The *Pseudomonas aeruginosa* *rmlBDAC* operon, encoding dTDP-L-rhamnose biosynthetic enzymes, is regulated by the quorum-sensing transcriptional regulator RhlR and the alternative sigma factor $\sigma^S$.

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*Pseudomonas aeruginosa* produces as biosurfactants rhamnolipids, containing one (mono-rhamnolipid) or two (di-rhamnolipid) L-rhamnose molecules. The rhamnosyltransferase RhlB catalyses the synthesis of mono-rhamnolipid using as precursors dTDP-L-rhamnose and 3-(3-hydroxyalkanoyloxy)alkanoic acids (HAAs) produced by RhlA, while the rhamnosyltransferase RhlC synthesizes di-rhamnolipid using mono-rhamnolipid and dTDP-L-rhamnose as substrates. The Las and Rhl quorum-sensing systems coordinately regulate the production of these surfactants, as well as that of other exoproducts involved in bacterial virulence, at the transcriptional level in a cell density-dependent manner. In this work we study the transcriptional regulation of the *rmlBDAC* operon, encoding the enzymes involved in the production of dTDP-L-rhamnose, the substrate of both rhamnosyltransferases, RhlB and RhlC, and also a component of *P. aeruginosa* lipopolysaccharide. Here we show that the *rmlBDAC* operon possesses three promoters. One of these transcriptional start sites (P2) is responsible for most of its expression and is dependent on the stationary phase sigma factor $\sigma^S$ and on RhlR/C4-HSL through its binding to an atypical ‘las box’.

**INTRODUCTION**

*Pseudomonas aeruginosa* is an opportunistic human pathogen that causes serious nosocomial infections. The secretion of numerous toxic compounds and hydrolytic enzymes that are involved in its pathogenicity is coordinately regulated at the transcriptional level by the so-called quorum-sensing response (QSR) (von Bodman *et al.*, 2008; Williams & Câmara, 2009). These exoproducts include proteases, phospholipase C, exotoxins, pyocyanin and rhamnolipids (Lazdunski *et al.*, 2004; Williams & Câmara, 2009).

In liquid culture, *P. aeruginosa* mainly produces two forms of rhamnose-containing glycolipid biosurfactant: rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate (mono-rhamnolipid) and rhamnosyl-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate (di-rhamnolipid). The biosynthesis of these tensio-active molecules proceeds by two sequential rhamnosyl transfer reactions, each catalysed by a specific rhamnosyltransferase (RhlB and RhlC, respectively), with deoxythymidine diphospho-L-rhamnose acting as rhamnosyl donor in both reactions and 3-(3-hydroxyalkanoyloxy)alkanoic acids (HAAs), the product of RhlA catalysis (Déziel *et al.*, 2003; Cabrera-Valladares *et al.*, 2006), or mono-rhamnolipid acting as the respective recipients. The general biosynthetic pathway of rhamnolipids has been reviewed (Soberón-Chávez *et al.*, 2005b; Müller & Hausmann, 2011), and evidence of their role as virulence factors has been published (Zulianello *et al.*, 2006).

Besides being a rhamnolipid precursor, dTDP-L-rhamnose is a lipopolysaccharide (LPS) precursor in a variety of bacteria, including *P. aeruginosa* (Rahim *et al.*, 2000). This activated sugar has been implicated in bacterial pathogenicity (Ma *et al.*, 1997, 2001; Bozue *et al.*, 2005; Engels-Deutsch *et al.*, 2003). The dTDP-L-rhamnose biosynthetic pathway consists of the conversion of glucose 1-phosphate to dTDP-L-rhamnose via dTDP-glucose, dTDP-6-deoxy-D-xylo-4-hexulose and dTDP-6-deoxy-D-xylo-4-hexulose.

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Abbreviations: C4-HSL, butanoyl-homoserine lactone; 3O-C12-HSL, 3-oxo-dodecanoyl homoserine lactone; RNAP, RNA polymerase; QS, quorum sensing; QSR, quorum-sensing response.

A supplementary table, showing oligonucleotides used in this study, is available with the online version of this paper.

