**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 σ^54^-dependent promoter, *gbdR* transcription can be activated by NtrC 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 σ^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 hyposomatic 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 phosphorylcholine, 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...
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 σ54-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 (µg 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 Kit (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⁻ 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⁻ (P1-Up/DnRpoN⁻), as indicated in Table S1 (available in the online Supplementary Material). The PCR products were digested with SpeI and Xhol, 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⁻, respectively. Each of these plasmids and pTNS2 were co-transformed into *P. aeruginosa* by electroperoration [38], and the resulting strains containing the integrated DNA fragments were termed WT-P1, WT-Up1, WT-Up2, WT-Up3 and WT-RpoN⁻. Colony PCR using the primers Pt7R and Pgms-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⁻, R-Beti⁻ and P1-Dn (Table S1) were employed for BetI consensus substitution in the gbdR promoter, obtaining the fragment fP1-Beti⁻, which was cloned in the pUC18-mini-Tn7T-Gm-lacZ vector between the SpeI and Xhol restriction sites to generate the vector pP1-Beti⁻. To be sure that no errors were introduced during the PCR or subcloning 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* PA01 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 µg ml⁻¹ ampicillin at 37 °C and shaken at 250 r.p.m. until it reached OD₅₅₀ ≈ 0.6–0.7. Protein
Table 1. Bacterial strains and plasmids

<table>
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<tr>
<th>Strains</th>
<th>Description</th>
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<tbody>
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<td></td>
</tr>
<tr>
<td>XL10-Gold</td>
<td>Tet^R Δ(mcrA) 183 Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lacI121 (F' proAB lacIqZ ΔM15 Tn10(Tet^R) Amy Cam^R)</td>
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</tr>
<tr>
<td>S17-1 λ pir</td>
<td>pro thi5ΔR70 'Ts'::Sm^R, chromosome::RP4-2 Tc::Mu-Kan::Tn7/hpir</td>
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<tr>
<td>ccdBSurvival</td>
<td>F mcrA Δ(mrr-hsdRS-mcrBC) Φ80lacZAM15 ΔlacX74 recA1 araΔ139 Δ(ara-leu)7697 galU galK rpsL (Str^R) endA1 nupG nhaA::IS2</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>PAO1</td>
<td></td>
</tr>
<tr>
<td>PAO1-WT</td>
<td>Prototrophic wild-type strain</td>
<td></td>
</tr>
<tr>
<td>WT-P1</td>
<td>PAO1 with a chromosomal integration of mini-Tn7T carrying the translational fusion fP1::lacZ</td>
<td>This study</td>
</tr>
<tr>
<td>WT-Up1</td>
<td>PAO1 with a chromosomal integration of mini-Tn7T carrying the translational fusion fUp1::lacZ</td>
<td>This study</td>
</tr>
<tr>
<td>WT-Up2</td>
<td>PAO1 with a chromosomal integration of mini-Tn7T carrying the translational fusion fUp2::lacZ</td>
<td>This study</td>
</tr>
<tr>
<td>WT-Up3</td>
<td>PAO1 with a chromosomal integration of mini-Tn7T carrying the translational fusion fUp3::lacZ</td>
<td>This study</td>
</tr>
<tr>
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<td>PAO1 with a chromosomal integration of mini-Tn7T carrying the translational fusion fP1-Bet^-::lacZ</td>
<td>This study</td>
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<td>This study</td>
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<td>ΔrpoN</td>
<td>PAO1 ΔrpoN</td>
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<td>ΔcbrB</td>
<td>PAO1 ΔcbrB</td>
<td>[26]</td>
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<td>This study</td>
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<tr>
<td>ΔntrC</td>
<td>PAO1 ΔntrC</td>
<td>[26]</td>
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<td>PAO1 Δbet1</td>
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<tr>
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<td>ΔhimA</td>
<td>ΔhimA-P1</td>
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<td>ΔhimA-P1</td>
<td>ΔhimA-P1 with a chromosomal integration of mini-Tn7T carrying the translational fusion fP1::lacZ</td>
<td>This study</td>
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Plasmids

<table>
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<tr>
<th>Plasmids</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pUC18-mini-Tn7T-Gm-lacZ</td>
<td>Gm^R on mini-Tn7T; lacZ transcriptional fusion vector</td>
<td>[38]</td>
</tr>
<tr>
<td>pTNS2</td>
<td>Ap^R; helper vector encoding the site-specific Tn7 transposition pathway</td>
<td>[38]</td>
</tr>
<tr>
<td>pFPL2</td>
<td>Ap^R; Flp recombinase-encoding vector</td>
<td>[38]</td>
</tr>
<tr>
<td>pEX18ApGW</td>
<td>Ap^R; gene replacement vector, compatible with the gateway system</td>
<td>[40]</td>
</tr>
<tr>
<td>pP1</td>
<td>Gm^R, Ap^R; pUC18-mini-Tn7T-Gm-lacZ harbouring gbdR promoter sequences fP1 introduced using SpeI and Xhol sites</td>
<td>This study</td>
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<tr>
<td>pUp1-3</td>
<td>Gm^R, Ap^R; pUC18-mini-Tn7T-Gm-lacZ harbouring gbdR promoter sequences fUp1-Up3 introduced using SpeI and Xhol sites</td>
<td>This study</td>
</tr>
<tr>
<td>pP1-Bet^-</td>
<td>Gm^R, Ap^R; pUC18-mini-Tn7T-Gm-lacZ harbouring gbdR promoter sequences fP1-Bet^- introduced using SpeI and Xhol sites</td>
<td>This study</td>
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<tr>
<td>pRpoN^-</td>
<td>Gm^R, Ap^R; pUC18-mini-Tn7T-Gm-lacZ harbouring gbdR promoter sequences fRpoN^- introduced using SpeI and Xhol sites</td>
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<td>pRpoN2</td>
<td>Gm^R, Ap^R; pUC18-mini-Tn7T-Gm-lacZ harbouring gbdR promoter sequences fRpoN2 introduced using SpeI and Xhol sites</td>
<td>This study</td>
</tr>
<tr>
<td>pET-Bet1</td>
<td>Ap^R; pET15b harbouring the betl gene</td>
<td>This study</td>
</tr>
</tbody>
</table>

