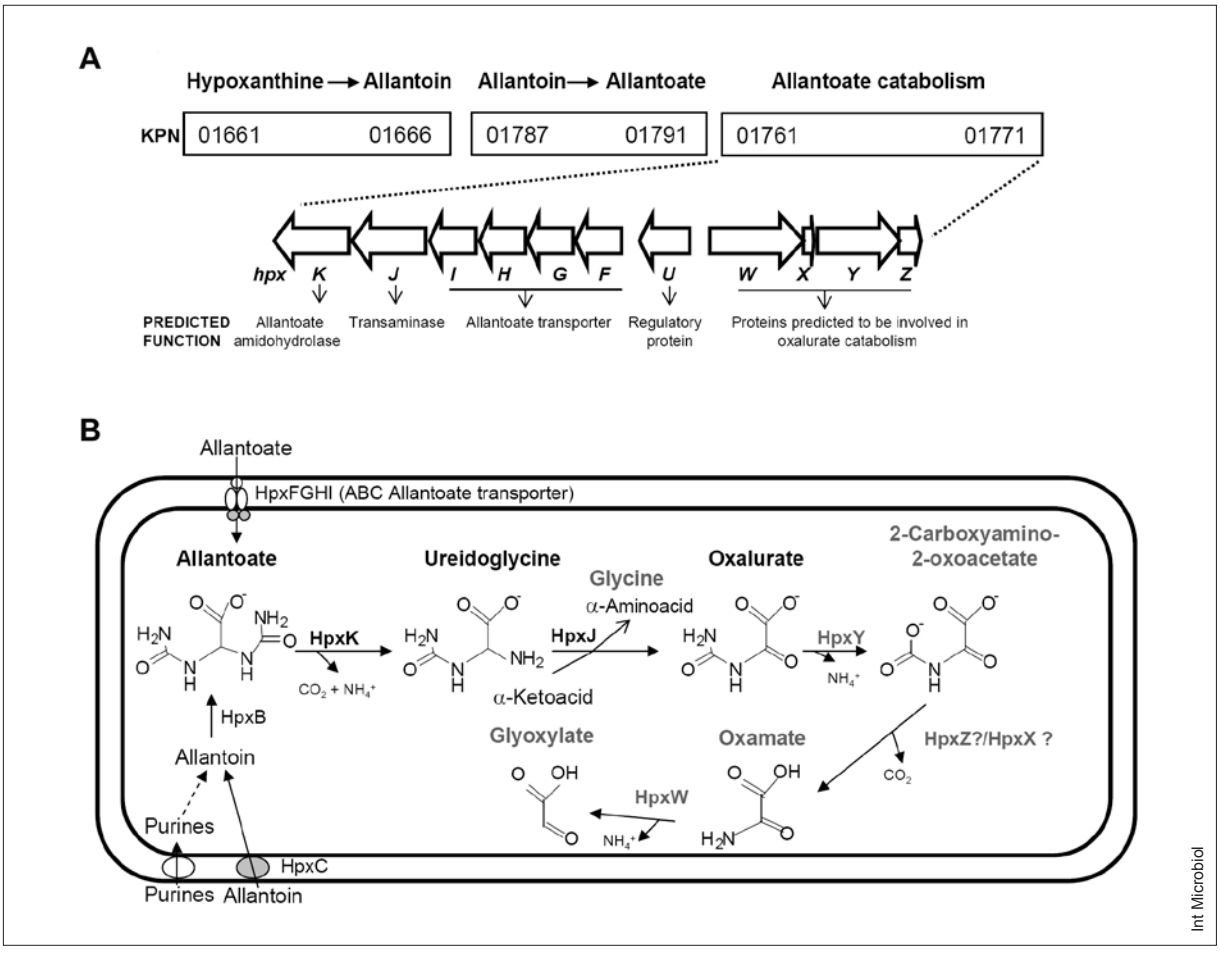

Fig. 1. Gene organization and metabolic map for allantoate catabolism in *K. pneumoniae* strain KC2653. (**A**) Scheme of the gene organization in cluster KPN_01761 to KPN_01771. The arrows indicate the extent and direction of the transcription of these genes. The predicted function of the *hpx*-encoded proteins is indicated below each gene. (**B**) Metabolic pathway for allantoate metabolism. Metabolites and proteins with putative functions are in gray.

20 min, and loaded onto a pre-run gel of 5 % native polyacrylamide, containing 10 % glycerol in 1× TBE (Tris-borate-EDTA buffer). Blotting was performed using a Biorad electro-blotting system (model Trans blot) according to the manufacturer's instructions. The chemiluminescence of DIG-labeled DNA-protein complexes on the nylon membranes was detected using Hyperfilm ECL (Amersham Pharmacia).

