

# *Pseudomonas aeruginosa* *gbdR* gene is transcribed from a $\sigma^{54}$ -dependent promoter under the control of NtrC/CbrB, IHF and BetI

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## Abstract

*Pseudomonas aeruginosa* uses choline as a source of carbon and nitrogen, and also for the synthesis of glycine betaine, an osmoprotectant under stress conditions such as drought and salinity. The transcription factor GbdR is the specific regulator of choline metabolism and it belongs to the Arac/XylS family of transcriptional regulators. Despite the link between choline catabolism and bacterial pathogenicity, *gbdR* regulation has not been explored in detail. In the present work, we describe how *gbdR* transcription can be initiated from a  $\sigma^{54}$ -dependent promoter. *gbdR* transcription can be activated by NtrC in the absence of a preferential nitrogen source, by CbrB in the absence of a preferential carbon source, and by the integration host factor favouring DNA bending. In addition, we found that BetI negatively regulates *gbdR* expression in the absence of choline. We identified two overlapping BetI binding sites in the *gbdR* promoter sequence, providing an additional example of  $\sigma^{54}$ -promoter down-regulation. Based on our findings, we propose a model for *gbdR* regulation and its impact on choline metabolism.

## INTRODUCTION

*Pseudomonas aeruginosa* is found in a wide range of environments, from hyperosmotic dry soils to aqueous hyposmotic media. It is an opportunistic pathogen with high nutritional versatility that is controlled by numerous transcriptional regulators (nearly 9 % of its genome) [1]. *P. aeruginosa* uses many substrates as carbon (C) and nitrogen (N) sources, including amino acids, polyamines, agmatine and quaternary amines, such as choline (cho) [2–6]. In addition to its use as a source of C and N, cho is the main source for the synthesis and accumulation of glycine betaine (GB), an osmoprotectant under stress caused by drought and salinity. Most of the available cho is present in macromolecules such as phosphatidylcholine, a major component of the plasma membrane of eukaryotic cells and the mammalian pulmonary surfactant [7, 8], and acetylcholine, which is abundant in the cornea [9]. Several studies performed on this issue have suggested a relationship between cho metabolism and virulence factors in *P. aeruginosa* [4, 6, 10–12] and *P. syringae* [13].

*P. aeruginosa* synthesizes several enzymes, such as hemolytic phospholipase C (PlcH), phosphorylcholine phosphatase (PchP) and cholinesterase (ChoE), to acquire cho from phosphatidylcholine, phosphocholine and acetylcholine, respectively [14–18]. The uptake of cho in *P. aeruginosa* occurs through various specific and non-specific transporter systems [16, 19]. When cho enters the cell, it is first converted – via a two-step oxidation process – into GB by the action of BetAB enzymes. *betA* and *betB* genes encode choline dehydrogenase and betaine aldehyde dehydrogenase, respectively. GB can be accumulated as an osmoprotectant, or it can be metabolized to obtain C and/or N [20]. GB, dimethylglycine (DMG) or cho itself induce the synthesis of cho catabolic enzymes in the absence of other C and/or N preferential sources [6, 21]. Furthermore, the enzymes involved in the initial steps of cho conversion into GB (BetA–BetB) are inducible at the transcriptional level by cho, but not by osmotic stress [22]. The *betA* and *betB* genes, together with *betI*, belong to the *betIBA* operon [22]. BetI, a 21.8 kDa protein that is a transcriptional repressor of

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**Abbreviations:** Ap, ampicillin; C, carbon; Cb, carbenicillin; CERELA, Centro de Referencia para Lactobacilos; cho, choline; ChoE, cholinesterase; CONICET, Consejo Nacional de Investigaciones Científicas y Técnicas; DMG, dimethylglycine; EBP, enhancer-binding protein; EMSA, electrophoretic mobility shift assay; FonCyT, Fondo para la Investigación Científica y Tecnológica; GB, glycine-betaine; Gm, gentamycin; HPI-BSM, high phosphate basal salt medium; IHF, integration host factor; MU, Miller units; N, nitrogen; NH<sub>4</sub>, ammonium chloride; PchP, phosphorylcholine phosphatase; PlcH, hemolytic phospholipase C; SECYT-UNRC, Secretaría de Ciencia y Técnica de la Universidad de Río Cuarto; suc, succinate; UAS, upstream activator sequence.

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Two supplementary figures and one supplementary table are available with the online Supplementary Material.

the TetR family, is involved in the regulation of the *bet* genes [23–25]. In the presence of cho, BetI is freed from the promoter and transcription is stimulated [19, 24].

On the other hand, the catabolism of cho is under the control of two global component systems, CbrA/CbrB and NtrB/NtrC [26], in a similar fashion to the regulation of amino acid metabolism [5, 27, 28]. In addition, cho metabolism in *P. aeruginosa* is specifically regulated by GbdR, which belongs to the AraC/XylS family of transcriptional factors [29]. Twelve GbdR-responsive transcriptional units that comprise 26 genes – 11 of which have measurable binding to GbdR *in vitro* – have already been identified [21]. Some of these proteins (PlcH, PchP and ChoE) are involved in the cho acquisition system, while others (BetX and the CbcXWV quaternary amine transport proteins) are involved in the import of cho, and there are also enzymes for GB, DMG, sarcosine, glycine and serine catabolism [6, 19, 21].

In *P. aeruginosa*, some key aspects related to the regulation of cho catabolism have previously been described [21, 30–32]. However, neither *gbdR* regulation itself nor the effects of such regulation on cho metabolic machinery expression have been analysed until now. A shared feature of many regulators within the AraC/XylS family is that their synthesis is strictly regulated to ensure that they perform their functions properly [33–35]. Taken together, our findings demonstrate that *gbdR* is regulated at the transcriptional level by: (i) a  $\sigma^{54}$ -dependent promoter; (ii) NtrC, in the absence of preferential N sources; (iii) CbrB, in the absence of preferential C sources; (iv) integrating host factor (IHF), by favouring DNA bending; and (v) BetI, by repressing *gbdR* expression in the absence of cho. Finally, we propose a model to understand *gbdR* regulation and its impact on cho or GB catabolism when *P. aeruginosa* grows in a complex medium with other non-preferential C and N sources.

## METHODS

### Strains, plasmids, and growth conditions

The strains and plasmids used in this study are listed in Table 1. *Escherichia coli* and *Pseudomonas aeruginosa* strains were routinely grown in a Luria–Bertani liquid culture or in high-phosphate basal salt medium (HPi-BSM) [36] at 37 °C. C and N sources were added at 20 mM. The antibiotic concentrations ( $\mu\text{g ml}^{-1}$ ) were: ampicillin (Ap) 100 or 150 and gentamycin (Gm) 15 for *E. coli* strains; Gm 30 and carbenicillin (Cb) 100 or 200 for *P. aeruginosa*.