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(Marumo et al., 1992). The enzymes in this pathway are highly conserved among bacteria and are absent from eukaryotic cells, thus making them potential targets for antibacterial drugs (Ma et al., 1997, 2001).

The transcriptional regulation of the genes encoding the enzymes directly involved in rhamnolipid synthesis has been reported. Both the rhlAB (Ochsner et al., 1994) and PA1131-rhlC (Rahim et al., 2001) operons are positively regulated by RhlR, which belongs to the family of LuxR transcription activators (Lazdunski et al., 2004; von Bodman et al., 2008; Williams & Câmara, 2009). The members of this family respond to the so-called bacterial autoinducers, N-acylated homoserine lactones that harbour acyl constituents of various lengths. These small signalling molecules accumulate and activate gene expression at high bacterial densities through the interaction with specific transcriptional activators.

RhlR activates gene expression when coupled with butanoyl-homoserine lactone (C₄-HSL) (Medina et al., 2003c) through binding to a specific DNA sequence. 'las boxes' are conserved DNA sequences recognized by RhlR or LasR, the second quorum sensing (QS) transcription activator present in P. aeruginosa (Whiteley & Greenberg, 2001). These sequences determine whether LasR, RhlR or both regulatory proteins regulate a gene, and subtle changes may represent a big difference in the regulatory protein binding affinity (Whiteley & Greenberg, 2001; de Kievit et al., 2002; Schuster & Greenberg, 2007). LasR is only able to dimerize and bind to its target DNA sequence in the presence of the autoinducer 3-oxo-dodecanoyl homoserine lactone (3O-C₁₂-HSL) (Kiratisin et al., 2002), while RhlR binds to rhlA las box in both the absence and the presence of C₄-HSL (Medina et al., 2003c). In the former case it functions as a repressor, while in the latter it activates transcription (Medina et al., 2003c).

After a mutagenesis screen for genes regulated by QS, it was postulated that genes activated only by LasR/3O-C₁₂-HSL possessed las boxes with the sequence NNCTNᵦAGNN, where nucleotides in bold type are invariable (Whiteley et al., 1999). However, it has been reported that las boxes with 11 nt instead of 12 nt between the invariable nucleotides are also functional and recognized by LasR (Medina et al., 2003a; Croda-García et al., 2011). It has also been proposed that genes regulated by RhlR and LasR possess the sequence NNCTNᵦANᵦTNᵦAGNN (Whiteley et al., 1999). These sequences are called las boxes because they bind both QS regulatory proteins, albeit with different affinities (Whiteley & Greenberg, 2001; Soberón-Chávez et al., 2005a). Following an in silico analysis of genes that seem to be directly regulated by RhlR, the existence was proposed of an RhlR-specific DNA-binding sequence (rhl box) with the sequence Nᵦ₂CTNCCAGNᵦ₂TTNGNAGN, where nucleotides in bold type are invariable (Schuster & Greenberg, 2007). However, no experimental evidence has been reported on the existence of an RhlR-specific box that lacks the conserved AG dinucleotide at positions 17 and 18.

In P. aeruginosa, the stationary phase sigma factor σ₂₅ has been shown to influence the expression of different QS-regulated traits, showing a very complex interaction with this system (Schuster et al., 2004a; Schuster & Greenberg, 2007). It has been reported that the genes that encode lectins have a σ₂₅-dependent promoter (Winzer et al., 2000), and that the transcription of the rhlAB promoter itself is partially dependent on this sigma factor (Medina et al., 2003b).

The enzymes involved in the dTDP-l-rhamnose biosynthetic pathway are encoded by rmlA, rmlB, rmlC and rmlD in P. aeruginosa (Rahim et al., 2000). These genes are arranged as an operon (rmlBDAC) (Rahim et al., 2000). Following transcriptome analysis, the rmlBDAC operon has been suggested to be induced by QS (Schuster et al., 2003), but this proposal has not been further addressed experimentally, and the precise mechanism of this regulation has not been reported.

Another P. aeruginosa gene involved in LPS biosynthesis, called migA, has been shown to be regulated by the QSR, specifically by RhlR (Yang et al., 2000), although the precise role of MigA in LPS formation has not been determined.

