Transcriptional regulation of *Pseudomonas aeruginosa* *rhIR*: role of the CRP orthologue Vfr (virulence factor regulator) and quorum-sensing regulators LasR and RhIR

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The production of many virulence factors by *Pseudomonas aeruginosa* is regulated by the quorum-sensing (QS) response. In this regulatory network LasR and RhIR, bound to their corresponding autoinducers, play a central role. The QS response has a hierarchical structure: LasR/3O-C12-HSL activates the transcription of *rhIR*, and RhIR/C4-HSL activates the transcription of several genes, including the *rhIAB* operon, which encodes the enzymes responsible for rhamnolipid synthesis. The *rhIAB* operon is located immediately upstream of the *rhIR* gene. *rhIR* has four transcription start sites, two of which are located in the *rhIB* coding region. Vfr directly activates transcription of *lasR*, and has been reported to be also involved in *rhIR* expression. The aim of this work was to characterize the details of the mechanism of *rhIR* transcriptional regulation. We show that Vfr directly regulates *rhIR* transcription through its binding to several Vfr-binding sites (VBSs) present in the *rhIR* promoter region, one of which has a negative effect on transcription. Two of the VBSs overlap with *las* boxes where LasR/3O-C12-HSL binds to activate *rhIR* transcription. We also show that *rhIR* transcription is subject to positive-feedback autoregulation through RhIR/C4-HSL activation of the *rhIA* promoter. This positive autoregulation plays a major role in *rhIR* expression.

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INTRODUCTION

Pseudomonas aeruginosa is a ubiquitous Gram-negative bacterium that can survive and proliferate in diverse environments due to its great metabolic versatility. This bacterium is an opportunistic pathogen that can cause infections in a diverse range of organisms, including mammals, plants, insects and nematodes. The infection produced by *P. aeruginosa* generally involves the production of a wide array of cell-associated and extracellular virulence factors. The extracellular virulence factors produced by *P. aeruginosa* include pyocyanin, elastase, rhamnolipids, alkaline protease, lectins, exotoxin A and HCN. The production of these virulence factors is under the control of quorum sensing (QS) (Williams & Cámara, 2009). QS is a process that allows bacteria to regulate gene expression in response to self-produced intercellular signalling molecules known as autoinducers and to have

a population density-dependent response (Williams & Cámara, 2009).

In *P. aeruginosa* there are two QS systems dependent on acylhomoserine lactone (AHL) autoinducers, the *las* and *rhl* systems. In the *las* system, the transcriptional regulator LasR coupled with 3-oxododecanoylhomoserine lactone (3O-C12-HSL), whose synthesis is catalysed by the LasI protein, activates the expression of genes for several virulence factors such as elastase, exotoxin A and alkaline protease, and also the gene encoding the transcriptional regulator RhIR (Latifi *et al.*, 1996). In the *rhl* system, the complex formed by RhIR and butyrylhomoserine lactone (C4-HSL, the autoinducer whose synthesis is catalysed by RhII), promotes the expression of genes responsible for the production of pyocyanin, PA-I lectin (encoded by *lecA*), and the *rhIAB* operon coding for enzymes involved in the production of the biosurfactant rhamnolipids (Williams & Cámara, 2009).

The *rhIR* gene has four promoters, two of which are located in the upstream *rhIB* coding region (Medina *et al.*, 2003a). LasR, which belongs to the LuxR family of transcriptional regulators, whose members bind to *lux* boxes, was

Abbreviations: C4-HSL, butyrylhomoserine lactone; 3O-C12-HSL, 3-oxododecanoylhomoserine lactone; CRP, cAMP receptor protein; QS, quorum sensing; VBS, Vfr-binding site.

A supplementary table of primers is available with the online version of this paper.

identified as the protein responsible for *rhlR* transcriptional activation (Latifi *et al.*, 1996; Medina *et al.*, 2003a). LasR/3O-C12-HSL transcriptional regulation of *rhlR* was shown to be due to its activation of promoter 1 through its binding to a *las* box (the *rhlR*-1 *las* box), and possibly also of promoter 4 (Medina *et al.*, 2003a). A second LasR/3O-C12-HSL-binding site was identified upstream of *rhlR* promoter 1 by the analysis of DNA sequences after immunoprecipitation of DNA–LasR complexes (Gilbert *et al.*, 2009). Transcription initiation at promoter 2 was shown to be constitutive. The *rpoN* gene product appears to be involved in the activation of promoter 3, although its effect seems to be indirect due to the absence of a consensus sequence for the binding of this sigma factor. *rhlR* activation is regulated by Vfr, a member of the cAMP receptor protein (CRP) family, but it has not been demonstrated whether this regulation is direct. There are data suggesting the direct interaction of this protein, since transcription initiation at *rhlR* promoter 4 is affected by Vfr, and a putative Vfr-binding sequence (VBS) has been identified in this region (Medina *et al.*, 2003a).

Vfr acts at the top of the regulatory QS cascade, since this is the only protein known to be directly involved in the transcriptional activation of *lasR* (Albus *et al.*, 1997); the transcriptional activation of *lasR* by Vfr is independent of cAMP binding by this protein (Fuchs *et al.*, 2010). It has been reported that RpoN is involved in Vfr transcription (Heurlier *et al.*, 2003), so the effect of this sigma factor in *rhlR* transcription might be indirect.

The aim of this work was the detailed characterization of the mechanism of *rhlR* transcriptional regulation, specifically in relation to the role of Vfr in this process. We show that Vfr directly regulates *rhlR* transcription by binding to several *vfr* boxes present in the *rhlR* promoter region, one of which has a negative effect on transcription. We also show that *rhlR* transcription is subject to a positive feedback autoregulatory loop through RhlR/C4-HSL activation of the *rhlA* promoter. This positive autoregulation plays a major role in RhlR expression, and thus in *P. aeruginosa* QS.