### DNA methodology

DNA manipulation and PCR amplification were performed as previously described [37]. Chromosomal and plasmid DNA purification was performed using the QIAamp DNA Minikit and QIAprep Spin Miniprep Kitm (Qiagen), respectively. DNA fragments were purified from agarose gels using a QIAquick Kit (Qiagen). For the construction of plasmids harbouring the putative promoter region of *gbdR*, DNA fragments fP1, fUp1, fUp2, fUp3 and fRpoN<sup>-</sup> were amplified from genomic

DNA templates with the following primers: fP1 (P1-Up/P1-Dn); fUp1 (Up-1/P1-Dn); fUp2 (Up-2/P1 Dn); fUp3 (Up-3/P1-Dn); and fRpoN<sup>-</sup> (P1-Up/DnRpoN<sup>-</sup>), as indicated in Table S1 (available in the online Supplementary Material). The PCR products were digested with SpeI and XhoI, after which they were individually cloned between the same sites in the pUC18-mini-Tn7T-Gm-*lacZ* vector to obtain pP1, pUp1, pUp2, pUp3 and pRpoN<sup>-</sup>, respectively. Each of these plasmids and pTNS2 were co-transformed into *P. aeruginosa* by electroporation [38], and the resulting strains containing the integrated DNA fragments were termed WT-P1, WT-Up1, WT-Up2, WT-Up3 and WT-RpoN<sup>-</sup>. Colony PCR using the primers PTn7R and PglmS-down was used to confirm the chromosomal Tn7 insertions. The Gm marker was excised as described in [38]. For site-directed mutants, the one-step overlap extension PCR method was used, as described in [39]. The primers P1-Up, F-BetI<sup>-</sup>, R-BetI<sup>-</sup> and P1-Dn (Table S1) were employed for BetI consensus substitution in the *gbdR* promoter, obtaining the fragment fP1-BetI<sup>-</sup>, which was cloned in the pUC18-mini-Tn7T-Gm-*lacZ* vector between the SpeI and XhoI restriction sites to generate the vector pP1-BetI<sup>-</sup>. To be sure that no errors were introduced during the PCR or sub-cloning procedures, all of the PCR products and constructs were sequenced at the CERELA Institute (Tucuman, Argentina).

### Construction of mutant strains

Deletion mutant strains were obtained as described in [40] and modified to introduce changes to obtain plasmid-borne deletions. Gene-specific fragments (gene-Up and gene-Dn) were amplified using the primers UpF-BetI, UpR-BetI, DnF-BetI, DnR-BetI, UpF-*himA*, UpR-*himA*, DnF-*himA* and DnR-*himA*. The Gm resistance gene cassette was obtained by PCR amplification using the Gm-F and Gm-R primers (Table 1). The construction was performed in two steps. (i) pEX18ApGW (digested with PstI and KpnI), Up fragments (restricted with KpnI and BamHI) and Dn fragments (restricted with PstI and BamHI) were ligated to generate the pEX::Up-Dn vector. *E. coli* cells were transformed with the ligation product and selected with Ap. The presence of pEX::Up-Dn was verified by PCR using the UpF and DnR primers. (ii) The pEX::Up-Dn vector and Gm cassette were cut with BamHI and then ligated. The ligation product was introduced into competent *E. coli* and then Gm-resistant bacteria were selected to obtain the pEX::Up-Gm-Dn vector. The Up-Gm-Dn fragment was confirmed by PCR using the primers UpF and DnR.

### Cloning and expression of recombinant BetI

*betI* was amplified from *P. aeruginosa* PAO1 with the primers BetI-Up and BetI-Dn. The PCR product was digested with NdeI/BamHI and ligated into the pET15b plasmid (Novagen) to generate the plasmid pET-BetI.

Expression of 6-His-BetI was induced in *E. coli* strain BL-21 Rosetta cells. Briefly, cells were grown in LB medium containing 200  $\mu\text{g ml}^{-1}$  ampicillin at 37 °C and shaken at 250 r.p.m. until it reached  $\text{OD}_{550} \cong 0.6\text{--}0.7$ . Protein synthesis was

Table 1. Bacterial strains and plasmids

Strains	Description	Reference or source
<i>E. coli</i>		
XL10-Gold	Tet <sup>r</sup> Δ( <i>mcrA</i> ) 183 Δ( <i>mcrCB-hsdSMR-mrr</i> )173 <i>endA1 supE44 thi-1 recA1 gyrA96 relA1 lacHte</i> [F' <i>proABlacIqZ</i> Δ <i>M15</i> Tn10 (Tet <sup>r</sup> ) Amy Cam <sup>r</sup> ]	
S17-1 λ <i>pir</i>	pro <i>thihsdR</i> <sup>+</sup> Tp <sup>r</sup> Sm <sup>r</sup> ; chromosome::RP4-2 Tc::Mu-Kan::Tn7/λ <i>pir</i>	[57]
<i>ccdBSurvival</i> 2 T1 <sup>R</sup>	F <sup>-</sup> <i>mcrA</i> Δ( <i>mrr-hsdRMS-mcrBC</i> ) Φ80 <i>lacZ</i> Δ <i>M15</i> Δ <i>lacX74 recA1 araΔ139</i> Δ( <i>ara-leu</i> )7697 <i>galU galK rpsL</i> (Str <sup>R</sup> ) <i>endA1 nupG fhuA::IS2</i>	Invitrogen
<i>P. aeruginosa</i>		
PAO1		
PAO1-WT	Prototrophic wild-type strain	
WT-P1	PAO1 with a chromosomal integration of mini-Tn7T carrying the translational fusion fP1::lacZ	This study
WT-Up1	PAO1 with a chromosomal integration of mini-Tn7T carrying the translational fusion fUp1::lacZ	This study
WT-Up2	PAO1 with a chromosomal integration of mini-Tn7T carrying the translational fusion fUp2::lacZ	This study
WT-Up3	PAO1 with a chromosomal integration of mini-Tn7T carrying the translational fusion fUp3::lacZ	This study
P1-BetI <sup>-</sup>	PAO1 with a chromosomal integration of mini-Tn7T carrying the translational fusion fP1-BetI <sup>-</sup> ::lacZ	This study
WT-RpoN <sup>-</sup>	PAO1 with a chromosomal integration of mini-Tn7T carrying the translational fusion fRpoN <sup>-</sup> ::lacZ	This study
WT-RpoN2	PAO1 with a chromosomal integration of mini-Tn7T carrying the translational fusion fRpoN2::lacZ	This study
Δ <i>rpoN</i>	PAO1 Δ <i>rpoN</i>	[58]
Δ <i>rpoN</i> -P1	PAO1 Δ <i>rpoN</i> with a chromosomal integration of mini-Tn7T carrying the translational fusion fP1::lacZ	This study
Δ <i>cbrB</i>	PAO1 Δ <i>cbrB</i>	[26]
Δ <i>cbrB</i> -P1	PAO1 Δ <i>cbrB</i> with a chromosomal integration of mini-Tn7T carrying the translational fusion fP1::lacZ	This study
Δ <i>ntrC</i>	PAO1 Δ <i>ntrC</i>	[26]
Δ <i>ntrC</i> -P1	PAO1 Δ <i>ntrC</i> with a chromosomal integration of mini-Tn7T carrying the translational fusion fP1::lacZ	This study
Δ <i>betI</i>	PAO1 Δ <i>betI</i>	This study
Δ <i>betI</i> -P1	PAO1 Δ <i>betI</i> with a chromosomal integration of mini-Tn7T carrying the translational fusion fP1::lacZ	This study
Δ <i>himA</i>	Δ <i>himA</i> -P1	This study
Δ <i>himA</i> -P1	Δ <i>himA</i> -P1 with a chromosomal integration of mini-Tn7T carrying the translational fusion fP1::lacZ	This study