The aim of this work is the study of the transcriptional regulation of the rmlBDAC operon in a culture medium where high levels of rhamnolipids are produced, and to determine the role of the QSR in this regulation. We show that this operon has three promoters and that the expression of one of these (P₂) is dependent on the stationary phase sigma factor σ₂₅ and on the QS regulator RhlR. This promoter has an atypical las box (rmbB box) centred at −44.5 nt from the P₂ transcriptional start site, which is shown to be functional for RhlR/C₄-HSL binding and to be responsible for most of the transcriptional activation of this operon. We show that the rml box is a specific RhlR DNA-binding sequence (an rhl box), since it is not recognized by LasR/3O-C₁₂-HSL at a detectable level. This is believed to be the first reported case of a DNA sequence which is recognized solely by RhlR and not by LasR/3O-C₁₂-HSL.

**METHODS**

**Microbiological procedures.** Bacterial strains and plasmids used in this work are shown in Table 1. The sequences of the oligonucleotides used are shown in Supplemental Table S1. P. aeruginosa strains were grown on PPGAS (Zhang & Miller, 1992) or on Luria–Bertani (LB) (Miller, 1972) medium at 37 °C and 225 r.p.m. Antibiotic concentrations used were (in μg ml⁻¹): ampicillin (Ap) 200, carbenicillin (Cb) 200, gentamicin (Gm) 300 and tetracycline (Tc) 150. β-Galactosidase activity was determined as reported by Miller (1972). One Miller unit will convert 1 nanomole of ONPG hydrolysed per minute per OD₆₀₀ unit. IPTG was used at a concentration of 1 mM, while C₄-HSL and 3O-C₁₂-HSL were both used at 10 μM in the experiments presented in Table 2. Expression of plasmid pMPRML-3 in Escherichia coli was induced in the presence of 10 μM C₄-HSL or 1 μM 3O-C₁₂-HSL (Fig. 3).
Table 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Reference or source</th>
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<tr>
<td><strong>Strains</strong></td>
<td></td>
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<tr>
<td>PAO1</td>
<td><em>P. aeruginosa</em> wild-type strain</td>
<td>Hancock &amp; Carey (1979)</td>
</tr>
<tr>
<td>PAO1ArhlR</td>
<td>PAO1 derivative, <em>ArhlR</em>::Gm</td>
<td>Rahim et al. (2001)</td>
</tr>
<tr>
<td>PAS1</td>
<td>PAO1 derivative, <em>rpoS</em>::Gm</td>
<td>Medina et al. (2003b)</td>
</tr>
<tr>
<td>MC4100</td>
<td>E. coli, araD139 Δ(argF-lacU169) rpsL150 thiA1 relA1 fib5301 deoC ptsF25 rbsR</td>
<td>Casadaban (1976)</td>
</tr>
<tr>
<td>ABI-rhlA</td>
<td>MC4100 derivative with a chromosomal Φ(pZrhlA-lacZ)</td>
<td>This work</td>
</tr>
<tr>
<td>ABI-rmlB1</td>
<td>MC4100 derivative with a chromosomal Φ(pZrmlB1-lacZ)</td>
<td>This work</td>
</tr>
<tr>
<td>ABI-lasl</td>
<td>MC4100 derivative with a chromosomal Φ(pZlasI-lacZ)</td>