Results and Discussion

Physical organization of the *hpxFGHIJK-hpxU-hpxWYXZ* cluster. The gene cluster encompassing genes KPN_01761 to KPN_1771 in *K. pneumoniae* has been proposed to encode proteins involved in allantoate metabolism [28], namely, genes KPN_01761 to KPN_01766 (*hpxFGHIJK*) in the conversion of allantoate to oxalurate, genes KPN_01768 to KPN_01771 (*hpxWXYZ*) in oxalurate metabolism, and gene KPN_01769 (*hpxU*) in the regulation of this cluster (Fig. 1). To determine whether these genes were also present in strain KC2653, the corresponding region was PCR-amplified using primers designed according to the genome sequence of *K. pneumoniae* strain MGH78578. This analysis confirmed the presence of these genes (KPN_01768 to KPN_01771) in the KC2653 genome.

Information available in databases suggested that this gene cluster is present in different strains of the genus *Klebsiella*. However, no experimental evidence for the transcriptional regulation of these genes is currently available. We therefore performed an in silico analysis of the *hpxF* 5′-flanking region and the *hpxU-hpxW* intergenic region using the Footprint and Promscan programs [http://www.promscan.uklinux.net]. Putative σ^{70}-promoters were identified upstream of *hpxF, hpxU*, and *hpxW*.

The 5′-ends of the *hpxFGHIJK, hpxU*, and *hpxWXYZ* transcripts were experimentally determined by the 5′-RACE method. Total RNA was obtained from aerobic cultures of strain KC2653 on glucose-glutamine (nitrogen-limiting conditions). For *hpxFGHIJK*, the transcriptional start site (tss) was identified at 89 nucleotides upstream of the *hpxF* start codon. Inspection of the sequences upstream of nucleotide +1 revealed the presence of –10 (TTTAAT) and –35 (TTGTCC) sequences, similar to the σ^{70} consensus sequence, separated by 15 bp (positions matching the consensus are underlined) (Fig. 2). For *hpxU*, the tss was identified 50 nucleotides upstream of the ATG codon. Inspection of the sequences upstream of nucleotide +1 revealed the presence of –10 (TAAGGC) and –35 (TTCCCA) sequences similar to the σ^{70} consensus and separated by 15 bp (Fig. 2). Although these spacers do not fit the optimal 17 ± 1 nucleotides, functional spacers ranging in size from 15 to 21 bp have been reported previously [16,17]. For *hpxWXYZ*, the tss was identified 20 bp upstream of the start codon for *hpxW*. In this case, sequences similar to σ^{70} RNA polymerase consensus were found at –10 (TGTTCT) and at –35 (TTCAGA), separated by 18 bp (Fig. 2).

Functional assignment of HpxK as an allantoate amidohydrolase. Genes involved in the conversion of allantoate to oxalurate are clustered together, forming the *hpxFGHIJK* operon. Sequence analysis by PBLAST showed that HpxK displays similarity to allantoate amidohydrolases. HpxK is 34 % identical and 49 % similar to *E. coli* AllC [10,36], and 40 % identical and 55 % similar to *Pseudomonas fluorescens* PuuE [29]. In AllC, residues involved in allantoate binding are conserved in the *K. pneumoniae* HpxK sequence (His219, Asn268, Arg281, and His379). This finding suggested that HpxK catalyzes the conversion of allantoate to ureidoglycine (Fig. 1B). The involvement of HpxK in allantoate metabolism was assessed by phenotypic characterization of strain JB101, a KC2653 derivative *hpxK::cat* knockout mutant (Table 1). This strain was unable to grow on allantoate as a nitrogen source but displayed normal growth on ureidoglycollate or oxamate.

To confirm HpxK function, the *hpxK* gene of strain KC2653 was cloned into plasmid pMal-c2X, and the protein was purified by affinity chromatography. Enzyme activity of the purified enzyme was assayed using allantoate as substrate. Since allantoate amidohydrolase from various sources is a Mn^{2+}-dependent enzyme [43], we measured the allantoate amidohydrolase activity of HpxK in the absence or presence of the divalent cations Mn^{2+}, Ca^{2+}, Mg^{2+}, Cu^{2+}, Zn^{2+}, and Co^{2+} (at final concentrations of 0–0.2 mM). Maximum activity was obtained with Mn^{2+} at 0.1 mM (100 % activity). At this concentration, the other divalent cations yielded significantly lower activity: Co^{2+} (31 %), Ca^{2+} (9 %), Mg^{2+}, and Cu^{2+} (<1 %), with no activity occurring in the presence of Zn^{2+}. In the absence of divalent cations, the activity was around 10 % of that obtained with Mn^{2+}. Therefore, Mg^{2+}, Cu^{2+}, and Zn^{2+} inhibited enzyme activity. The kinetic parameters for allantoate were determined in the presence of 0.1 mM $MnCl_2$ from the double reciprocal Lineweaver-Burk plot. This analysis yielded a K_m of 0.105 mM and a V_{max} of 0.042 μmol/min/mg.