Plasmids	Description	Reference
pUC18-mini-Tn7T-Gm-lacZ	Gm <sup>r</sup> on mini-Tn7T; lacZ transcriptional fusion vector	[38]
pTNS2	Ap <sup>r</sup> ; helper vector encoding the site-specific Tn7 transposition pathway	[38]
pFLP2	Ap <sup>r</sup> ; Flp recombinase-encoding vector	[38]
pEX18ApGW	Ap <sup>r</sup> ; gene replacement vector, compatible with the gateway system	[40]
pP1	Gm <sup>r</sup> , Ap <sup>r</sup> ; pUC18-mini-Tn7T-Gm-lacZ harbouring <i>gbdR</i> promoter sequences fP1 introduced using <i>SpeI</i> and <i>XhoI</i> sites	This study
pUp1-3	Gm <sup>r</sup> , Ap <sup>r</sup> ; pUC18-mini-Tn7T-Gm-lacZ harbouring <i>gbdR</i> promoter sequences fUp1-Up3 introduced using <i>SpeI</i> and <i>XhoI</i> sites	This study
pP1-BetI <sup>-</sup>	Gm <sup>r</sup> , Ap <sup>r</sup> ; pUC18-mini-Tn7T-Gm-lacZ harbouring <i>gbdR</i> promoter sequences fP1-BetI <sup>-</sup> introduced using <i>SpeI</i> and <i>XhoI</i> sites	This study
pRpoN <sup>-</sup>	Gm <sup>r</sup> , Ap <sup>r</sup> ; pUC18-mini-Tn7T-Gm-lacZ harbouring <i>gbdR</i> promoter sequences fRpoN <sup>-</sup> introduced using <i>SpeI</i> and <i>XhoI</i> sites	This study
pRpoN2	Gm <sup>r</sup> , Ap <sup>r</sup> ; pUC18-mini-Tn7T-Gm-lacZ harbouring <i>gbdR</i> promoter sequences fRpoN2 introduced using <i>SpeI</i> and <i>XhoI</i> sites	This study
pET-BetI	Ap <sup>r</sup> ; pET15b harbouring the <i>betI</i> gene	This study

induced by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM, incubation overnight at 18 °C and shaking at 100 r.p.m. Then, cells were centrifuged at 5000 g for 15 min at 4 °C, re-suspended in lysis buffer [300 mM NaCl, 20 mM phosphate buffer (pH 8.0) and 2 % glycerol] and disrupted by sonication. After that, the suspension was centrifuged at 5000 g for 15 min, and the supernatant was applied to a Ni-nitrilotriacetic acid column (Qiagen). Purification under native conditions was carried out

according to the manufacturer's instructions. The elution buffer was 10 % glycerol, 0.1 % Triton X-100, 200 mM imidazole and 20 mM phosphate buffer (pH 8.0). The purified protein was stored at 4 °C. The protein concentration was estimated by measuring its absorbance at 280 nm.

### Electrophoretic mobility shift assay (EMSA)

Plasmids P1 and pP1-BetI<sup>-</sup> (lacking BetI binding site consensus) were used as templates for PCR amplification with

the primers: Up: CCTGGTGCTACCGAAATG and Dn: AGCAAGGCAAATGCCATGCC. Approximately 100 ng of PCR-generated DNA fragments (550 pb) was used for each assay. The fragments carry the promoter region of *gbdR*, and they were mixed with increasing concentrations of purified 6-His-BetI protein in a buffer containing 50 mM Tris-HCl (pH 8.0); 50 mM KCl; 1.25 mM MgCl<sub>2</sub>; 0.1 mg ml<sup>-1</sup> BSA; 0.975 mM dithiothreitol; and 6.5 % glycerol. Samples were incubated for 20 min at room temperature and then separated by electrophoresis in 6 % polyacrylamide gels in Tris-acetate-EDTA buffer for 60 min, 100 V, at room temperature. The DNA bands were stained with Gel Green and visualized with a Fujifilm LAS-3000 system.

### β-Galactosidase activity

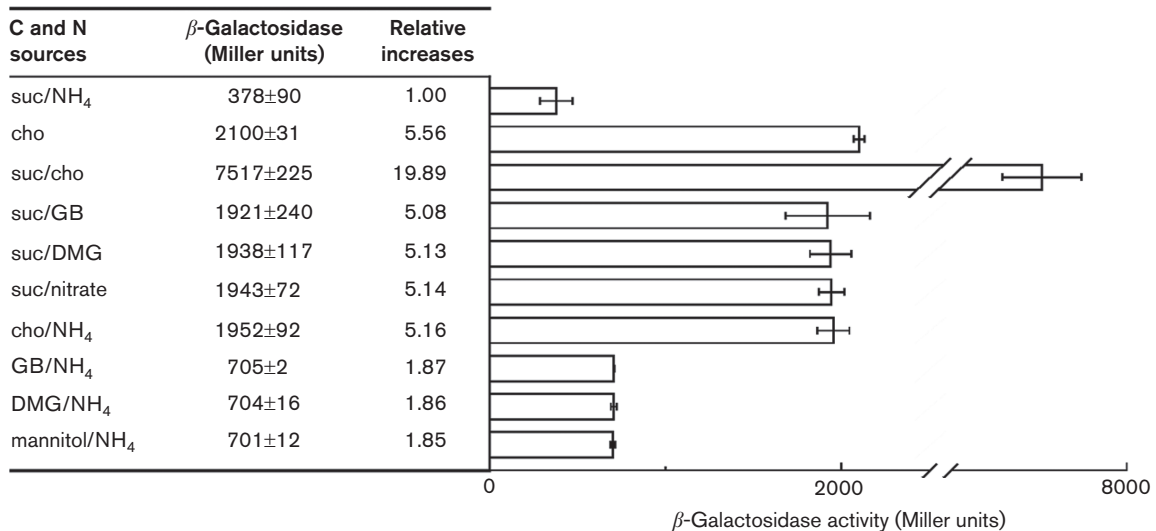
β-Galactosidase assays were performed according to the procedure described by Miller [41].

## RESULTS

### Expression of *gbdR* under various nutritional conditions

To understand how *gbdR* is regulated when cho or its derivatives are used as C or N sources, a 700 bp P1 fragment (620 bp upstream and 80 bp downstream of the ATG initiation codon of the *gbdR* gene) was fused to *lacZ* and integrated into the bacterial chromosome of PAO1-WT, giving rise to the strain WT-P1 :: *lacZ* (henceforth termed WT-P1). In this strain, the activity of the promoter was evaluated in cells grown in HPI-BSM medium supplemented with

succinate/ammonium (suc/NH<sub>4</sub>) as preferential C and N sources (control or basal activity). Mannitol and nitrate were used as the controls for non-preferential C and N sources, respectively. Other substrates, such as cho, GB and dimethylglycine (DMG) were also evaluated as non-preferential N or C sources. In fact, when these compounds were used as a N donor, succinate was added as the C source (suc/cho, suc/GB, suc/DMG), whereas when they were used as a C source, NH<sub>4</sub> was added as the N donor (cho/NH<sub>4</sub>, GB/NH<sub>4</sub>, DMG/NH<sub>4</sub>). Fig. 1 shows that: (i) the *gbdR* expression increased ≈20-fold in cells grown with cho as the N source (7517±225 MU) compared to cells grown in suc/NH<sub>4</sub> (378±90 MU); (ii) no differences were observed in the reporter activity of cells grown with GB or DMG (both molecules related to cho) in comparison with cells grown in nitrate (a compound that is not related to cho) as the N source, with this activity being around 5 fold higher compared with that for cells grown in suc/NH<sub>4</sub>; (iii) the activity determined in cells grown in cho alone or supplemented with another preferential N source (with cho being the C source) was ≈5.5-fold higher than the basal level; (iv) no differences were found between cells grown with GB, DMG or mannitol (a molecule that is not related to cho) as C sources, despite the fact that in these conditions *gbdR* expression was double that of the basal level (cells grown in suc/NH<sub>4</sub>). Taken together, these results indicate that *gbdR* expression is induced in the absence of preferential C and/or N sources; nevertheless, when cho is the C or N source, the highest level of transcriptional activity is reached.