<td>This work</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
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<tr>
<td>pLP170</td>
<td>Transcriptional lacZ fusion vector that contains an origin of replication for both <em>E. coli</em> and <em>P. aeruginosa</em>; Ap&lt;sup&gt;T&lt;/sup&gt;</td>
<td>Pearson et al. (1997)</td>
</tr>
<tr>
<td>pRML-P1</td>
<td>pLP170 derivative with a rmlB-lacZ fusion containing the upstream tRNA gene with its promoter region as well as the complete 5' rmlB region; Ap&lt;sup&gt;T&lt;/sup&gt;</td>
<td>Cabrera-Valladares et al. (2006)</td>
</tr>
<tr>
<td>pRML-2</td>
<td>Deletion derivative of pRML-P1 containing the entire rmlB upstream region, starting at an internal position of the upstream tRNA gene, Ap&lt;sup&gt;T&lt;/sup&gt;. Oligonucleotides rmlB62 and rmlBm (Supplementary Table S1) were used to construct this plasmid</td>
<td>This work</td>
</tr>
<tr>
<td>pRML-3</td>
<td>Deletion derivative of pRML-P1 containing only the sequences of the rml box and the downstream sequences, Ap&lt;sup&gt;T&lt;/sup&gt;. Oligonucleotides rmlB62 and rmlBc (Supplementary Table S1) were used to construct this plasmid</td>
<td>This work</td>
</tr>
<tr>
<td>pRML-4</td>
<td>pUCP18 derivative encoding the rmlBDAC operon (6.3 kb) under its own promoter; Ap&lt;sup&gt;T&lt;/sup&gt;</td>
<td>Cabrera-Valladares et al. (2006)</td>
</tr>
<tr>
<td>pRML-P1(AA)</td>
<td>pRML-P1 derivative containing a mutation in the dinucleotide GC to AA at positions 3 and 4 of the rml box. Constructed with oligonucleotides lasI1AA and lasI1Aar (Supplementary Table S1)</td>
<td>This work</td>
</tr>
<tr>
<td>pRML-P1(CC)</td>
<td>pRML-P1 derivative containing a mutation (changing AA at positions −89 and −90 to CC) in the −10 sequence of promoter P2. Constructed with oligonucleotides P210CC and P210CC-r (Supplementary Table S1)</td>
<td>This work</td>
</tr>
<tr>
<td>pRML-2(CC)</td>
<td>pRML-2 derivative containing a mutation (changing AA at positions −151 and −150 to CC) in the −10 sequence of promoter P3. Constructed with oligonucleotides P310CC and P310CC-r (Supplementary Table S1)</td>
<td>This work</td>
</tr>
<tr>
<td>pMP220</td>
<td>Vector to construct transcriptional lacZ fusions, Tc&lt;sup&gt;T&lt;/sup&gt;</td>
<td>Spank et al. (1987)</td>
</tr>
<tr>
<td>pMPRML-3</td>
<td>pMP220 derivative with a rmlB-lacZ fusion containing the same insert as pRML-3, Tc&lt;sup&gt;T&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>pGMYC</td>
<td>pUCP20 derivative encoding RhlR, expressed under the lac promoter; Ap&lt;sup&gt;T&lt;/sup&gt;</td>
<td>Medina et al. (2003c)</td>
</tr>
<tr>
<td>pMT1</td>
<td>pUCP20 derivative encoding LasR, expressed under the lac promoter; Ap&lt;sup&gt;T&lt;/sup&gt;</td>
<td>Medina et al. (2003a)</td>
</tr>
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</table>