The ureidoglycine produced by the action of HpxK on allantoate is further metabolized by HpxJ. The functional assignment of *K. pneumoniae* HpxJ was previously reported by French and Ealick [12]. The protein acts as an aminotransferase catalyzing the conversion of ureidoglycine to oxalurate,

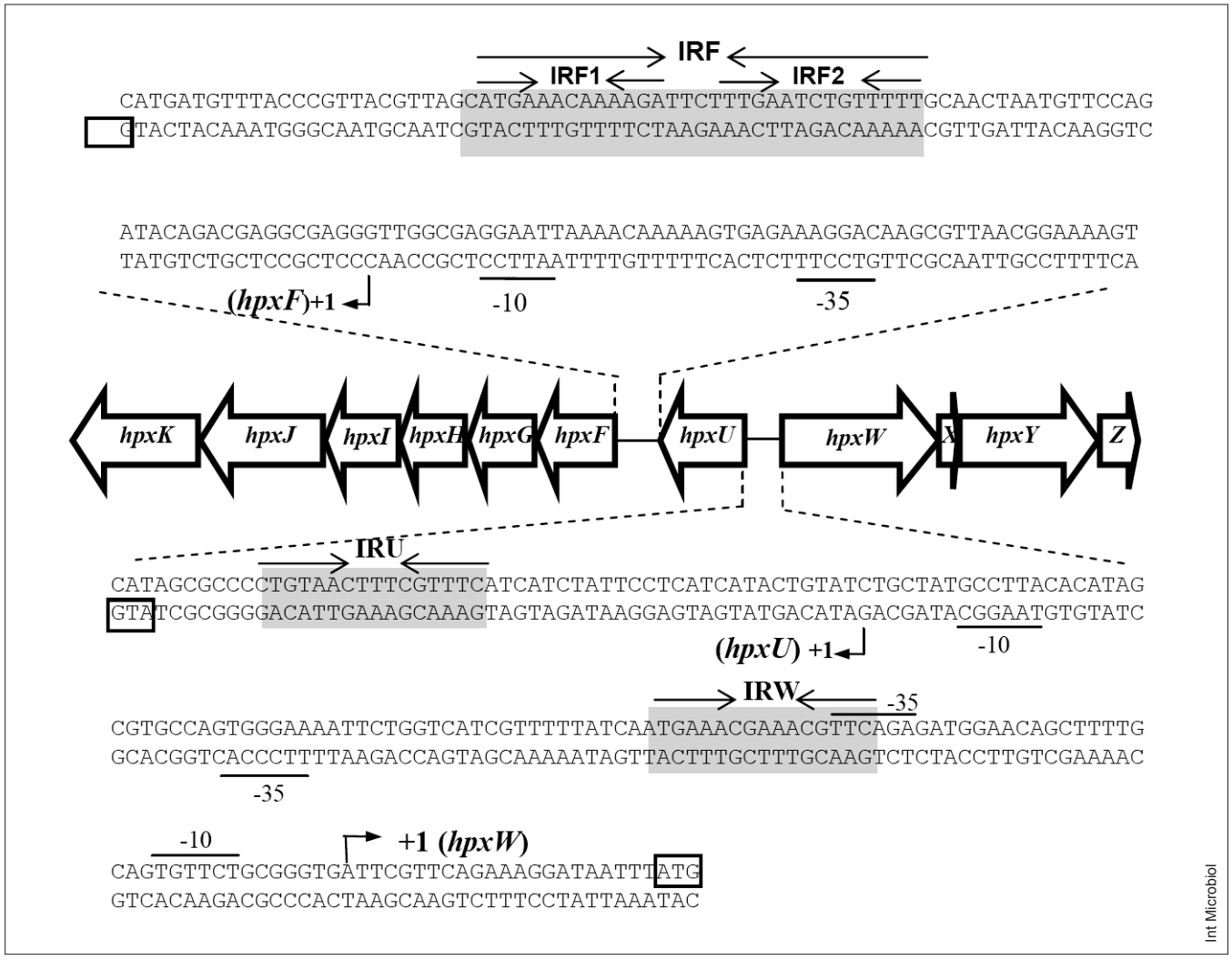

Fig. 2. Promoter sequences of *hpxF*, *hpxU* and *hpxW* genes. For each gene, the ATG initiation codon is boxed, the consensus sequence for RNA polymerase (–10 and –35 sequences) is indicated, and the transcriptional start site (tss) is shown by a black arrowhead labeled +1. Binding sites recognized by HpxU are boxed in gray. IRF, IRF1, IRF2, IRU, and IRW inverted repeats are indicated by arrows below the corresponding sequence.

