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

Marisela Aguirre-Ramírez,^{1†} Gerardo Medina,² Abigail González-Valdez,¹ Victoria Grosso-Becerra¹ and Gloria Soberón-Chávez¹

¹Departamento de Biología Molecular y Biotecnología, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México (UNAM), Ciudad Universitaria, México D. F., México

²Universidad Autónoma de Baja California, Mexicali, BC, México

Correspondence

Gloria Soberón-Chávez
gloria@biomedicas.unam.mx

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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 *rmIBDAC* 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 *rmIBDAC* 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 σ^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 & Cámara, 2009). These exoproducts include proteases, phospholipase C, exotoxins, pyocyanin and rhamnolipids (Lazdunski *et al.*, 2004; Williams & Cámara, 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 (dTDP-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-L-xylo-4-hexulose

[†]Present address: Departamento de Ciencias Químico-Biológicas, Instituto de Ciencias Biomédicas, Universidad Autónoma de Ciudad Juárez, Mexico.

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.

(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 & Cámara, 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 NNCTN₁₂AGNN, 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 NNCTN₃AN₄TN₃AGNN (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 N₂CTNCCAGN₂TTNGNAGN, 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 σ^S 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 σ^S -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 σ^S and on the QS regulator RhlR. This promoter has an atypical las box (*rmlB* 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 Supplementary 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 $\mu\text{g ml}^{-1}$): 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 corresponds to 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

Strain or plasmid	Relevant characteristics	Reference or source
Strains		
PAO1	<i>P. aeruginosa</i> wild-type strain	Hancock & Carey (1979)
PAO1 Δ rhlR	PAO1 derivative, Δ rhlR::Gm	Rahim <i>et al.</i> (2001)
PAS1	PAO1 derivative, <i>rpoS</i> ::Gm	Medina <i>et al.</i> (2003b)
MC4100	<i>E. coli</i> , araD139 Δ (argF-lacU169) <i>rpsL150 thiA1 relA1 fib5301 deoC ptsF25 rbsR</i>	Casadaban (1976)
ABI-rhlA	MC4100 derivative with a chromosomal Φ (pZrhlA-lacZ)	This work
ABI-rmlB1	MC4100 derivative with a chromosomal Φ (pZrmlB1-lacZ)	This work
ABI-lasI	MC4100 derivative with a chromosomal Φ (pZlasI-lacZ)	This work
Plasmids		
pLP170	Transcriptional <i>lacZ</i> fusion vector that contains an origin of replication for both <i>E. coli</i> and <i>P. aeruginosa</i> ; Ap ^r	Pearson <i>et al.</i> (1997)
pRML-P1	pLP170 derivative with a <i>rmlB-lacZ</i> fusion containing the upstream tRNA gene with its promoter region as well as the complete 5' <i>rmlB</i> region, Ap ^r	Cabrera-Valladares <i>et al.</i> (2006)
pRML-2	Deletion derivative of pRML-P1 containing the entire <i>rmlB</i> upstream region, starting at an internal position of the upstream tRNA gene, Ap ^r . Oligonucleotides rmlB62 and rmlBm (Supplementary Table S1) were used to construct this plasmid	This work
pRML-3	Deletion derivative of pRML-P1 containing only the sequences of the <i>rml</i> box and the downstream sequences, Ap ^r . Oligonucleotides rmlB62 and rmlBc (Supplementary Table S1) were used to construct this plasmid	This work
pRML-4	pUCP18 derivative encoding the <i>rmlBDAC</i> operon (6.3 kb) under its own promoter, Ap ^r	Cabrera-Valladares <i>et al.</i> (2006)
pRML-P1(AA)	pRML-P1 derivative containing a mutation in the dinucleotide GC to AA at positions 3 and 4 of the <i>rml</i> box. Constructed with oligonucleotides lasIAA and lasIAAr (Supplementary Table S1)	This work
pRML-P1(CC)	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)	This work
pRML-2(CC)	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)	This work
pMP220	Vector to construct transcriptional <i>lacZ</i> fusions, Tc ^r	Spaink <i>et al.</i> (1987)
pMPRML-3	pMP220 derivative with a <i>rmlB-lacZ</i> fusion containing the same insert as pRML-3, Tc ^r	This work
pGMYC	pUCP20 derivative encoding RhlR, expressed under the <i>lac</i> promoter, Ap ^r	Medina <i>et al.</i> (2003c)
pMT1	pUCP20 derivative encoding LasR, expressed under the <i>lac</i> promoter, Ap ^r	Medina <i>et al.</i> (2003a)