**Nucleic acid procedures.** DNA manipulations were done as reported by Sambrook & Russell (2001). DNA sequencing reactions were done using the Thermo Sequenase kit (Amersham Life Science). Oligonucleotides were radioactively labelled with T4 polynucleotide kinase (Amersham Life Science) using [γ-<sup>32</sup>P]ATP [3000 Ci mmol<sup>−1</sup>] (111 TBq mmol<sup>−1</sup>), Perkin Elmer Life Science) as substrate. The construction of a point mutation was done using a QuikChange II XL DNA Cycle Sequencing System (Promega) using pRML-4 (Cabrera-Valladares et al., 2006) as template.

**Primer extension analysis.** *P. aeruginosa* strains were grown to OD<sub>600</sub> 0.6 or 1.5 and their total RNA was extracted using an RNeasy kit (Qiagen). Primer extension reactions were done using the SuperScript III reverse transcriptase (Invitrogen) according to the manufacturer’s instructions. Reactions were carried out at 55 °C. The experiments were done using oligonucleotide rmlB·+87 (Supplementary Table S1). The sequencing reaction was done with a fmol DNA Cycle Sequencing System (Promega) using pRML-4 (Cabrera-Valladares et al., 2006) as template.

**Determination of RhlR DNA-binding ability.** To measure RhlR binding to different DNA sequences (las boxes) in vivo, we followed the procedure described previously (Croda-García et al., 2011) to construct hybrid promoters with consensus −10 and −35 RNA polymerase (RNAP) recognition sequences separated by 19 bp of the sequence corresponding to a las box. We used either an rhlA or a lasI las box as a control (Table 2).

Briefly, the procedure was as follows. Complementary oligonucleotides with synthentic promoter sequences and an rhlA, rmlB or lasI las box between the −10 and −35 RNAP recognition sites flanked by EcoRI and BamHI restriction sites, were hybridized to form a dsDNA fragment and cloned into plasmid pRS551 (Simons et al., 1987) to produce transcriptional lacZ fusions. These plasmid-encoded lacZ fusions were introduced into the *E. coli* MC4100 chromosome by the method of Simons et al. (1987). Plasmids encoding RhlR (pGMYC; Medina et al., 2003c) or LasR (pMT1; Medina et al., 2003a) were introduced into *E. coli* strains (ABI-rhlA, ABI-rmlB1 and ABI-lasl, Table 1) harbouring the chromosomally encoded lasZ fusions with the synthentic promoters. The extent of lacZ repression in the presence...
Table 2. Ability of RhlR and LasR to bind different DNA sequences, as determined by repression of the lacZ expression of synthetic promoters carrying the sequences shown below the −10 and −35 consensus RNAP binding sequences

Values shown are percentage repression, expressed using as 100% the β-galactosidase activity of the chromosomal synthetic lacZ fusion in the presence of the empty vector pUCP20. Plasmid pGMYC expresses the rhlR gene and pMTI expresses the lasR gene. Data shown are the mean from three independent experiments and enzymic activity was determined in triplicate. Bold type shows nucleotides which have been reported to be conserved in las boxes recognized by LasR and RhlR; italic type shows the atypical nucleotides in the rml box.

<table>
<thead>
<tr>
<th>Box</th>
<th>Sequence (5’-3’)</th>
<th>RhlR</th>
<th>LasR</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>C4-HSL (10 μM)</td>
<td>No C4-HSL</td>
</tr>
<tr>
<td>lasl</td>
<td>ATCTATCATCATTGCTAGTT</td>
<td>&gt;1</td>
<td>&gt;1</td>
</tr>
<tr>
<td>rhlA</td>
<td>TCCTGTGAATCTGGGAGTT</td>
<td>50 ± 1.5</td>
<td>50 ± 1.5</td>
</tr>
<tr>
<td>rml</td>
<td>ACCTACCAGATCTGGGTTG</td>
<td>50 ± 5</td>
<td>50 ± 5</td>
</tr>
</tbody>
</table>

of RhlR, RhlR/C4-HSL or LasR/3O-C12-HSL corresponds to the binding of these proteins with or without their corresponding autoinducers (Table 2).

RESULTS AND DISCUSSION

Transcriptional regulation of the rmlBDAC operon

P. aeruginosa produces high rhamnolipid levels, starting at the early stationary phase when cultivated under phosphate-limited conditions (Zhang & Miller, 1992). This increased production is due to an increase in RhlR-dependent rhlAB and rhlC transcription (Ochsner et al., 1994; Rahim et al., 2001; Medina et al., 2003c). A positive autoregulatory loop regulates RhlR expression from the rhlAB promoter (Croda-Garcia et al., 2011). To determine whether rhamnolipid production was accompanied by an increase in the synthesis of dTDP-L-rhamnose, we measured the expression of the rmlBDAC operon using an rmlB-lacZ transcriptional fusion (pRML-P1, Table 1) during growth of strain PAO1 on phosphate-limited PPGAS medium. The results showed that rmlBDAC expression and presumably dTDP-L-rhamnose synthesis were high throughout the P. aeruginosa growth curve, and that they were further induced at the start of the stationary phase (Fig. 1a). The dTDP-L-rhamnose synthesized during the exponential phase of growth is presumably used in LPS synthesis, while the induction of rmlBDAC transcription coincides with rhamnolipid biosynthesis.