**Fig. 1.** Expression of *gbdR* promoter under different nutritional conditions. WT-P1 strain (WT-P1 :: *lacZ*) was grown in HPI-BSM medium supplemented with the indicated C and N sources (left) at a concentration of 20 mM. β-galactosidase activity (expressed as Miller units, MU) was measured in cells at OD<sub>660</sub> ≈ 0.5, when maximal promoter expression occurred. The relative increase was calculated as a multiple of the reporter activity obtained in cells grown in suc/NH<sub>4</sub> conditions (378 MU). The bar graph (right) represents the β-galactosidase activity under each set of culture conditions. The average values for at least three independent assays are shown. The error bars indicate standard errors. References: suc, succinate; NH<sub>4</sub>, ammonium chloride; cho, choline; GB, glycine betaine; DMG, dimethylglycine.

### ***gbdR* gene regulation by a $\sigma^{54}$ -dependent promoter**

The findings derived from physiological experiments suggest that *gbdR* expression is under the control of complex regulatory mechanisms. To investigate these mechanisms, we performed an analysis, by visual inspection, of the regulatory *gbdR* region. Interestingly, by examining 620 bp upstream of the *gbdR* start codon, we found two consensus regions for RpoN-binding motifs (Fig. S1). Both motifs are overlapped and located between -131 and -113 pb upstream of the *gbdR* start codon (<sup>-132</sup>GGCATGGCATTTTGCCTTGC<sup>-113</sup>). We termed them 'proximal' or 'RpoN1' (<sup>-127</sup>GGCATTTTGC<sup>-113</sup>) and 'distal' or 'RpoN2' (<sup>-132</sup>GGCATGNTTGC<sup>-118</sup>)  $\sigma^{54}$ -promoters. The most highly conserved nucleotides are in contour and are underlined for RpoN1, while they are underlined and in bold type for RpoN2.

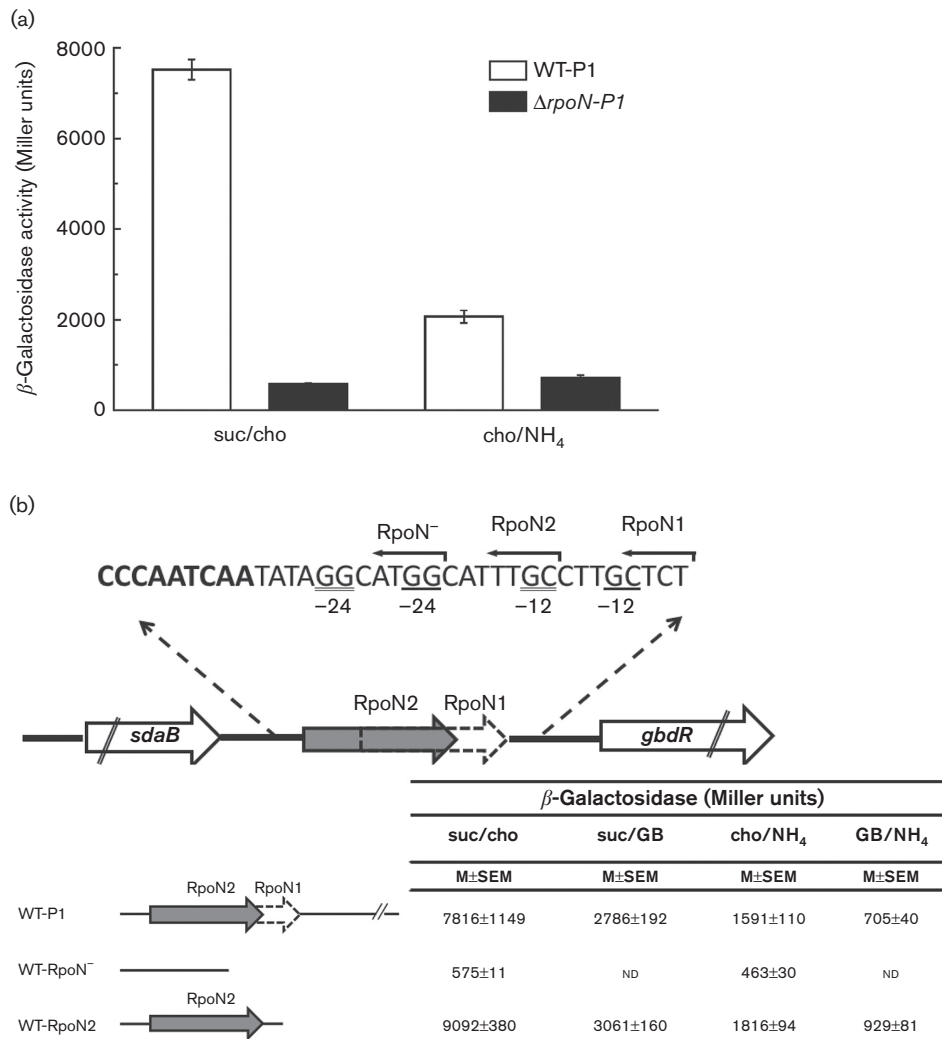
To determine whether *gbdR* expression is truly dependent on RpoN, we generated the  $\Delta rpoN$ -P1 mutant strain (Table 1). The results obtained with this mutant were compared with WT-P1 bacteria. The  $\beta$ -galactosidase activity of the mutant strain that was grown with cho as the C or N source was significantly reduced (93 and 65 %, respectively) compared with that of wild-type (WT) cells grown in similar conditions (Fig. 2a). The two putative RpoN binding sites (RpoN1 and RpoN2) are spaced by five nucleotides (Fig. 2b). Hence, to assess the relevance of each RpoN binding site, we performed a ClustalX analysis, taking into account the regions upstream of the start codon of *gbdR* in different *Pseudomonas* strains (Fig. S2). Multiple alignment analysis showed that only the RpoN2 promoter is conserved in all strains tested, suggesting that RpoN1 promoter may be less relevant than RpoN2 in *gbdR* regulation. To determine whether the RpoN2 consensus binding site was sufficient for the activation of *gbdR* expression, RpoN1 and RpoN2 consensus sequences were modified by shortening the P1 fragment 3' end. These constructs were fused to *lacZ* and inserted into the chromosome of WT bacteria. The WT-RpoN<sup>-</sup> strain was obtained when the -12 element consensus sequences of both RpoN binding sites were deleted. On the other hand, the WT-RpoN2 strain was obtained when the -12 element of RpoN1 was deleted. The WT-P1 strain was used as the control bacteria. As shown in Fig. 2(b), the reporter activity of WT-RpoN<sup>-</sup> cells grown with cho as the C or N source was similar ( $\approx 500$  MU) to the basal activity (WT-P1 grown in suc/NH<sub>4</sub>). The RpoN2 strain displayed similar values of reporter activity to the WT-P1 bacteria when both cells were grown in the same conditions. Data analysis of the  $\Delta rpoN$ -P1, WT-RpoN<sup>-</sup> and RpoN2 strains indicated that the *gbdR* promoter belongs to the class of promoters that depend on the alternative  $\sigma^{54}$  factor.