coupled to the conversion of an α-keto acid to the corresponding amino acid (Fig. 1B). Aminotransferases are rather promiscuous with respect to their amino donors and acceptors. For example, HpxJ displays specificity for ureidoglycine as a donor but can use different substrates as acceptors, including pyruvate, glyoxylate, and oxaloacetate [12]. Glyoxylate could be provided by the action of enzymes encoded by genes belonging to the *hpxWXYZ* operon. PBLAST analysis showed that HpxY shared 85 % similarity with amidohydrolases belonging to the AtzE family [7]. The best protein characterized in the Atz family was biuret hydrolase from *Pseudomonas* sp. [24], which catalyzes the hydrolysis of biuret to allophanate plus ammonium in the atrazine catabolic pathway [9]. Since biuret is structurally related to oxalurate, we hypothesize that HpxY would be involved in the conversion of oxalurate to ammonium plus 2-carboxyamino-2-oxoacetate. In fact, *K. oxytoca* HpxY has been classified as a putative oxalurate amidohydrolase (information available from GenBank, reference EU884423). Information provided in this entry suggest that *K. oxytoca* HpxW is a putative oxamate amidohydrolase able to catalyze the hydrolysis of oxamate to ammonium plus glyoxylate. This reaction would provide the glyoxylate used in the transamination reaction catalyzed by HpxJ [12], thereby avoiding the toxicity due to glyoxylate accumulation [27]. A similar mechanism of detoxification was proposed for the catabolism of purines in *Bacillus subtilis* [30].

As discussed above, allantoate is endogenously generated from purine metabolism. The presence of genes encoding

transport proteins in the *hpxFGHIJK* operon suggested that allantoate is also used as an extracellular substrate. An in silico analysis of the *hpxFGHI* gene products evidenced the similarity of these proteins to proteins of the histidine and glutamine ABC transport systems [8], which suggests that they are components of the allantoate uptake system.

Transcriptional regulation of the *hpxFGHIJK-hpxU-hpxWYXZ* unit by nitrogen availability and pathway metabolites. To determine whether the expression of this gene cluster is regulated by nitrogen availability, the expression of the promoter fusions ϕ(*hpxF-lacZ*), ϕ(*hpxW-lacZ*), and ϕ(*hpxU-lacZ*) was analyzed against the genetic background of strain KC2653 grown in glucose-minimal medium with nitrogen excess or nitrogen limitation. As shown in Fig. 3, nitrogen limitation resulted in weak repression of HpxU expression and weak activation of HpxW expression, suggesting that the transcription of these genes is somewhat regulated by nitrogen. To study this regulation, the expression of the three promoter fusions was analyzed in the genetic background of the *ntrC* mutant strain KC2738 [3]. NtrC activates the transcription of another transcriptional regulator, NAC, described in Introduction [2]. No changes in the β-galactosidase expression pattern were observed for any of these promoter constructs in strain KC2738 vs. the wild-type strain (not shown). These results ruled out the involvement of the Ntr system in the transcriptional regulation of this gene cluster. Moreover, chromatin immnoprecipitation studies have not identified these genes as NAC targets in *K. pneumoniae* [13].

The expressions of ϕ(*hpxF-lacZ*), ϕ(*hpxW-lacZ*), and ϕ(*hpxU-lacZ*) were also analyzed in nitrogen-limiting medium in the presence of either allantoin or downstream pathway metabolites such as allantoate or oxamate. The expression of ϕ-(*hpxF-lacZ*) was induced 13-fold by allantoin or allantoate, whereas no induction was obtained with oxamate (Fig. 3). Since allantoin metabolism generates allantoate, this latter metabolite may be the effector molecule needed for *hpxFGHIJK* induction, in agreement with the proposed role of this operon in allantoate dissimilation. Regarding *hpxU* expression, a 40–50 % increase in β-galactosidase activity in the presence of allantoin or allantoate but not with oxamate was observed, indicating that this transcriptional unit was also regulated by the presence of allantoate (Fig. 3). Assays of *hpxW* showed that the addition of allantoin or allantoate to nitrogen- limiting cultures did not significantly modify β-galactosidase expression, but a 50 % increase in β-galactosidase activity was achieved in the presence of oxamate (Fig. 3). Induction by oxamate is compatible with the involvement of *hpxWXYZ* in the metabolism of this compound.