Using primer extension analysis of the rmlBDAC upstream region we detected two transcription initiation sites (Fig. 2a). One of these promoters (P1), located at −28 nt from the translational start site (Fig. 2b), seems to be expressed at a low level (Fig. 2a) and has the characteristics of promoters expressed by RNAP containing a σ54 subunit, since it has the sequence 5’-GGGACCTGTGCTGAGC-3’, where nucleotides in bold type correspond to typical −24 (GG) and −12 (GC) dinucleotides, present on promoters transcribed by RNAP containing this alternative sigma factor (Fig. 2b). Upstream of this putative σN promoter, we identified sequences compatible with FleQ and integration host factor (IHF) binding (data not shown). The functionality of these factors for P1 expression was not further investigated. The other transcriptional start site detected by primer extension (P2), located at −81 nt from the translational start site (Fig. 2a, b), seems to be responsible for most of the transcription of the rmlBDAC operon (Fig. 1a), as it shows a high level of expression (Fig. 2a).

The putative −10 (5’-TACTAA-3’) and −35 (5’-TTGGCA-3’) sequences corresponding to the P2 promoter are separated by 18 bp (Fig. 2b). Adjacent to the P2 promoter and centered at −44.5 bp from this start site we detected a sequence resembling a las box, but with an atypical sequence (5’-ACCTACGATCTGGGTTG-3’) that did not contain the AG sequence at positions 17 and 18 that has been reported to be invariable for LasR and RhlR recognition sequences (Whiteley & Greenberg, 2001; Whiteley et al., 1999). The functionality of this atypical las box is addressed below.

Transcription of the rmlBDAC operon from the P2 start site is RhlR- and σ54-dependent

To determine whether rmlBDAC expression was RhlR-dependent, we measured the expression of the rmlB-lacZ fusion encoded on plasmid pRML-P1 in the PAO1ΔrhlR mutant. We found that in this mutant the rmlBDAC operon was still expressed at a constitutive level throughout the growth curve, but that the induction of its expression at the beginning of the stationary phase was lost (Fig. 1a). The analysis of the rmlBDAC transcriptional start sites in the PAO1ΔrhlR mutant showed that the P2 promoter was dependent on RhlR activity (Fig. 2a). These results suggest that the induction of the expression of the rmlBDAC operon in early stationary phase is dependent on RhlR through its activation of the P2 promoter.

Promoters that contain 18 bp between the −10 and −35 sequences for the recognition of RNAP have been shown in E. coli to be preferentially transcribed by RNAP containing a σ54 subunit (TYPAS & HENGGE, 2006). To determine whether P2 expression was transcribed by RNAP containing this alternative sigma subunit we determined the
expression of the rmlB–lacZ fusion encoded in plasmid pRML-P1 in a PAO1-derived rpoS mutant (PAS1, Table 1). We found that the kinetics of rmlBDAC expression were very similar in the PAS1 (rpoS mutant) and PAO1ΔrhlR mutants (Fig. 1a), and that a P2 transcriptional start site could not be detected in either mutant (Fig. 2a). These results show that P2 promoter expression is both RhlR- and RpoS-dependent. It is possible that we were unable to detect the residual expression of the P2 promoter in exponential phase in the PAS1 and PAO1ΔrhlR mutants using primer extension analysis.

Identification of the rml box as an RhlR-specific binding site (rhl box)

The data regarding the RhlR-dependent expression of the P2 promoter suggest that the rml box is functional, since it is positioned in the typical place where this transcriptional regulator binds to promoter sequences (~44.5 nt from the transcription start site) (Fig. 2b). To test this hypothesis we constructed a lacZ transcriptional fusion which started immediately upstream of the rmlB-las box (pRML-3, Table 1), eliminating 494 bp from the 885 bp insert of plasmid pRML-P1. The lacZ fusion encoded on plasmid pRML-3 showed the same pattern of expression as that encoded on plasmid pRML-P1 in PAO1 and its rhlR mutant derivative, albeit at a lower level (Fig. 1b), showing that the insert in this plasmid contains the sequences required for RhlR-dependent expression.

To further confirm the functionality of the rml box for RhlR binding and P2 promoter activation we used several different strategies.

