Transcriptional regulation of Pseudomonas aeruginosa rhlR, encoding a quorum-sensing regulatory protein

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The Pseudomonas aeruginosa rhlR gene encodes the transcriptional regulator RhlR which has a central role in the quorum-sensing response. Different gene products involved in bacterial pathogenesis are regulated at the transcriptional level by two quorum-sensing response systems, Las and Rhl. The expression of rhlR has been reported to be under the control of the Las system, but its transcriptional regulation has not been studied in detail. Here, the rhlR promoter region has been characterized and shown to present four different transcription start sites, two of which are included in the upstream gene (rhlB) coding region. It was found that rhlR expression is not only dependent on LasR but also on different regulatory proteins such as Vfr and RhlR itself, and also on the alternative sigma factor σ54. It is reported that rhlR expression is partially LasR-independent under certain culture conditions and is strongly influenced by environmental factors.

INTRODUCTION

Pseudomonas aeruginosa can be isolated from many different habitats, including water, soil and plants (Costerton, 1980; Hardal & Edberg, 1997), but it is also an opportunistic human pathogen that causes serious nosocomial infections (Costerton, 1980). The production of several virulence-associated traits, including rhamnolipids, by this species is coordinately regulated by a mechanism known as quorum sensing (QS) (Van Delden & Iglesias, 1998). The QS response depends on the production of two autoinducers, butyryl-homoserine lactone (C4-HSL) and 3-oxo-dodecanoylhomoserine lactone (3-O-C12-HSL), which coupled with the proteins RhlR and LasR, respectively, activate gene expression. Rhl synthesizes C4-HSL and Las synthesizes 3-O-C12-HSL [see reviews by Fuqua et al. (2001) and Pesci & Iglesias (1997)]. The transcriptional activator LasR coupled with 3-O-C12-HSL promotes the expression of several virulence-associated traits (Pesci & Iglesias, 1997; Whiteley et al., 1999) including the gene encoding the transcriptional regulator RhlR (Latifi et al., 1996; Pesci et al., 1997). The second QS genetic circuit responds to RhlR (Ochsner et al., 1994b). RhlR coupled with C4-HSL (Ochsner & Raiser, 1995) promotes the expression of the rhlAB operon, encoding the enzyme rhamnosyltransferase 1 (Ochsner et al., 1994a), and the rhlC gene, encoding rhamnosyltransferase 2 (Rahim et al., 2001); it also promotes the expression of other genes (Brint & Ohman, 1995). The rhlAB- and rhlC-encoded rhamnosyltransferases are responsible for rhamnolipid biosynthesis (Maier & Soberón-Chávez, 2000). Besides 3-O-C12-HSL and C4-HSL, P. aeruginosa produces as a QS signal 2-heptyl-3-hydroxy-quinolone (PQS) which affects the expression of at least some of the genes regulated by QS and has been proposed to be a link between the las and rhl systems (McNight et al., 2000). On the one hand, PQS synthesis and activity have only been analysed in P. aeruginosa mutants defective in key elements of the QS response, so its real physiological role is unknown. On the other hand, PQS seems to be only produced at the late-stationary phase of growth, so its role in QS during other phases of growth is limited (McNight et al., 2000).

The LasR and RhlR proteins belong to the LuxR family of transcription regulators whose members bind to specific DNA sequences, called lux boxes (Fuqua et al., 2001). In the case of P. aeruginosa, these specific sequences have been called las boxes (Whiteley et al., 1999), and the sequences determining whether a certain promoter is regulated by QS, quorum sensing.
LasR, RhlR or both regulators (Whiteley et al., 1999) are starting to be understood (De Kievit et al., 2002; Whiteley & Greenberg, 2001). LasR forms a multimer and binds las boxes only in the presence of 3O-C12-HSL (Kiratissi et al., 2002), while RhlR dimerizes (Ventre et al., 2003) and binds DNA (Medina et al., 2003b) both in the presence and in the absence of C4-HSL.