synthesis was 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-nitrioltriacetic 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-Bet^- (lacking Betl binding site consensus) were used as templates for PCR amplification with the primers: Up: CCTGGTGCTACCGAAATG and Dn:...
AGCAAGGGCAATGCCATGCC. 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 MgCl₂; 0.1 ng ml⁻¹ 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/NH₄) 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, NH₄ was added as the N donor (cho/NH₄, GB/NH₄, DMG/NH₄). 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/NH₄ (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/NH₄; (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/NH₄). 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 OD₆00 ≥ 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/NH₄ 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; NH₄, ammonium chloride; cho, choline; GB, glycine betaine; DMG, dimethylglycine.](image-url)
**gbdr gene regulation by a σ54-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 620bp 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 (\(1^{−132}\)GGCATG/TTGGC/TTGC\(^{−113}\)). We termed them 'proximal' or 'RpoN1' (\(1^{−132}\)GGCATG/TTGGC/TTGC\(^{−113}\)) and 'distal' or 'RpoN2' (\(1^{−132}\)GGCATGN/TTGC\(^{−113}\)) \(σ^{54}\)-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 \(ΔrpoN\)-P1 mutant strain (Table 1). The results obtained with this mutant were compared with WT-P1 bacteria. The \(β\)-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\(^{−}\) 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\(^{−}\) cells grown with cho as the C or N source was similar (\(500 \text{ MU}\)) to the basal activity (WT-P1 grown in suc/NH\(_4\)). 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 \(ΔrpoN\)-P1, WT-RpoN\(^{−}\) and RpoN2 strains indicated that the gbdR promoter belongs to the class of promoters that depend on the alternative \(σ^{54}\) factor.

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

The presence of the \(σ^{54}\) 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 \(^{−357}\)CGTGTCGT/C\(^{−338}\) (the nucleotides in bold match the CbrB consensus), similarly to the consensus site \(cTGTACcN_{3/12}\) cGTACAG reported in [42]. In addition, we found an NtrC binding site at position \(^{−391}\)GGCCACN\(_5\) GGCGCA\(^{−374}\) (the nucleotides in bold match the NtrC consensus), in agreement with the data described for the NtrC consensus in *P. putida* (CGCAC-N\(_5\)-GGTGC) [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 ΔcbrB and ΔntrC *P. aeruginosa* mutants, giving rise to the ΔcbrB-P1:: lacZ and ΔntrC-P1:: lacZ strains, respectively (henceforth termed the ΔcbrB-P1 and ΔntrC-P1 strains). The results obtained with the mutant strains were compared with those for the WT-P1 strain. The \(β\)-galactosidase activity was determined in cells grown in HPi-BSM medium supplemented with suc/NH\(_4\) (control), with cho as the C source (cho/NH\(_4\)) or with cho as the N source (suc/cho). As shown in Fig. 3(a), the different strains grown in suc/NH\(_4\) displayed similar reporter activity. In contrast, the reporter activity was markedly reduced (\(70\%\)) in Δcbrb-P1 cells (729±189 MU) when cho was the C source compared to that in the WT-P1 or Δntrc-P1 cells (\(2400 \text{ MU}\)) grown in suc/cho compared with that in WT-P1 cells (7838±497 MU). A functional and efficient interaction of 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\(_4\) (\(1691±143\) versus 1836±183 MU, respectively) or in suc/cho media (\(7642±461\) versus \(8071±824\) MU, respectively). Interestingly, when it was cultured in suc/cho media, WT-Up2 reporter activity was strongly reduced (\(90\%\)) compared with that for WT-P1 bacteria grown in similar culture conditions. However, in WT-Up2 cells grown in cho/NH\(_4\), the reporter activity was similar or slightly reduced when compared with that for the WT-P1 strain (1253±166 versus 1691±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\(_4\) (\(500 \text{ MU}\)). A functional and efficient interaction of \(σ^{54}\)-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
analysis revealed a potential IHF-binding site located at \(-175\) GCTAAAN\(_4\)TCC\(^{-163}\) 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\(_4\)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\(_4\). 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 =70 and =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\(_4\) or GB/NH\(_4\) media, the reporter activity of the mutant strain was reduced by =35 and =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.
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 ΔbetI-P1 mutant strain was constructed. Fig. 5(a) shows the reporter activity measured in WT-P1 and ΔbetI-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, β-galactosidase activity was similar in both strains. However, when nitrate was the N source, the reporter activity in ΔbetI-P1 was higher (>70%) than in WT-P1 bacteria. Interestingly, ΔbetI-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 \[\text{CTTTGATTGA*AGGCCCAATC}^{138}\] and Bet-C1 \[\text{GATTGAAGCC*CCAATCAATA}^{134}\] (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
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 ς^{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 ΔntrC-P1 and ΔcbrB-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 ΔhimA-P1 strain grown with cho as the N source (Fig. 4). The remaining expression of gbdR in the ΔhimA-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 ς^{70}-dependent promoters by binding to the operator (−35 box) [24, 50]. On the other hand, there are few examples of ς^{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 ς^{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 ς^{54}-dependent promoter (Fig. 5a, c). Further studies are currently being carried out in our laboratory to unravel the contribution of both ς^{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 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 Betl. 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.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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