### **Role of CbrB and NtrC in the expression of *gbdR***

The presence of the  $\sigma^{54}$  promoter in the regulatory region of *gbdR* prompted us to search binding sites for enhancers such as CbrB and NtrC. By visual inspection, a putative CbrB binding site was identified at <sup>-357</sup>CGGTCGTCN<sub>3</sub>

CGTCACAC<sup>-338</sup> (the nucleotides in bold match the CbrB consensus), similarly to the consensus site cTGTTACcN<sub>3/12</sub>cGTAACAG reported in [42]. In addition, we found an NtrC binding site at position <sup>-391</sup>CGCCACN<sub>5</sub>GGCGCA<sup>-374</sup> (the nucleotides in bold match the NtrC consensus), in agreement with the data described for the NtrC consensus in *P. putida* (CGCACC-N<sub>5</sub>-GGTGCA) [43]. To assess whether these putative binding sites are functional, the P1 fragment of the *gbdR* promoter was fused to *lacZ* and integrated into the bacterial chromosome of the  $\Delta cbrB$  and  $\Delta ntrC$  *P. aeruginosa* mutants, giving rise to the  $\Delta cbrB$ -P1 :: *lacZ* and  $\Delta ntrC$ -P1 :: *lacZ* strains, respectively (henceforth termed the  $\Delta cbrB$ -P1 and  $\Delta ntrC$ -P1 strains). The results obtained with the mutant strains were compared with those for the WT-P1 strain. The  $\beta$ -galactosidase activity was determined in cells grown in HPI-BSM medium supplemented with suc/NH<sub>4</sub> (control), with cho as the C source (cho/NH<sub>4</sub>) or with cho as the N source (suc/cho). As shown in Fig. 3(a), the different strains grown in suc/NH<sub>4</sub> displayed similar reporter activity. In contrast, the reporter activity was markedly reduced ( $\approx 70$  %) in  $\Delta cbrB$ -P1 cells (729 $\pm$ 189 MU) when cho was the C source compared to that in the WT-P1 or  $\Delta ntrC$ -P1 cells ( $\approx 2400$  MU) grown in similar culture conditions. On the other hand,  $\beta$ -galactosidase activity was reduced by more than 85 % in  $\Delta ntrC$ -P1 cells (1161 $\pm$ 199 MU) grown in suc/cho compared with that in WT-P1 cells (7838 $\pm$ 497 MU). These results strongly suggest that *gbdR* expression is controlled by CbrB and NtrC. In order to experimentally characterize the putative binding sites for CbrB and NtrC that we had previously identified by visual inspection, we constructed three mutant strains: WT-Up1, WT-Up2 and WT-Up3. They bear the following DNA fragments: Up1, which lacks the upstream region up to the NtrC binding site; Up2, which lacks the upstream region up to the CbrB binding site, and is thus without the NtrC binding site; and Up3, which has neither the NtrC nor the CbrB binding site (Fig. 3b). All of the fragments were fused to *lacZ* and integrated into the bacterial chromosome of the PAO1-WT.

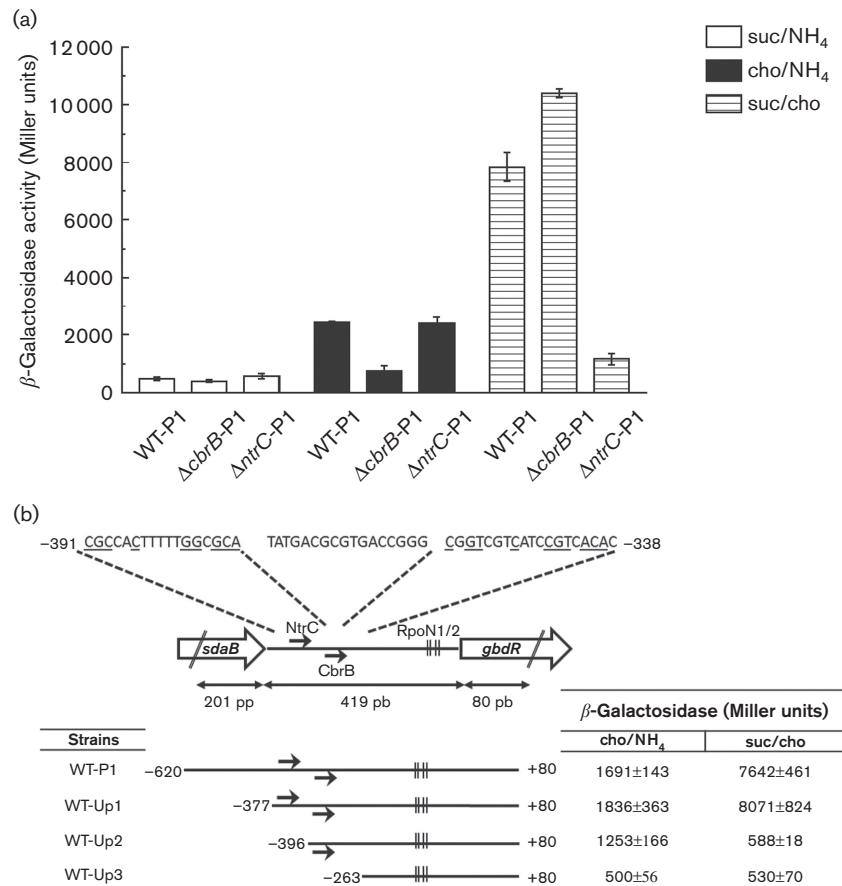
WT-P1 and WT-Up1 cells displayed similar reporter activity when they were grown in cho/NH<sub>4</sub> (1691 $\pm$ 143 versus 1836 $\pm$ 363 MU, respectively) or in suc/cho media (7642 $\pm$ 461 versus 8071 $\pm$ 824 MU, respectively). Interestingly, when it was cultured in suc/cho media, WT-Up2 reporter activity was strongly reduced ( $\approx 90$  %) compared with that for WT-P1 bacteria grown in similar culture conditions. However, in WT-Up2 cells grown in cho/NH<sub>4</sub>, the reporter activity was similar or slightly reduced when compared with that for the WT-P1 strain (1253 $\pm$ 166 versus 1691 $\pm$ 461 MU, respectively). The WT-Up3 strain showed basal reporter activity under both conditions, similar to the results obtained for WT-P1 bacteria grown in suc/NH<sub>4</sub> ( $\approx 500$  MU). A functional and efficient interaction of  $\sigma^{54}$ -RNAPol with the enhancer-binding protein (EBP) requires DNA bending. This bending may be the result of intrinsic bends or the action of the IHF, a heterodimer of two basic peptides encoded by the *himA* and *himD* [44–46]. Visual inspection