The rhlR gene is encoded immediately downstream of the rhlAB operon and has been reported to be activated by LasR (Latifi et al., 1996; Pearson et al., 1997). An rhlR-specific promoter has been predicted within the rhlB–rhlR intergenic region (Ochsner et al., 1994b). The role of LasR in rhlR transcription was concluded from two different studies (Latifi et al., 1996; Pesci et al., 1997). First, a P. aeruginosa lasR mutant did not express an rhlR::lacZ fusion when grown on Luria–Bertani (LB) medium and, second, Escherichia coli expressed an rhlR::lacZ fusion at a low, but significant, level in the presence of LasR and 3O-C12-HSL. These reports show that QS-regulated genes are mainly, but not exclusively, expressed at the early-stationary phase of growth and that this regulon is greatly influenced by environmental conditions. These results show that QS in P. aeruginosa is a very complex and fine-tuned genetic regulatory circuit that seems to be very important for both the pathogenesis of P. aeruginosa and its environmental life style.

The aim of this work was to characterize in detail the rhlR promoter region of the P. aeruginosa genome and to identify the different elements that participate in the regulation of expression of this region, determining whether environmental conditions modulate its transcription. One of the conditions studied was P. aeruginosa growth on a phosphate-limited peptone/glucose/ammonium salts (PPGAS) medium where rhamnolipid production is high (Zhang & Miller, 1992) – we presumed that rhlR expression would also be high under this condition. Using primer extension analysis of RNA obtained from cells grown on PPGAS medium, we detected four rhlR transcription start sites, two of which were within the rhlB-coding region. It was also apparent from our results that cells grown on PPGAS medium expressed rhlR at a significant level in the absence of LasR and that different transcription activators, such as Vfr and RhlR itself, as well as the alternative sigma factor σ24, participate in the expression of rhlR from these multiple promoters. However, when P. aeruginosa PAO1 was grown on LB medium, rhlR expression was found to be completely dependent on LasR and the gene was found to be transcribed solely from the previously predicted promoter (Ochsner et al., 1994b). The role in rhlR transcriptional regulation of the two putative las boxes detected in the rhlR regulatory region was also explored.

**METHODS**

**Bacterial strains, plasmids and culture conditions.** Bacterial strains and plasmids used in this study are listed in Table 1.

**Table 1. Strains and plasmids used in this study**

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Relevant characteristic(s)*</th>
<th>Reference/source</th>
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<tbody>
<tr>
<td><strong>P. aeruginosa</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAO1</td>
<td>Wild-type strain</td>
<td>Hancock &amp; Carey (1979)</td>
</tr>
<tr>
<td>PAOR1</td>
<td>PAO1 lasR::Tc mutant</td>
<td>Gambello &amp; Iglewski (1991)</td>
</tr>
<tr>
<td>PAO9001</td>
<td>PAO1 vfr::Gm mutant</td>
<td>Runyen-Janecky et al. (1997)</td>
</tr>
<tr>
<td>PG201</td>
<td>Wild-type strain</td>
<td>Ochsner et al. (1994b)</td>
</tr>
<tr>
<td>6SE12</td>
<td>PG201 rhlR frameshift mutant</td>
<td>Ochsner et al. (1994b)</td>
</tr>
<tr>
<td>PAK</td>
<td>Wild-type strain</td>
<td>Ishimoto &amp; Lory (1989)</td>
</tr>
<tr>
<td>PAKN1</td>
<td>PAK rpoN::Tc mutant</td>
<td>Ishimoto &amp; Lory (1989)</td>
</tr>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH5α</td>
<td>sup44 ΔlacU169 hsdR17 recA1 endA1 gyrA96 thiI relA1</td>
<td>Sambrook et al. (1989)</td>
</tr>
<tr>
<td><strong>Plasmid</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pSB1075</td>
<td>p_ lac–lasR, lasI::luxCDABE; Ap'</td>
<td>Winson et al. (1998)</td>
</tr>
<tr>
<td>pMP220</td>
<td>Vector to construct transcriptional lacZ fusions; Tc'</td>
<td>Spaink et al. (1987)</td>
</tr>
<tr>
<td>pRDS8-1</td>
<td>pMP220 derivative with 288 bp upstream of the rhlR ATG start codon</td>
<td>This study</td>
</tr>
<tr>
<td>pRDS8-2</td>
<td>pMP220 derivative with 498 bp upstream of the rhlR ATG start codon</td>
<td>This study</td>
</tr>
<tr>
<td>pPCS1002</td>
<td>Contains an rhlR::lacZ fusion; Ch'</td>
<td>Pearson et al. (1997)</td>
</tr>
<tr>
<td>pUCP20</td>
<td>Cloning vector able to replicate in P. aeruginosa with p_ lac; Ch'</td>
<td>West et al. (1994b)</td>
</tr>
<tr>
<td>pMT1</td>
<td>pUCP20 derivative with p_ lac–lasR</td>
<td>This study</td>
</tr>
</tbody>
</table>