First we constructed an rml box mutant derivative of plasmid pRML-P1, changing the CG to AA at positions 3 and 4 of the box [plasmid pRML-P1(AA), Table 1], and found that RhlR-dependent induction of the lacZ fusion encoded on this plasmid was completely abolished (Fig. 1c), suggesting that this sequence is functional for RhlR binding and activation of rmlBDAC transcription from the P2 promoter.

To determine RhlR binding to the rml box and to further confirm its ability to activate transcription from the P2 promoter, we measured the expression of different lacZ fusions in an E. coli background. The functionality of the rml box for RhlR binding in vivo was determined using an artificial lacZ fusion inserted in the E. coli MC4100 chromosome. This artificial lacZ fusion was constructed by inserting the 19 nt sequence of this putative RhlR-binding site (rml box, Table 2) between −210 and −35 RNAP consensus binding sequences. Using this construct we were able to measure the binding of a protein to the DNA sequence between the RNAP binding sequences by repression of lacZ expression. In this experiment we used as a positive control the las box of rhlA, which is preferentially bound by RhlR, both in the presence and in the absence of C4-HSL [in the former case RhlR activates transcription, while in the latter case it acts as a repressor (Medina et al., 2003b)]. As a negative control for RhlR binding and control of binding specificity, we used the

Fig. 1. Kinetics of rmlBDAC operon expression during growth of P. aeruginosa PAO1 and its mutant derivatives cultured on PPGAS medium. (a, b) Results obtained with plasmids pRML-P1 and pRML-3, expressed in the PAO1 wild-type strain and its mutant derivatives. Open symbols, OD600 values; closed symbols, β-galactosidase activity. Circles, PAO1 wild-type strain; squares, rhlR mutant derivative (PAO1ΔrhlR); triangles, PAO1 rpoS mutant PAS1. (c) Expression of plasmid pRML-P1 and its mutant derivatives pRML-P1(AA) and pRML-P1(CC) in P. aeruginosa strains at different time points. Black bars, plasmid pRML-P1 expressed in PAO1; grey bars, plasmid pRML-P1(AA), which inactivates the RhlR-binding site in the wild-type strain; white bars, the same plasmid in the PAO1ΔrhlR mutant; hatched bars, plasmid pRML-P1(CC), which carries mutations inactivating the −10 sequences of the P2 promoter on PAO1.
DNA sequence of the lasI las box, which is specifically bound by LasR/3O-C₁₂-HSL (Schuster et al., 2004b). The results obtained clearly showed that RhlR is able to bind to the rml box both in the presence and in the absence of C₄-HSL, as it does to the rhlA las box, and that LasR/3O-C₁₂-HSL is not able to bind to this sequence at a detectable level (Table 2).

The ability of LasR/3O-C₁₂-HSL to activate rhlA transcription in the *E. coli* background, albeit at a reduced level compared with the transcription of this gene by RhlR/C₄-HSL (Medina et al., 2003c), shows that LasR is able to bind to this sequence to some extent. To determine whether the rml box was also recognized by LasR/3O-C₁₂-HSL to some extent, we expressed plasmid pMPRML-3, which contains the same insert as plasmid pRML-3, but in a different vector plasmid (Table 1, Fig. 3), in the *E. coli* background in the presence of plasmids encoding genes expressing either RhlR (pGMYC, Table 1) or LasR (pMT1, Table 1), and by adding their corresponding autoinducers. The results showed that RhlR/C₄-HSL activates rmlB transcription, while LasR/3O-C₁₂-HSL has no activity towards this promoter (Fig. 3).

**Fig. 2.** Characterization of the transcriptional arrangement of the *P. aeruginosa* PAO1 rmlBDAC operon. (a) Identification of the transcription start sites by primer extension analysis of the rmlBDAC upstream region in the PAO1 (P) genetic background and in its rhlR (R) and rpoS (S) derived mutants (PAO1ΔrhlR and PAS1, respectively, Table 1). Lanes: 1, RNA purified from cells in the exponential phase (OD₆₀₀ 0.6); 2, RNA start sites obtained with RNA purified from PAO1 cells in early stationary phase (OD₆₀₀ 1.5). The DNA ladder used to obtain the DNA sequence is shown. (b) Schematic representation of the rmlBDAC promoter region, showing the three promoters and the rml box (bold type) described in this work. The transcription start sites (P₁, P₂ and P₃) are shown by arrows and the corresponding RNAP recognition sequences (−₁₀ and ₃₅, or −₁₂ and ₄₆) are underlined. A question mark next to P₃ denotes that this start site was not detected by primer extension and thus that its location is not certain.