**Fig. 2.** Expression of the *gbdR* gene in the WT-P1 and  $\Delta rpoN$ -P1 strains. (a)  $\beta$ -galactosidase activity in *P. aeruginosa* WT-P1 and the  $\Delta rpoN$ -P1 mutant strain. Cells were grown in HPI-BSM supplemented with choline as the C (cho/NH<sub>4</sub>) or N (suc/cho) source. Top: schematic diagram of the *gbdR* promoter showing the nucleotide sequences between -175/-109 bp upstream of the ATG start codon of the *gbdR* gene with the putative RpoN1 and RpoN2 binding sites. The -24/-12 elements [59] are underlined. Bottom: DNA fragments of different sizes from the *gbdR* promoter (indicated by lines) fused to *lacZ* (P1::lacZ, RpoN<sup>-</sup>::lacZ and RpoN2::lacZ) and then inserted into the bacterial chromosome of *P. aeruginosa* WT, giving rise to the WT-P1, WT-RpoN<sup>-</sup> and WT-RpoN2 strains, respectively. The RpoN binding sites are represented by arrows, while the grey colour indicates RpoN2 and the white with dotted lines indicates RpoN1. Right:  $\beta$ -galactosidase activity was measured in cells grown in suc/cho, suc/GB, cho/NH<sub>4</sub> or GB/NH<sub>4</sub>. Average values of at least three independent experiments are shown. References: suc, succinate; NH<sub>4</sub>, ammonium chloride; cho, choline; GB, glycine betaine.

analysis revealed a potential IHF-binding site located at <sup>-175</sup>GCTAAAN<sub>4</sub>TCC<sup>-163</sup> upstream of the ATG of the *gbdR* start site (highly conserved nucleotides are in bold type). This IHF binding site was similar to the consensus 5'-A/TATCAAN<sub>4</sub>TTA/G-3' [47]. To assess the contribution of IHF in the expression of the promoter *gbdR*, P1::lacZ fusions were examined in IHF-proficient (WT-P1) and IHF-deficient ( $\Delta himA$ -P1) strains. These strains were cultured in: (i) cho or GB as the sole N source plus succinate, or (ii) cho or GB as the only C source plus NH<sub>4</sub>. As shown in Fig. 4, when the strain  $\Delta himA$ -P1 was grown in suc/cho

or in suc/GB, the reporter activity decreased by  $\approx 70$  and  $\approx 60\%$ , respectively, compared with the activity of WT-P1 cells grown in the same culture conditions. In cases in which cho or GB were used as the C source, and thus there were cho/NH<sub>4</sub> or GB/NH<sub>4</sub> media, the reporter activity of the mutant strain was reduced by  $\approx 35$  and  $\approx 33\%$ , respectively, compared with that for the WT-P1 strain (Fig. 4). These data indicated that IHF was necessary to generate the highest *gbdR* expression. Nevertheless, the  $\Delta himA$ -P1 mutant retained some *gbdR* transcriptional activity, suggesting an intrinsic bend mechanism.



**Fig. 3.** Expression of *gbdR* promoter in WT-P1,  $\Delta cbrB$ -P1 and  $\Delta ntrC$ -P1 strains. (a)  $\beta$ -galactosidase activity in cells grown in HPI-BSM supplemented with suc/NH<sub>4</sub>, cho/NH<sub>4</sub> or suc/cho as the C and N sources, respectively. The average values of at least three independent experiments are shown. The error bars indicate standard errors. (b) Schematic diagram of the promoter showing the nucleotide sequences (top) between -338/-391 bp upstream of the ATG start codon of the *gbdR* gene with the putative CbrB and NtrC binding sites. The nucleotides match the consensus sequences described for NtrC [43] and CbrB [42]. The binding sites are underlined. DNA fragments of different sizes (indicated by lines) were fused to *lacZ* (P1 :: *lacZ*, Up1 :: *lacZ* and Up3 :: *lacZ*) and then inserted into the bacterial chromosome of *P. aeruginosa* WT. The  $\beta$ -galactosidase activity was measured in cells grown in cho/NH<sub>4</sub> or suc/cho. The CbrB and NtrC binding sites are represented by arrows. The RpoN1 and RpoN2 binding sites are shown as small vertical lines. The average values of at least three independent experiments are shown. References: suc, succinate; NH<sub>4</sub>, ammonium chloride; cho, choline.

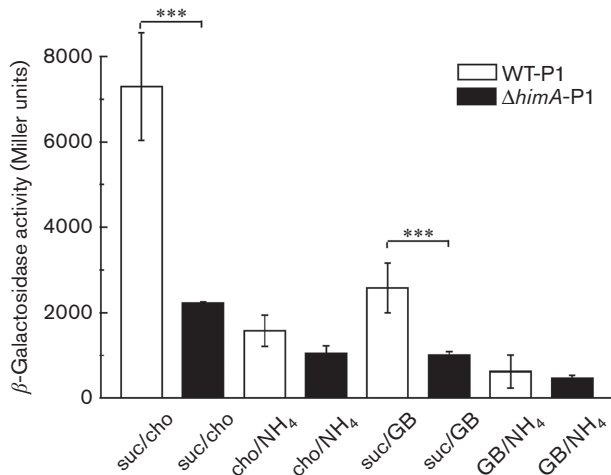
### BetI represses the expression of *gbdR*

Our findings that CbrB and NtrC were involved in the regulation of *gbdR* expression did not explain the high transcription level displayed in bacteria grown in the presence of cho compared with that for cells grown in other non-preferential sources of N or C as nitrate, GB, DMG or mannitol (Fig. 1). This prompted us to investigate whether cho by itself could act as an inductor or as a derepressor of *gbdR* transcription.

BetI has been described as a transcriptional repressor that controls the expression of *bet* genes [19, 23]. To evaluate whether BetI regulates *gbdR* expression, a  $\Delta betI$ -P1 mutant strain was constructed. Fig. 5(a) shows the reporter activity measured in WT-P1 and  $\Delta betI$ -P1 strains grown in either cho or nitrate as the N source, with succinate being added

as the C source. In the presence of cho,  $\beta$ -galactosidase activity was similar in both strains. However, when nitrate was the N source, the reporter activity in  $\Delta betI$ -P1 was higher ( $\approx 70\%$ ) than in WT-P1 bacteria. Interestingly,  $\Delta betI$ -P1 mutant grown in either suc/cho or suc/nitrate media displayed similar transcriptional activity (Fig. 5a).

Two putative BetI binding sites were identified in the *gbdR* promoter: Bet-C2 <sup>-158</sup>**TTTTGATTGA**\*ACGCCCAATC<sup>-138</sup> and Bet-C1 <sup>-153</sup>GATTGAACGC\*CCAATCAATA<sup>-134</sup> (conserved nucleotides are in contour and underlined for the proximal site and are in boldtype for the distal one) (Fig. 5b). Each site has two overlapped sequences of dyad symmetry, and the asterisks show the centres of symmetry [19, 24]. They were located at <sup>-158</sup>**TTTTGATTGA**ACGCCCAATCAA<sup>-136</sup> from the ATG start codon of *gbdR* (Figs 5c and S1). To assess the relevance of BetI binding sites in *gbdR* expression, we



**Fig. 4.** Expression of the *gbdR* gene in *P. aeruginosa* WT-P1 and  $\Delta$ himA-P1 strains. The strains were grown in HPI-BSM supplemented with 20 mM of cho or GB as the N source and succinate as the C source (suc/cho and suc/GB), or with cho or GB as the C source and ammonium as the N source (cho/NH<sub>4</sub> and GB/NH<sub>4</sub>). The average values of at least three independent experiments are shown. The error bars indicate standard errors. References: suc, succinate; NH<sub>4</sub>, ammonium chloride; cho, choline; GB, glycine betaine.