*Tc, tetracycline; Gm, gentamicin; Ap, ampicillin; Ch, carbenicillin.
Bacterial strains were cultured on PPGAS medium pH 7-2 (Zhang & Miller, 1992) containing NH4Cl (0·02 M), KCl (0·02 M), Tris/HCl (0·12 M), MgSO4 (0·0016 M), glucose (0·5%, w/v) and peptone (1%, w/v), or on LB medium (Miller, 1972), as stated. Bacto agar was added to 1·5% for solid media. When necessary, IPTG (0·1 mM) was added to the media. Antibiotics (Sigma) were used to supplement selection media at the following concentrations: for E. coli, 150 μg ampicillin ml−1 and 13 μg tetracycline ml−1; for P. aeruginosa, 250 μg carbenicillin ml−1, 100 μg tetracycline ml−1 and 200 μg gentamicin ml−1. β-Galactosidase activity was determined as reported by Miller (1972); one Miller unit corresponds to 1 nM ONPG hydrolysed per minute and per optical density unit at 600 nm. The presented data represent the triplicate measurements of two independent experiments.

Enzymes and reagents. Plasmids were purified using the Wizard plus miniprep DNA purification system (Promega). DNA bands were cut from agarose gels and purified using the Geneclean III kit (BIO 101). Enzymes were purchased from New England Biolabs or Roche Diagnostics and were used according to the manufacturer’s instructions. DNA sequencing reactions were done using the Sequenase kit (Amersham Life Science). Oligonucleotides were radioactively labelled with the T4 polynucleotide kinase (Amersham Life Science). Oligonucleotides were purchased from Bio 101. Enzymes were purchased from New England Biolabs or plus minipreps DNA purification system (Promega). DNA bands of DNA polymerase I (Amersham Life Science). 3O-C12-HSL was purchased from Quorum Sciences.

Nucleic acid techniques. DNA manipulations were performed following standard procedures (Sambrook et al., 1989). Plasmids were electroporated into P. aeruginosa as described by Smith & Igleski (1989).

Primer extension analysis. The different P. aeruginosa strains analysed were grown to an OD600 value of 1·5 and their total RNA was extracted using a GlassMAX RNA Microsolation Reagent Assembly (Life Technologies). Primer extension reactions were done using the two-step RT-PCR kit (Roche Diagnostics) according to the manufacturer’s instructions. Reactions were carried out at 60°C. These experiments were performed using two different oligonucleotides: Oligo-1 (5'-CATCTCGCTACGAAAAACGCCTCCCACC-3') is located at +25 bp with respect to the rhlR translational start site; Oligo-2 (5'-CGCGCATTCCTCCCTATGTACAAAC-3') is located at −146 bp with respect to the rhlR translation start site.

Plasmid construction. Plasmid pRD58-1, containing 288 bp of the rhlR upstream region fused to a promoterless lacZ gene, was constructed by isolating a 580 bp DNA fragment containing the 288 bp of the rhlR regulatory region and part of the coding region by PCR, using the oligonucleotides 5'-GGCAGGCTTGTGGCGC- TTGCTGAGGACC-3' and 5'-CGCTCCAGACCACCATTTCC-3'. The PCR product was digested with HindIII and BamHI and cloned into the vector pMP220 (Spanik et al., 1987). Plasmid pRD58-2, which contains 498 bp of the rhlR regulatory region fused to the lacZ gene of the vector pMP220, was constructed by ligating into this vector a fragment of 741 bp which included the rhlR upstream region and part of the coding region, which was purified from a PsrI/BamHI digestion of pUO58, a plasmid containing the entire rhlA, rhlB, rhlR and rhlI gene cluster (Ochsner et al., 1994a).

Plasmid pMT1 contains the lasR gene under the control of the lac promoter; it was constructed by isolating a PCR fragment of 860 bp containing lasR, using pSB1075 (Winson et al., 1998) as template. The oligonucleotides 5'-CGGGATTCTGTACGCGTAAAC-3' and 5'-GAAGGGCGAATTCCGCCGAG-3' were used for the amplification. The PCR product was cloned into pMO8Blue (Amersham Life Science), which was later digested with KpnI and HindIII. The insert was then subcloned into pUCP20 (West et al., 1994b).