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 -³²P]ATP [3000 Ci mmol⁻¹ (111 TBq mmol⁻¹), Perkin Elmer Life Science] as substrate. The construction of a point mutation was done using a QuikChange II XL site directed mutagenesis kit (Stratagene). All the constructions were verified by automated sequencing using oligonucleotide lacZr (Supplementary Table S1).

Primer extension analysis. *P. aeruginosa* strains were grown to OD₆₀₀ 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 synthetic 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-lasI, Table 1) harbouring the chromosomally encoded *lacZ* fusions with the synthetic 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 between 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 pMT1 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.

Box	Sequence (5'–3')	RhlR		LasR	
		C ₄ -HSL (10 μ M)	No C ₄ -HSL	3O-C ₁₂ HSL (10 μ M)	No 3O-C ₁₂ -HSL
<i>lasI</i>	ATCTATCTCATTTGCTAGTT	>1	>1	70 $\pm$ 10	>1
<i>rhlA</i>	TCCTGTGAAATCTGGCAGTT	50 $\pm$ 1.5	50 $\pm$ 1.5	5 $\pm$ 3	>1
<i>rml</i>	ACCTACCAGATCTGGGGTTG	50 $\pm$ 5	50 $\pm$ 5	>1	>1

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-García *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 σ^{54} subunit, since it has the sequence 5'-GGGACGTGTCGAAGC-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 σ^N 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 centred at –44.5 bp from this start site we detected a sequence resembling a las box, but with an atypical sequence (5'-ACCTACCAGATCTGGGGTTG-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 σ^S -dependent

To determine whether *rmlBDAC* expression was RhlR-dependent, we measured the expression of the *rmlB-lacZ* fusion encoded on plasmid pRLM-P1 in the PAO1 Δ *rhlR* 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 PAO1 Δ *rhlR* 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 σ^S subunit (Typas & Hengge, 2006). To determine whether P2 expression was transcribed by RNAP containing this alternative sigma subunit we determined 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 (PAO1 Δ *rhlR*); 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 PAO1 Δ *rhlR* mutant; hatched bars, plasmid pRML-P1(CC), which carries mutations inactivating the -10 sequences of the P2 promoter on PAO1.

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 PAO1 Δ *rhlR* 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 PAO1 Δ *rhlR* 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 -10 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 the 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. 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 (PAO1 Δ *rhlR* 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 -24) 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.

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. 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 μ M C₄-HSL (C4); black bars, pMT1 encoding LasR, with the addition of 3O-C₁₂-HSL (C12). -, No addition.

Fig. 4. The P3 promoter is only functional in the absence of the RhlR-dependent QSR. Data show the expression in the PAO1 background of plasmid pRML-2 (white bars), containing the complete *rmlB* promoter region, its mutant derivative pRLM-2(CC) with mutations that inactivate the -10 sequences of promoter P3 (grey bars), pRML-2 in a PAO1 Δ *rhlR* mutant background (hatched bars), and pRML-2(CC) in the same *rhlR*-deficient strain (black bars).

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 σ^S -independent expression

The expression of the *lacZ* fusion encoded on plasmid pRML-P1 in the PAO1 Δ *rhlR* 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 σ^S 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 PAO1 Δ *rhlR* 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 PAO1 Δ *rhlR* 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 PAO1 Δ *rhlR* 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-L-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 σ^S -dependent. This pattern of regulation permits the high basal expression of the genes that encode enzymes involved in dTDP-L-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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