performed multiple nucleotide substitutions in the *gbdR* P1 promoter, changing both potential BetI binding sites (Fig. 5b). The fP1-BetI<sup>-</sup> fragment was fused to the *lacZ* gene and inserted into the chromosome of the WT strain (termed the P1-BetI<sup>-</sup> strain). The WT-P1 and P1-BetI<sup>-</sup> strains showed similar reporter activity when the cells were grown in suc/cho ( $\approx$ 7500 MU) or in cho/NH<sub>4</sub> ( $\approx$ 2000 MU). However, when cells grew in non-preferential C or N sources (different from cho), such as suc/nitrate, suc/GB or GB/NH<sub>4</sub>, the reporter activity in the P1-BetI<sup>-</sup> strain was at least 60% higher than in the WT-P1 strain (Fig. 5c). On the other hand, EMSA assays were performed to determine the binding of BetI to the *gbdR* promoter *in vitro*. Briefly, DNA fragments carrying the *gbdR* promoter, with and without the BetI binding site, were incubated with an increasing amount of purified 6-His-BetI protein. Accordingly, BetI only bound to the *gbdR* fragment that included the BetI binding site (Fig. 5d). Taken together, these results demonstrate that BetI regulates *gbdR* expression by direct interaction with its promoter, repressing *gbdR* transcription in the absence of cho.

## DISCUSSION

Choline constitutes an important substrate for *P. aeruginosa* adaptation to hyperosmotic environments and for the infection of different hosts. In the present work, we describe the transcriptional regulation of *gbdR*, the specific controller of cho catabolism. Our findings indicate that *gbdR* transcription can be initiated from a  $\sigma^{54}$ -dependent promoter. We show that *gbdR* expression is activated by CbrB when cho is the C source. Similarly, NtrC promotes *gbdR*

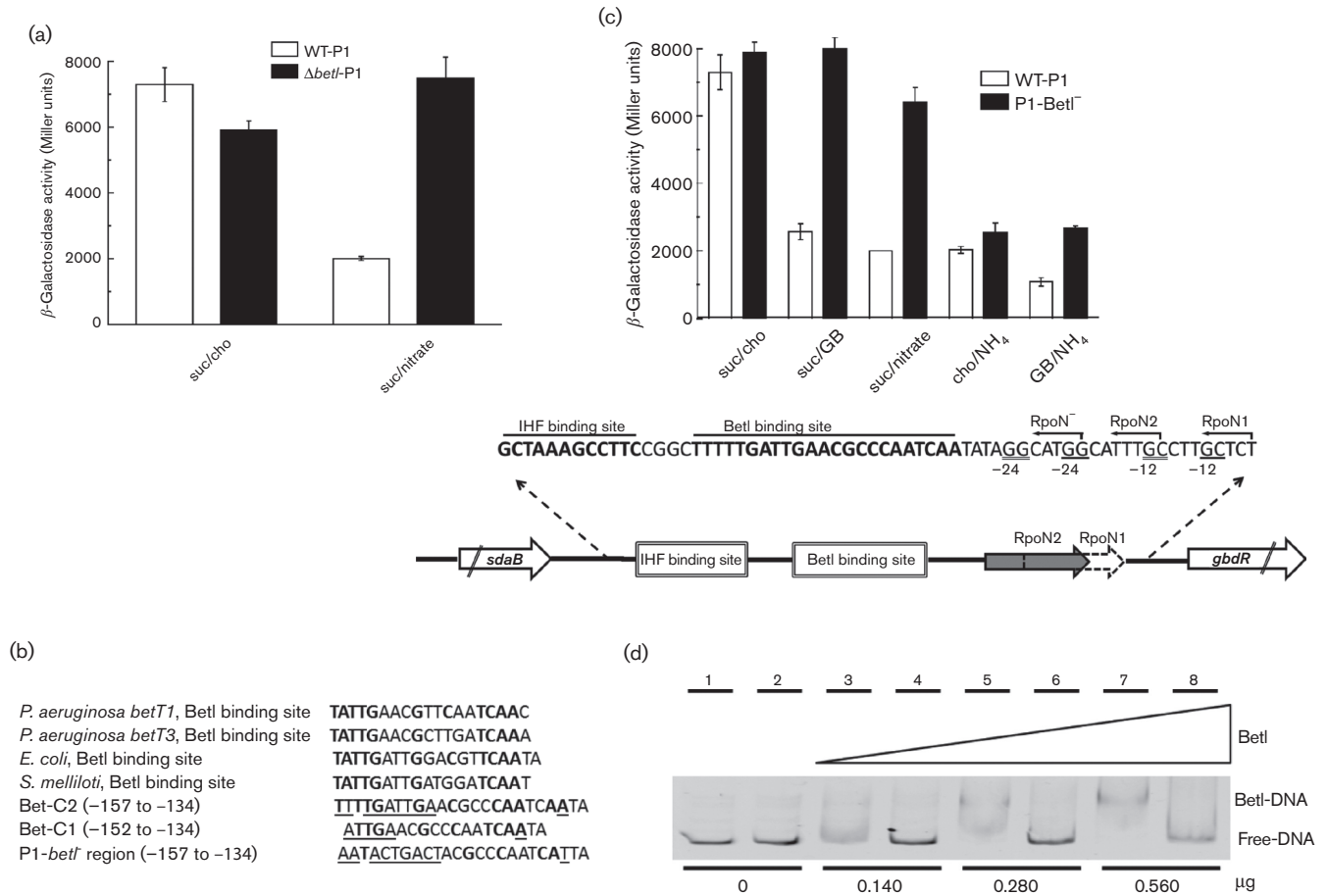
transcription when cho is used as the N source. In addition, IHF is required for NtrC-induced *gbdR* transcription. Interestingly, we demonstrate that BetI directly inhibits the expression of *gbdR* in the absence of cho.

The participation of NtrC and CbrB in the regulation of *gbdR* was assessed by physiological experiments (Fig. 1) and by measuring the reporter activity in the  $\Delta$ ntrC-P1 and  $\Delta$ cbrB-P1 strains (Fig. 3a). Furthermore, the direct participation of NtrC in *gbdR* regulation was demonstrated by the measurement of the reporter activity in WT-Up2 lacking the NtrC binding site (Fig. 3b). Although our results indicate that CbrB is required for *gbdR* transcriptional activation (Fig. 2a), it is possible that the CbrB binding site in the *gbdR* promoter may be dispensable (Fig. 3b). This fact is not surprising, since the association of EBP with an upstream activator sequence (UAS) is not a strict requirement for transcriptional activation [48, 49]. On the other hand, our data demonstrate that IHF plays an important role in the activation of *gbdR* by NtrC, as indicated by the strong decrease in *gbdR* transcription in the  $\Delta$ himA-P1 strain grown with cho as the N source (Fig. 4). The remaining expression of *gbdR* in the  $\Delta$ himA-P1 strain can be explained by additional mechanisms, such as intrinsic curvature or protein-protein interactions (Fig. 4). Further studies are needed to unravel the contribution of the CbrB binding site to the activation of *gbdR* transcription.