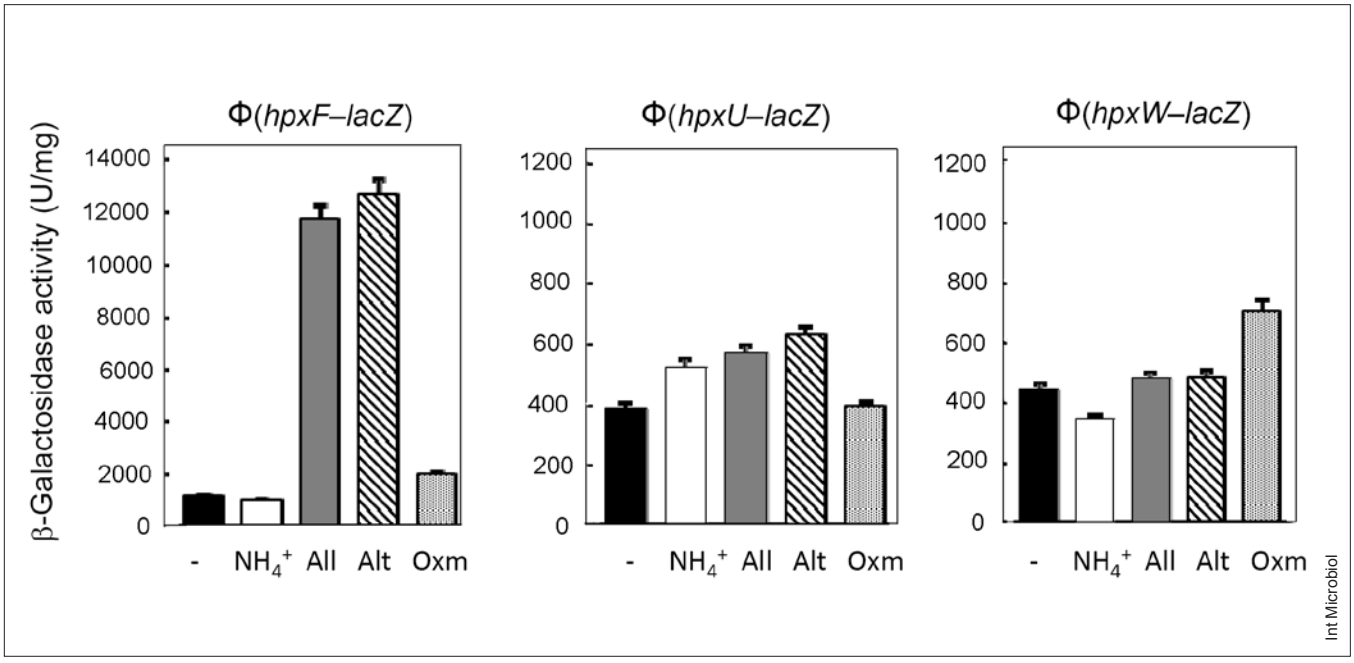

Fig. 3. Expression analysis of ϕ(*hpxF-lacZ*), ϕ(*hpxU-lacZ*), and ϕ(*hpxW-lacZ*). Cells of strain KC2653 bearing the indicated promoter fusions were grown in medium containing excess nitrogen (indicated as NH_4^+), with 0.4 % glucose and 0.2 % each of ammonium sulfate and glutamine, or in a nitrogen-limiting medium (indicated by a minus sign) supplemented with 0.4 % glucose and 0.04 % glutamine. Where indicated, 0.05 % allantoin (All), allantoate (Alt), or oxamate (Oxm) was added to the nitrogen-limiting medium.