RESULTS AND DISCUSSION

rhlR has four transcription start sites when P. aeruginosa PAO1 is grown on PPGAS medium

To study P. aeruginosa PAO1 rhlR transcriptional regulation, we determined the transcription start sites of rhlR using primer extension analysis of total RNA extracted from P. aeruginosa PAO1 that had been grown on PPGAS medium, where high rhamnolipid levels were produced. However, with this experiment, we could only detect some faint bands and we were unable to assess whether they were real transcription start sites (data not shown). To enhance the sensitivity of the primer extension assay and to enable us to measure rhlR expression in the same experimental conditions, we transferred pPCS1002 (Pearson et al., 1997; Table 1) carrying an rhlR::lacZ fusion to the different P. aeruginosa strains studied (Figs 1 and 2). There are some bands that can be seen in Figs 1, 2 and 4 that were discarded as transcription start sites based on the lack of reproducibility for the four primer extension experiments done for each strain. Results of primer extension analyses reported here (Figs 1, 2 and 4) correspond to the detected rhlR expression at an OD600 value of 1·5 shown in Fig. 5, as determined by β-galactosidase activity expressed from the rhlR::lacZ fusion encoded on pPCS1002. All transcription start sites detected using pPCS1002 were present when the expression of the chromosomal gene was studied (data not shown), but we cannot rule out that certain points of rhlR

![Fig. 1. Primer extension analysis of RNA extracted from different P. aeruginosa strains containing plasmid pPCS1002, grown on PPGAS medium to an OD600 value of 1·5. Oligo-1 was used as the primer for the reverse transcriptase reaction and also to determine the DNA sequence [the ladder used to determine this sequence is shown in (a) and (b)]. Lanes correspond to RNA extracted from the following strains. (a) Lanes: 1, PAO1; 2, PAOR1; 3, PAK; 4, PAKN1. (b) Lanes: 1, PG201; 2, 65E12; 3, PAKN1; 4, PAK; 5, PAO9001.](image-url)
transcriptional regulation might be overlooked when using a plasmid-encoded version of the gene instead of the chromosomal gene to examine this process. However, the levels of two RhlR-regulated traits, rhamnolipid and pyocyanin, were the same in the different *P. aeruginosa* strains studied with or without pPCS1002 (data not shown), showing that the presence of *rhlR* regulatory sequences on this plasmid has no major effect on the expression of this central QS regulatory protein encoded in the chromosome.

The *P. aeruginosa* PAO1 *rhlR* gene presents four transcription start sites (P1–P3 in Fig. 1, and P4 in Fig. 2), two of which (P3 and P4) are within the upstream *rhlB*-coding region (Fig. 3). The analysis of the *rhlR* regulatory sequence (Fig. 3b) suggests that three of the detected start sites are recognized by the RNA polymerase containing the σ^70 subunit (P1, P2 and P4, Fig. 3a). This assumption is made based on the presence of conserved motifs at positions −10 and −35 from the detected transcription start sites (Fig. 3b), and in the maintenance of these transcription start sites in an *rpoS* mutant (data not shown). The previously predicted *rhlR* promoter sequence within the *rhlB–rhlR* intergenic region (Ochsner et al., 1994b) corresponds to the promoter designated by us as P2 (Fig. 3a). We suggest, based on the

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**Fig. 2.** Primer extension analysis of RNA extracted from strain PAO1 and its lasR- and vfr-derived mutants PAOR1 and PAO9001 (all containing plasmid pPCS1002), respectively, grown on PPGAS medium to an OD_{600} value of 1.5. Oligo-2 was used as primer for the reverse transcriptase reaction and also to determine the DNA sequence of the region (the ladder used to determine this sequence is shown). Lanes correspond to RNA extracted from the following strains. (a) Lanes: 1, PAO1; 2, PAOR1. (b) Lanes: 1, PAO1; 2, PAO9001.

**Fig. 3.** (a) Nucleotide sequence of the *rhlR* upstream region showing the four detected transcription start sites (bold letters) and their putative −10/−35 or −12/−24 promoter regions. The putative ‘las boxes’ and the Vfr box are also shown. The end of the *rhlB*-coding sequence is shown. The sequence underlined corresponds to the oligonucleotides used in the primer extension experiments. The point where the insert in pRD58-1 starts is shown. (b) Schematic representation of the *rhlR* promoter region showing the four transcription start sites and the elements that participate in their expression.
results described below, that the transcription start site at -159 (P3) is recognized by RNA polymerase containing a $\sigma^{54}$ subunit (P3, Fig. 3a).