BetI binds to DNA as a dimer or tetramer perpendicularly to the longitudinal DNA axis [24, 25]. It regulates the transcription of *betA* and *betB* and other *bet* genes in several bacteria [19, 50, 51]. Basically, it inhibits the transcription of  $\sigma^{70}$ -dependent promoters by binding to the operator (-35 box) [24, 50]. On the other hand, there are few examples of  $\sigma^{54}$  promoters that are negatively regulated. It has been proposed that repression occurs via anti-activation mechanisms, or through the prevention of open complex formation by competition with  $\sigma^{54}$  for binding to its recognition elements [48, 52]. In the present work, we demonstrate that BetI binds directly to the *gbdR* promoter (Fig. 5d). Furthermore BetI, in the absence of cho, negatively regulates *gbdR* expression from an  $\sigma^{54}$ -dependent promoter (Fig. 5a, c). Further studies are currently being carried out in our laboratory to unravel the contribution of both  $\sigma^{54}$  promoters (RpoN1 and RpoN2) to the transcription of *gbdR*, and also their role in the mechanism of repression by BetI.

Our data show that *gbdR* synthesis is tightly regulated, which is similar to what occurs with many regulators of the AraC/XylS family. As an example, MarA, Rob and SoxS synthesis regulation is crucial, since these three regulators may bind to the same promoters, depending on their intracellular concentration and binding affinities [53–56]. Taking into account our findings and the fact that a crucial point in the regulation of microbial genes is the supply of RNAPol, we have proposed a model to explain how different levels of *gbdR* expression could turn cho catabolism on and off (Fig. 6). In the presence



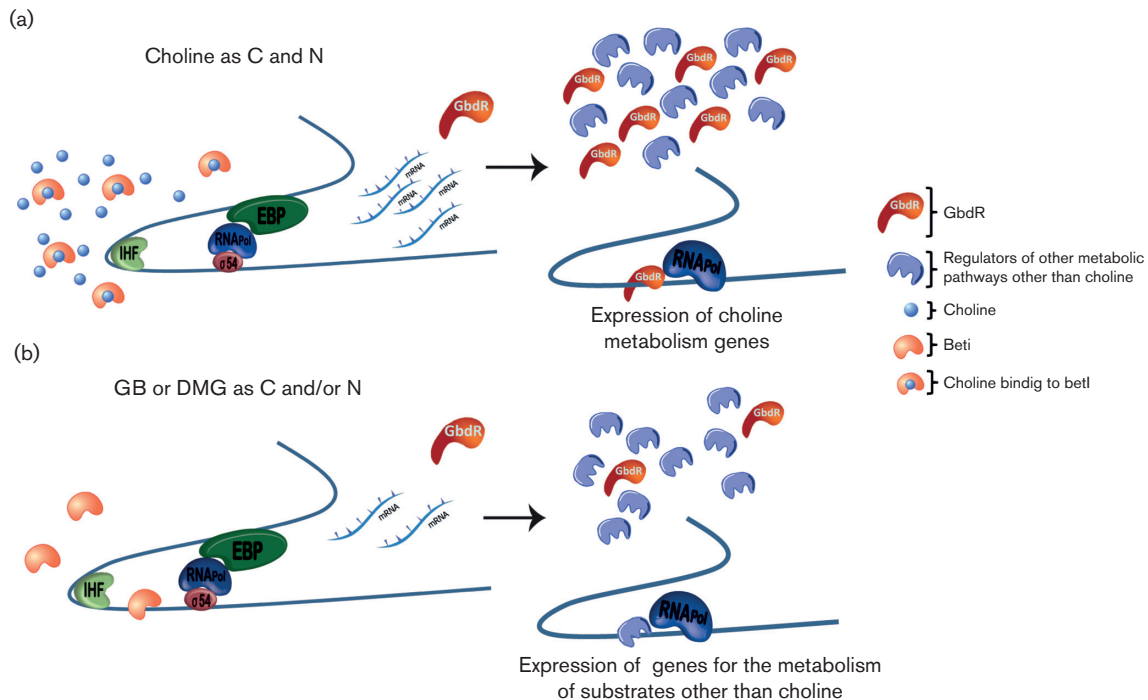


**Fig. 5.** The role of BetI as a repressor of *gbdR* expression in the absence of choline. (a) The results from the  $\beta$ -galactosidase assay in the WT-P1 and  $\Delta betI$ -P1 strains grown in HPI-BSM supplemented with suc/cho or suc/nitrate, as indicated in the figure. (b) Identification, by visual inspection, of the BetI binding site in the *gbdR* promoter and comparison with other previously described BetI binding sites [19, 23]. Conserved nucleotides that match with the BetI binding site are in bold type. The conserved nucleotides of the BetI binding site of the *gbdR* promoter in the P1 fragment were removed and changed by site-directed mutagenesis (underlined). (c)  $\beta$ -galactosidase activity in WT-P1 and P1-*BetI*<sup>-</sup> strains grown in HPI-BSM supplemented with suc/cho, suc/GB, suc/nitrate, cho/NH<sub>4</sub> or GB/NH<sub>4</sub>, as indicated in the figure, and a schematic diagram of the promoter showing the nucleotide sequences between -111/-175 bp upstream of the ATG start codon of the *gbdR* gene with the putative BetI and IHF binding sites. (d) Binding of BetI to *gbdR* promoter region. DNA fragments of 550 bp (100 ng) that include the *gbdR* promoter region were amplified by PCR from the WT-P1 and  $\Delta betI$ -P1 strains. DNA fragments with (lines 1, 3, 5 and 7) and without (lines 2, 4, 6 and 8) betI binding site consensus were incubated with increasing amounts of purified BetI protein for 20 min at room temperature. Subsequently, samples were separated by native polyacrylamide (6%) gel electrophoresis and DNA was labelled with Gel Green stain. The average values of at least three independent experiments are shown in (a) and (b). The error bars indicate standard errors. References: suc, succinate; NH<sub>4</sub>, ammonium chloride; cho, choline; GB, glycine betaine.

of cho, GB or other C or N sources, different regulators compete for the available RNAPol. Therefore, a greater abundance of GbdR favours the transcription of its regulon and eventually, cho catabolism. In this way, the level of GbdR could determine whether the cho catabolic machinery is expressed and at what magnitude. Moreover, the highest *gbdR* expression reached in bacteria grown in enriched cho media may be due to an evolutionary process in response to the higher bioavailability of cho compared with GB or DMG. In fact, cho is ubiquitous in nature, in many cases as part of a large number of more

complex molecules that are abundant in higher organisms (such as acetylcholine, lysophosphatidylcholine, etc.).

Overall, the metabolism of cho for use as an osmoprotectant or as a C, N and energy source involves two associated processes that are highly regulated. Our findings link these two processes through the regulation of *gbdR* by BetI. In addition, this work contributes to our understanding of how *gbdR* expression could determine whether bacteria in a complex nutritional medium prefer cho as a source of C and/or N.



**Fig. 6.** Scheme of putative *gbdR* regulation in bacteria grown in a complex medium without preferential C and/or N sources. (a) In the presence of cho as the N source, *gbdR* is expressed abundantly and competes strongly for the available RNApol, resulting in the induction of a choline catabolic pathway. (b) In the case where GB or DMG is present in a complex medium, *gbdR* is expressed moderately; consequently, GbdR is less competitive for RNApol. As a result, the choline catabolic pathway could be less favoured than the catabolism of other substrates.

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#### Conflicts of interest

The authors declare that there are no conflicts of interest.

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