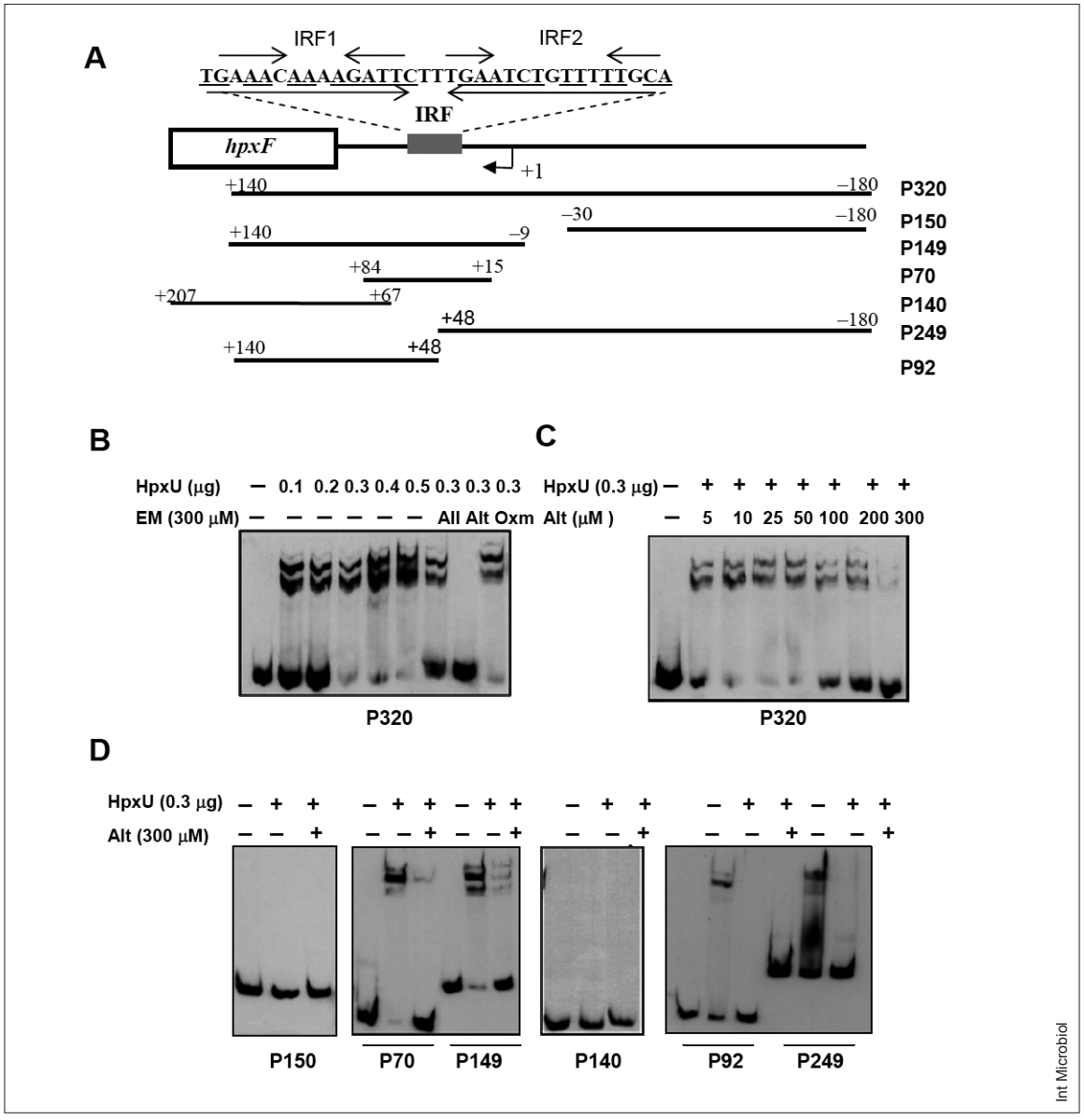

Fig. 4. Binding of HpxU to promoter fragments of the *hpxF* promoter region. (**A**) Diagram of the *hpxF* promoter region showing the tss. The promoter fragments used as probes and their end terminus positions with respect to position +1 of *hpxF* are shown below. The HpxU binding site is indicated by a gray box and the corresponding inverted repeat sequence (IRF) is shown above the diagram. The underlined bases indicate the nucleotides conserved in both halves of the palindromic sequence. The two palindromic sequences, IRF1 and IRF2, identified inside the IRF are also shown and delimited by arrows. (**B**) EMSA performed with probe P320 in the absence or presence of allantoin (All), allantoate (Alt), or oxamate (Oxm) as a putative effector molecule (EM). (**C**) EMSA performed with probe P320 in the presence of increasing concentrations of allantoate. (**D**) EMSA performed with the indicated probes, corresponding to the *hpxF* promoter fragments, in the absence or presence of 300 μM allantoate.

Analysis of HpxU binding to promoter regions of *hpxFGHIJK-hpxU-hpxWXYZ* cluster. PBLAST analysis of HpxU showed that this protein has high sequence identity with transcriptional regulators of the RpiR/AlsA family. Analysis of the 279 amino acid residues of HpxU revealed that it had the helix-turn-helix domain of the RpiR family between residues 2 and 115. In addition, proteins of the RpiR family have a C-terminal SIS domain (sugar isomerase domain, INTERPRO:IPR001347). In HpxU, the SIS domain is located between residues 123 and 250. Some members of this family (RpiR, IolR, and GlvR) control sugar phosphate metabolic pathways [11,18,39,45], although genes encoding Rpi-type

regulators have also been identified in gene clusters involved in the metabolism of nitrogenous compounds, such as agmatine [20]. Our analysis suggested that HpxU acts as a transcriptional regulator of genes of the allantoate metabolic pathway.

Binding of HpxU to the 5′-region of *hpxFGHIJK* was analyzed by electrophoretic mobility shift assays (EMSA). As seen in Fig. 4B, the binding of HpxU to probe P320 (Fig. 4A) is consistent with the proposed role for HpxU in the regulation of the *hpxFGHIJK* operon. Binding experiments performed in the presence of allantoin, allantoate, or oxamate showed that only allantoate abolished HpxU binding to probe P320 (Fig. 4B). We next incubated HpxU (0.3 μg) with probe P320 in the presence of increasing concentrations of allantoate. The amount of free probe increased with the allantoate