**rhlR is expressed from promoter P2 when *P. aeruginosa* PAO1 is grown on LB medium**

Primer extension analysis of the *rhlR* gene using total RNA extracted from *P. aeruginosa* PAO1(pPCS1002) grown to an OD$_{600}$ value of 1.5 on LB medium shows the presence of only one transcription start site corresponding to promoter P2 (Fig. 4). The transcription of *rhlR* from different promoters depending on culture conditions shows that environmental stimuli play a crucial role in modulating the expression of the *P. aeruginosa* QS response, and is in accordance with the results obtained using transcriptome analysis (Wagner et al., 2003). The different mode of *rhlR* transcriptional regulation on different culture media also explains, at least in part, discrepancies in results reported by different research groups [for an example, see Vasil (2003)].

**Expression from the P1 and P4 *rhlR* transcription start sites is LasR-dependent**

To correlate the detected transcription start sites with the level of *rhlR* expression, we measured $\beta$-galactosidase expression from the *rhlR::lacZ* fusion encoded on pPCS1002 by growing different *P. aeruginosa* strains on PPGAS and LB media (Fig. 5).

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**Fig. 4.** Primer extension analysis of RNA extracted from different *P. aeruginosa* strains containing plasmid pPCS1002, grown on LB medium to an OD$_{600}$ value of 1.5. Oligo-1 was used as primer for the reverse transcriptase reaction and also to determine the DNA sequence (the ladder used to determine this sequence is shown). Lanes correspond to RNA extracted from the following strains: 1, PAO1; 2, PAOR1; 3, PG201; 4, 65E12.

**Fig. 5.** Expression of the *rhlR::lacZ* fusion encoded in pPSC1002 along the growth curve of different *P. aeruginosa* strains grown on LB (open symbols) and PPGAS (closed symbols) media. (a) Shows strain PAO1 (diamonds), its lasR-derived mutant PAOR1 (triangles) and its vfr-derived mutant PAO9001 (circles). (b) Presents data obtained with strain PAK (squares) and its rpoN-derived mutant PAKN1 (triangles) grown on PPGAS medium. (c) Shows results obtained with strain PG201 (diamonds) and its rhlR-derived mutant 65E12 (circles).
It has been reported that rhlR transcription on LB medium depends on LasR activation (Latifi et al., 1996; Pesci et al., 1997). In accordance with the reported results, we found that rhlR was expressed at a very low level in the lasR mutant PAOR1 when grown on LB medium (Fig. 5a), even though we could still detect transcription from promoter P2 in strain PAOR1 at a level similar to that seen in P. aeruginosa PAO1 using primer extension analysis (Fig. 4). These results suggest that rhlR expression is subject to post-transcriptional regulation.

In contrast to the results obtained with cells grown on LB medium, we found that rhlR was expressed at a significant level in the lasR mutant PAOR1 when it was grown on PPGAS medium (Fig. 5a). This result shows that, when P. aeruginosa is grown on PPGAS medium, rhlR expression can be activated in a LasR-independent manner.

We found that start sites P1 and P4 were not detected when the lasR mutant PAOR1 was grown on PPGAS medium (Figs 1a and 2a). Promoter P1 has a characteristic structure of the promoters activated by regulators of the LuxR family (Whiteley & Greenberg, 2001), presenting the LasR-binding site (las box1) centred at −40 nucleotides from the transcription start site (Fig. 3a). Primer extension analysis in mutant strain PAOR1 confirms that rhlR expression from this promoter is completely dependent on LasR. However, there is not a clear explanation for the LasR-dependent transcription from promoter P4, since the position of the putative las boxes relative to this start site is not consistent with its direct activation by LasR. It is possible that this promoter is activated by LasR in an indirect manner.

The P. aeruginosa PAO1 lasR mutant (PAOR1) is completely unable to produce rhamnolipid when grown on PPGAS medium (data not shown). This result could be due to the lack of C4-HSL, considering that rhlI expression is strongly regulated by LasR (De Kievit et al., 2002). To test this hypothesis, we grew strain PAOR1 on PPGAS supplemented with 10 μM C4-HSL and found that it did not produce rhamnolipids. Recently, we have reported that the presence of RhlR and C4-HSL is a necessary, but not sufficient, condition for rhlAB expression (Medina et al., 2003a). It is therefore possible that, when P. aeruginosa PAOR1 is grown on PPGAS supplemented with 10 μM C4-HSL, the expression of the genes encoding rhamnosyltransferase 1 is limited by other factors. Another intriguing result that highlights the importance of additional regulatory factors in rhlAB expression, besides RhlR and C4-HSL, is the lack of rhamnolipid production by P. aeruginosa PAO1 grown on LB medium, even though rhlR is expressed (Figs 4 and 5) and C4-HSL is produced in this culture medium (Diggle et al., 2002).