**Fig. 3.** Expression of the rmlBDAC operon in an *E. coli* background. White bars, plasmid pMPRML-3 in the presence of plasmid pGMYC encoding RhlR, with the addition of 10 μM C₄-HSL (C₄); black bars, pMT1 encoding LasR, with the addition of 3O-C₁₂-HSL (C₁₂). ⌐, No addition.
Taken together these results show that the rmlBDAC operon is specifically activated by RhlR/C4-HSL and that LasR/3O-C12-HSL does not recognize the rml box.

**The P2 rmlB promoter shows some RhlR- and σ5-independent expression**

The expression of the lacZ fusion encoded on plasmid pRML-P1 in the PAO1ΔrhlR mutant showed a high level of expression throughout the growth curve (Fig. 1a), which was similar to that shown by the lacZ fusion encoded on plasmid pRML-P1(AA) in this mutant background (Fig. 1c). To determine whether the basal level of expression, which was independent of RhlR, was dependent on the P2 promoter, we constructed a mutant in plasmid pRML-P1 that inactivated the −10 region of this promoter [pRML-P1(CC), Table 1]. This mutation reduced rmlB::lacZ expression in the PAO1 background to much lower levels than the mutation that inactivates the rml box (Fig. 1c). This result strongly suggests that the rmlBDAC P2 promoter contributes most of the expression of this operon and that its dependence on RhlR and σ5 for activation is not absolute.

**The P3 start site contributes to rmlBDAC activation only under conditions where RhlR is not functional**

*In silico* analysis of the rmlB upstream region revealed the presence of a putative promoter (P3, Fig. 2b) at approximately −140 nt from the translational start site (the putative −10 region of this promoter spans between −150 and −156, and the −35 region between −171 and −176, Fig. 2b). To test the functionality of this putative promoter we constructed a mutation to change the AA dinucleotide at positions −152 and −151 to CC, thus inactivating the putative −10 RNAP recognition sequence [plasmid pRML-2(CC), Table 1]. This mutation was constructed in plasmid pRML-2, which is a pRLM-P1 deletion derivative with a 550 bp insert containing the whole 5′ rmlB untranslated region and a truncated upstream tRNA gene (Table 1).

We found that the lacZ fusion encoded on plasmid pRML-2 showed a similar pattern of expression to that of pRML-P1 both in PAO1 and the PAO1ΔrhlR mutant (Fig. 4), and that inactivation of the −10 sequence of promoter P3 had no effect on the wild-type background, although it considerably reduced rmlB expression in the PAO1ΔrhlR mutant background (Fig. 4). The molecular basis for the repression of the P3 promoter in the wild-type strain was not determined.

Promoter P3 was not detected by primer extension analysis (Fig. 2a). This is the expected result for the wild-type strain PAO1, since P3 expression is very low if it is expressed at all (Fig. 4a). However we do not have an explanation for our inability to detect by primer extension analysis the RNA start site that corresponds to promoter P3 in the PAO1ΔrhlR mutant. These results suggest that promoter P3 is important for the expression of the rmlBDAC operon under conditions where the QSR is not active. Under these circumstances the dTDP-1-rhamnose produced would be solely used for LPS synthesis.

In conclusion we have described how the rmlBDAC operon is expressed by three promoters, one of which (P2) is partially RhlR- and σ5-dependent. This pattern of regulation permits the high basal expression of the genes that encode enzymes involved in dTDP-1-rhamnose synthesis, which is fundamental to the production of this activated sugar used as an LPS precursor, and also the coordinated induction of these genes with the RhlR-dependent expression of genes encoding enzymes responsible for rhamnolipid biosynthesis (RhlA, RhlB and RhlC).

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REFERENCES


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