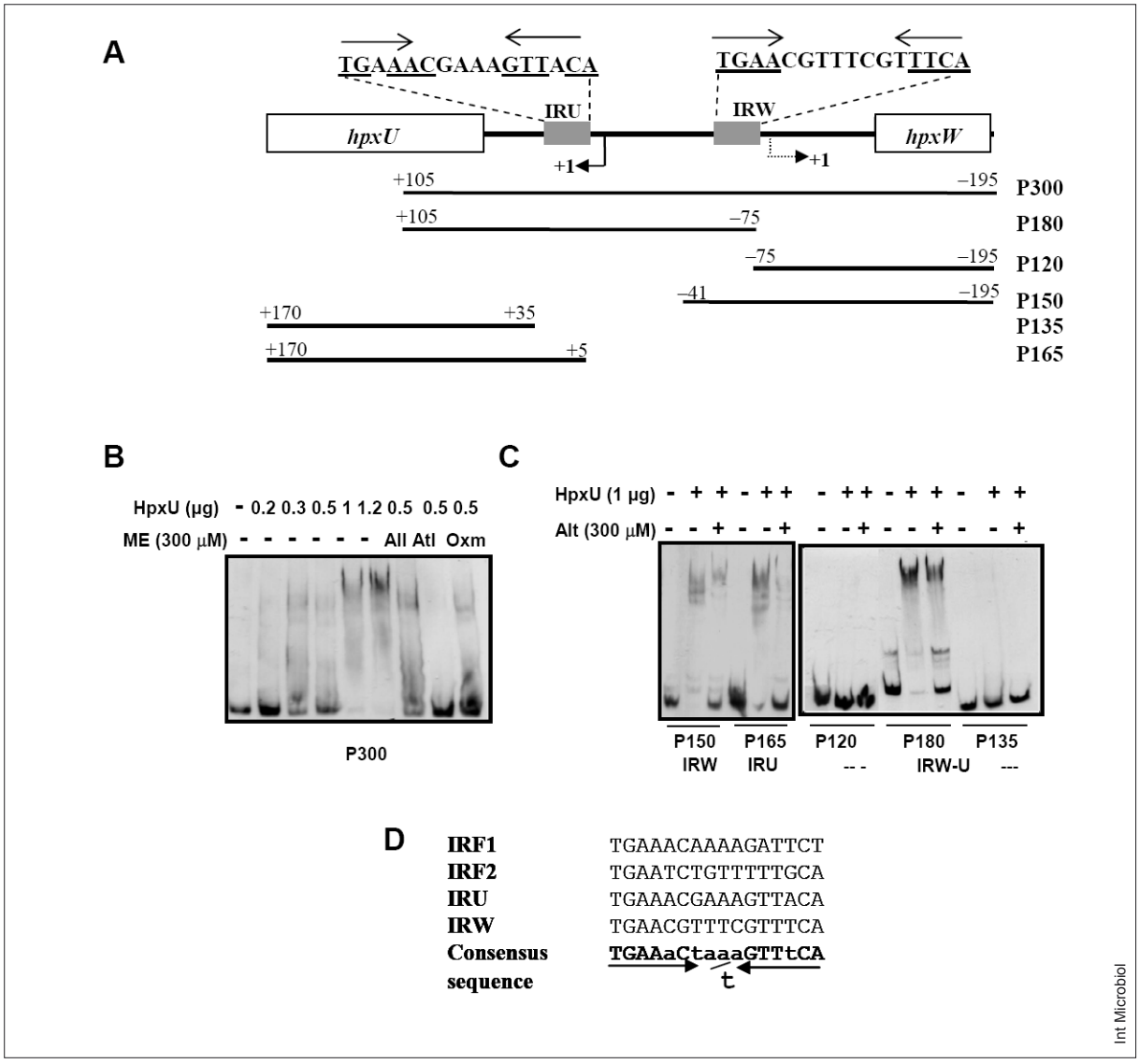

Fig. 5. Binding of HpxU to promoter fragments of the *hpxU-hpxW* intergenic region. (**A**) Diagram of the *hpxU-hpxW* intergenic region showing the tss for each gene. The promoter fragments used as probes and their end terminus positions with respect to position +1 of *hpxU* are shown below. The sequence of the two inverted repeats, IRU and IRW, are indicated above the diagram. The conserved residues in each inverted repeat are underlined. (**B**) EMSA performed with probe P300 in the absence or presence of allantoin (All), allantoate (Alt), and oxamate (Oxm) as putative effector molecules (EM). (**C**) EMSA performed with the indicated probes in the absence or presence of 300 μM allantoate. (**D**) Alignment of the palindromic sequences recognized by HpxU, identified in this study. The derived consensus sequence is indicated below. Nucleotides conserved in at least three of these sequences are indicated by capital letters, and those conserved in all four operators by asterisks.

concentration and complete dissociation of the HpxU-DNA complexes was obtained at 0.3 mM allantoate (Fig. 4C). Overall, these results suggested that allantoate was the effector molecule required to release the HpxU-mediated repression of the *hpxFGHIJK* operon.

To precisely locate the HpxU binding site, the *hpxF* promoter region was split into several fragments (Fig. 4A) that were tested as probes in subsequent experiments. HpxU-DNA complexes were detected with probes P149 and P70 but not with probe P150 or P140 (Fig. 4D). These studies allowed us to locate the HpxU binding site between positions +15 and +84 with respect to the *hpxF* tss. An in silico analysis of this region showed the presence of an inverted repeat (5′-TGAA-ACAAAAGATTCTTTGAATCTGTTTTTGCA-3′) between positions +35 and +67, which we refer to as the IRF (inverted repeat for *hpxF*) (Figs. 2 and 4A). To analyze whether IRF was the recognition motif for HpxU, two additional probes, P92 and P249, were designed, each containing only half of the IRF palindromic sequence. EMSA studies showed HpxU binding to both probes although only one protein-DNA complex was visible (Fig. 4D). More detailed inspection of these sequences revealed that each half of the IRF palindrome was itself an inverted repeat (IRF1 and IRF2), which in turn suggested that both IRF1 and IRF2 acted as HpxU binding sites. Analysis of the IRF1 and IRF2 operator sequences identified TGAA-N8-TTCT and TGAA-N8-TGCA as the putative sites recognized by HpxU in the *hpxF* promoter. These sequences display similarity with the operator sites recognized by other RpiR-like regulators [18].