**Vfr activates the expression of the P4 rhlR promoter**

It has been reported that Vfr, a P. aeruginosa Crp homologue that regulates the expression of different virulence-associated traits (West et al., 1994a), directly activates lasR expression (Albus et al., 1997). However, primer extension experiments done with RNA extracted from the PAO1-derived vfr mutant PAO9001 show that P1, the LasR-dependent transcription start site, is fully expressed (Fig. 1), while the P4 transcription start site is not (Fig. 2). Accordingly, with these results we found that rhlR expression is reduced in the vfr mutant PAO9001 (Fig. 5a).

Upstream of promoter P3 and overlapping the P4 transcription start site we detected a putative Vfr-binding site (Figs 3 and 6a). The relative position of this Vfr-binding site with respect to promoter P4, which is the detected point of Vfr regulation of rhlR transcription, is uncommon for a promoter activated by a Crp-like regulator. The Crp-activated promoters present a binding site centred at −40 or −60 nucleotides from the transcription start site, and when this protein binds downstream from the start site it acts as a repressor. It is possible, however, that even though Vfr and Crp have sequence homology and recognize similar consensus sequences, they have a different mode of transcriptional activation. To address this matter, additional evidence is needed, via Vfr-binding and footprinting analyses, before it can be concluded that Vfr directly activates the P4 rhlR promoter.

**Expression from the P3 rhlR transcription start site is σ54-dependent**

It has been reported that a P. aeruginosa rpoN mutant is unable to produce rhamnolipid (Ochsner et al., 1994a; Pearson et al., 1997), and we have recently reported that the rhlAB promoter is not dependent on σ54 (Medina et al., 2003b). However, nitrogen metabolism has been shown to play a crucial role in the modulation of the QS response in P. aeruginosa (Wagner et al., 2003).

To study the influence of σ54 on rhlR expression, we used the P. aeruginosa PAK-derivated rpoN mutant PAKN1 (Ishimoto & Lory, 1989) (Table 1). We found that strain PAKN1, when grown on PPGAS medium, expressed the rhlR::lacZ fusion at a significantly lower level than the wild-type strain PAK (Fig. 5b), demonstrating that σ54 is involved in rhlR expression.

By comparing the rhlR transcription start sites present in strains PAK and PAKN1 grown on PPGAS medium, we found that the only promoter that was completely dependent on σ54 was P3 [Fig. 1; promoter P4 was not affected by σ54 activity (data not shown)]. Upstream of the P3 transcription start site we found sequences with the nucleotides and positions required for this promoter to be recognized by the RNA polymerase containing a σ54 subunit (Fig. 3a).

All σ54-dependent promoters are activated by proteins belonging to the NtrC family. The activator protein of promoter P3 and its DNA-binding site remain to be identified.
Expression from the P2 rhlR transcription start site is repressed by RhlR

We detected an increased level of rhlR::lacZ expression in the rhlR mutant 65E12 (Ochsner et al., 1994b; Table 1) compared to the PG201 wild-type strain, when grown on LB medium (Fig. 5c). This enhanced expression, however, could not be visualized as an enhanced level of transcription from promoter P2 using primer extension analysis (Fig. 4). These data suggest that RhlR plays a negative role in the expression of the P2 transcription start site of its own gene, rhlR.

When mutant strain 65E12 was grown on PPGAS medium, we detected increased transcription starting from promoter P2 during the stationary phase of growth (Fig. 1), but this increment was not reflected in an increased level of expression of the rhlR::lacZ fusion (Fig. 5c). These data suggest that RhlR also regulates its own gene expression when cells are grown on PPGAS medium by reducing the level of transcription from promoter P2. It seems, though, that the contribution of promoter P2 to the expression of rhlR, when cells are grown on PPGAS medium, is not as important as that of the other transcription start sites.

It has been proposed that RhlR binds to the lasB OP2 las box and represses its LasR-dependent expression (Anderson et al., 1999). We found that rhlR las box1 is similar to this lasB las box (Fig. 6a). It is possible that RhlR exerts its negative auto-regulation on promoter P2 by binding to las box1. This possibility remains to be validated experimentally.