Binding analysis of HpxU to the *hpxU-hpxW* intergenic region was also undertaken. Computational analysis of this region allowed us to identify two palindromic sequences as putative HpxU binding sites, IRU (5′-TGAAACGAAA GTTACA-3′), located at positions +43 to +28 with respect to the *hpxU* tss, and IRW (5′-TGAACGTTTCGTTTCA-3′), at positions -51 to -35 with respect to the the putative *hpxW* tss (Fig. 2). These positions are in agreement with the repressor function proposed for HpxU. EMSA experiments performed with different probes were consistent with the binding of HpxU to probe P180, encompassing IRU and IRW, but not to probes P135 and P120, which lacked these sequences (Fig. 5). HpxU binding studies to P150 (containing only IRW) and P165 (containing only IRU) confirmed the functionality of both proposed HpxU binding sites. As expected, allantoate abolished HpxU binding to IRW and IRU sites. These sites were separated by 84 nucleotides, corresponding to approximately eight turns in the DNA helix. Interactions between HpxU proteins simultaneously bound to IRU and IRW might lead to the formation of a DNA loop responsible for the transcriptional repression of each transcriptional unit.

Sequence alignment of the four proposed HpxU operators (IRF1, IRF2, IRW, IRU) revealed a high degree of identity and a common inverted repeat consensus sequence, TGAA-N_8-TTCA (Fig. 5D), which most likely represented the HpxU binding motif (underlined nucleotides are conserved in all four operators). The right arm of this motif was less conserved and might determine the HpxU binding affinity of these sites.

Model for the coordinated regulation of the three clusters involved in the purine catabolic pathway. As noted above, the genes required for purine nitrogen assimilation are distributed in three separate clusters in the *K. pneumoniae* genome. Two clusters had been previously studied by our group [15,33]. In those studies, genes of the purine pathway are shown to be co-ordinately regulated through sequential transcriptional activation of gene subsets by upstream metabolic pathway intermediates. The first cluster (KPN_01661 to KPN_01666) comprises genes involved in hypoxanthine oxidation to allantoin. Genes encoding the hypoxanthine dehydrogenase subunits (*hpxDE*) are induced by hypoxanthine through the activator protein HpxR, encoded in the same cluster. Uric acid, the product of hypoxanthine oxidation, is required for induction of the *hpxPQT* operon encoding the enzymes involved in the conversion of uric acid to allantoin [33]. Allantoin itself is in turn required for the induction of the *hpxSAB* operon, located in the second cluster (KPN_01787 to KPN_01791), in which the gene *hpxB* encodes allantoinase. Full induction of *hpxSAB* by allantoin requires the specific regulator HpxS and the global nitrogen regulatory protein NAC [15]. Lastly, the results presented here show that allantoate, the product of allantoinase activity, is required for the induction of genes in the third cluster (KPN_01761 to KPN_01771), which in turn triggers the induction of the *hpxFGHIJK* operon, involved in the conversion of allantoate to oxalurate. The finding that the addition of allantoate to glucose-glutamine cultures of strain KC2653 bearing ϕ(*hpxW-lacZ*) did not yield any increase in β-galactosidase activity (Fig. 3) suggested that additional signals were needed for *hpxWXYZ* expression. Since each gene cluster encodes specific transport systems and regulatory proteins that recognize the cognate substrates, our findings were consistent with an integrated genetic system for the uptake and assimilation of purine-derived compounds present in the environment.

The assimilation pathway of purines as a nitrogen source is also transcriptionally regulated by nitrogen availability through different mechanisms. Genes *hpxPQT* (in the first

cluster) and *guaD* (in the second cluster), both with a σ^{54}-dependent promoter, are activated by the global regulatory protein NtrC. In genes with σ^{70}-dependent promoters, only *hpxDE* (in the first cluster) and *hpxSAB* (in the second cluster) are activated under nitrogen-limiting conditions. This nitrogen regulation is mediated by NAC in the case of the *hpxSAB* operon, whereas for *hpxDE* an as yet unidentified repression mechanism that is involved under nitrogen excess conditions has been proposed [15,33]. Transcriptional control by nitrogen is expected for genes involved in the catabolism of compounds that can be used as nitrogen sources [13,31]. Our results suggest that the genes involved in allantoate catabolism (third cluster) would not be significantly regulated by nitrogen availability, in accordance with additional roles for allantoate or its derived metabolites besides facilitating the supply of nitrogen. In the metabolic context of this bacterial species, this cluster might contribute to detoxifying intracellular glyoxylate or other α-ketoacids through HpxJ aminotransferase activity.

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Competing interests. None declared.

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