Role of the two putative rhlR ‘las box’ sequences

The analysis of the sequence of the rhlR regulatory region shows two putative las boxes (Figs 3a and 6). LasR and RhlR activate gene expression by binding to sequences located around 40 nucleotides upstream of the transcription start site (Whiteley & Greenberg, 2001). Only las box1 is located in the expected position to bind a transcription activator acting on promoter P1 (Fig. 3). As mentioned above, we found that this las box (Fig. 6) is similar to one (OP2) of the two present in the regulatory region of P. aeruginosa lasB. Both the lasB OP2 las box and rhlR las box1 have 11 nucleotides between the invariable CT and AG sequences (Fig. 6). It has been shown that the lasB OP2 las box is a suboptimal LasR–3O-C12-HSL-responsive sequence (Anderson et al., 1999). Our results (Figs 1 and 5) are compatible with the rhlR las box1 being the LasR–3O-C12-HSL-recognized sequence for activation of rhlR transcription from promoter P1.

The great majority of las boxes, including rhlR las box2, present 12 nucleotides between the invariable CT and AG sequences (Whiteley et al., 1999; Whiteley & Greenberg, 2001). It has been reported that on these canonical las boxes an A is present at position 8 and a T is present at position 13 on genes activated by both QS regulators (Whiteley & Greenberg, 2001), even though they might show some specificity towards one of them (Anderson et al., 1999; De Kievit et al., 2002; Pearson et al., 1997; Pesci et al., 1997). We found that none of the rhlR las boxes has the sequence requirements to be recognized by both LasR and RhlR (Fig. 6).

It has been reported that LasR is sufficient to activate rhlR expression in an E. coli background (Latifi et al., 1996; Pearson et al., 1997). We used this heterologous host to determine whether the LasR-dependent rhlR expression was affected by the presence of las box2. The LasR-dependent rhlR expression in the E. coli DH5α background was determined using pRD58-1 and pRD58-2 (Table 1), both of which have a transcriptional rhlR::lacZ fusion containing the four detected promoters, but the former plasmid lacks the most distal las box2. To do these experiments, LasR was expressed from pMT1 (Table 1) and 3O-C12-HSL was supplemented at 10 μM. It was found that strain DH5α(pRD58-1) showed nearly 50 % more rhlR::lacZ expression than strain DH5α(pRD58-2) (22.1 ± 2.07 Miller Units in the former case vs 14.7 ± 1.34 Miller Units in the latter). This difference in expression levels shows that the presence of las box2 in pRD58-2 has a significant negative effect on LasR-dependent rhlR expression in the E. coli background. However, this result also suggests that LasR–3O-C12-HSL binds to both rhlR las boxes.
Negative regulation by binding of transcriptional regulators to DNA-binding sites that are distant from the promoters has been reported in the case of Vibrio fischeri luxR (Shadel & Baldwin, 1992). One of the possible mechanisms for the negative effect of LasR binding to las box2 is the formation of multimers between the LasR molecules bound to this box and LasR molecules attached to las box1 that interfere with transcription initiation or elongation. LasR multimerization and gene activation have been shown to be dependent on binding of 30-C12-HSL (Kiratiseit al., 2002), but it is not known whether this regulator can form dimers or higher level multimers and whether this is a regulated process. The precise role of las box2 in P. aeruginosa rhlR expression remains to be determined.

In summary, we have shown that rhlR is subject to a complex transcriptional regulation that is greatly influenced by media composition. This gene presents multiple promoters and regulatory sequences in its upstream region (Fig. 3c). Its expression is tightly regulated by environmental stimuli and is not only dependent on bacterial density, as has also been shown to occur with the P. aeruginosa QS response studied as a whole by microarray analysis (Schuster et al., 2003; Wagner et al., 2003). The expression of rhlR is LasR-dependent when cells are grown on LB medium, but its expression is much less dependent on this QS regulator when they are grown on PPGAS medium. The transcription from some of the rhlR promoters is positively regulated by LasR (P1 and P4) and by Vfr (P4) and repressed by RhlR (P2), and one of the rhlR promoters (P3) is σ^54-dependent (Fig. 3c). To our knowledge, this is the most complex pattern of transcriptional regulation described for any of the genes encoding members of the LuxR family of transcriptional regulators.

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