METHODS

Bacterial strains, plasmids and growth conditions. Bacterial strains and plasmids used in this study are shown in Table 1. Bacterial strains were cultured at 37 °C; either on PPGAS medium pH 7.2 (Zhang & Miller, 1992), or on Luria–Bertani (LB) medium (Miller, 1972), as stated. Growth was monitored by measuring OD₆₀₀. When necessary, antibiotics were added at the following concentrations (µg ml⁻¹): ampicillin 200, kanamycin 75, and chloramphenicol 30 for *Escherichia coli*; or carbenicillin 200, tetracycline 150, and streptomycin 600 for *P. aeruginosa*.

Cells of *P. aeruginosa* strains PAO1, PAO1V, PW6882, PAO1-WV and PAO1-WA, containing a reporter *lacZ* gene fusion, or the expression vector, were grown overnight at 37 °C in LB medium (supplemented with carbenicillin) with shaking; they were then subcultured into PPGAS at a starting OD₆₀₀ of 0.05 and incubated at 37 °C with vigorous shaking (225 r.p.m.) until the culture reached the

late-exponential phase, when the system is already induced. β -Galactosidase activity was measured as described by Miller (1972). Each culture was assayed in triplicate; results were confirmed in at least three independent experiments. To test the ability of Vfr and LasR to activate the expression of *rhlR* in *E. coli*, cultures of *E. coli* strains TGC000, TGC001 and TGC002 harbouring plasmid pWNP28 or pMT1 or the empty vector were grown overnight in LB medium at 37 °C supplemented with ampicillin and subcultured into LB supplemented with 50 mM MOPS (pH 7.0) at a starting OD₆₀₀ of 0.05. After incubation at 37 °C with vigorous shaking (225 r.p.m.) until the cultures reached an OD₆₀₀ of 0.4, IPTG was added to a final concentration of 1 mM; 3O-C12-HSL was also added at this time to a final concentration of 5 µM for strains containing plasmid pMT1, which encodes LasR, and to its corresponding empty vector (Table 1). After the additions *E. coli* cultures were allowed to grow for an additional 1.5 h; then cells were collected and β -galactosidase activity was determined (Miller, 1972). The culture conditions to measure β -galactosidase activity were standardized to obtain similar levels of expression promoted by Vfr of a *lacZ* fusion that contains a CRP-binding site to that promoted by CRP itself (data not shown). The statistical significance of the results presented in this work was determined using Student's one-tailed *t*-test.

To generate a *vfr* mutant in the PAO1 background and a *vfr* mutant in the background of strain PW6882, we transduced the *vfr*:: Ω Sp/Sm marker from strain PAO6301 into strains PAO1 and PW6882, respectively, using bacteriophage E79tv-2 as described by Morgan (1979). Strain PAO1-WA was also constructed by transduction with phage E79tv-2 of the *rhlA*:: Ω Gm mutation described previously (Rahim *et al.*, 2001) into strain PW6882.

To determine pyocyanin concentration we used the method reported previously (Essar *et al.*, 1990). In summary this method consists in extracting 16 h PPGAS culture supernatant with 0.3 vol. chloroform, and acidifying the organic phase by addition of 0.1 vol. 0.2 M HCl to obtain a pink solution. Pyocyanin concentration was determined spectrophotometrically at a wavelength of 520 nm, using a factor of 17.072 to convert A₅₂₀ values to µg ml⁻¹.

DNA techniques. Standard recombinant DNA manipulation techniques were used (Sambrook *et al.*, 1989). Oligonucleotide synthesis and DNA sequencing were done at the facilities of the Instituto de Biotecnología, UNAM. Plasmids were introduced into *E. coli* and *P. aeruginosa* by transformation (Chung *et al.*, 1989; Irani & Rowe, 1997). The identification of putative VBS sequences was performed using a relaxed Vfr consensus sequence and the program fuzznuc of the EMBOSS package using the 500 bp sequence upstream of the *rhlR* translational start site.

Conditions for RT-PCR used to determine the presence of a transcript extending from *rhlA* to *rhlR* and the *rpoD* transcript (used as positive control in these experiments) were as follows. Strains PAO1 and PW6882 were grown in PPGAS medium to an OD₆₀₀ of 1.5. RNA was isolated using the RNeasy Protect Mini kit (Qiagen) according to the manufacturer's recommendations. Then RNA was treated with DNase I (Qiagen) and repurified using the hot phenol protocol (Aiba *et al.*, 1981). Synthesis of cDNA was performed using SuperScript II Reverse Transcriptase (Invitrogen) with 1.5 µg DNA-free total RNA and 2 pmol *rhlR*-specific primer (R-*rhlABR*; see Supplementary Table S1, available with the online version of this paper) or *rpoD*-specific primer (R-*rpoD*, Table S1).

The cDNA thus synthesized was used as template for PCR, using primers R-*rhlABR* and F-*rhlA* (Table S1). To further confirm that the PCR product contained the sequence corresponding to the mRNA extending from *rhlA* to *rhlR* we amplified by PCR an internal sequence using as primers R-*rhlABR* and an internal *rhlA* sequence (F'-*rhlA*, Table S1). Genomic DNA of PAO1 served as a positive control for the

Table 1. Bacterial strains and plasmids used in this work

Strain or plasmid	Relevant genotype	Source
<i>E. coli</i>		
SA2777	F ⁻ <i>his</i> <i>rpsL</i> <i>relA</i> <i>crp</i> :: <i>cat</i>	Nam <i>et al.</i> (2005)
DH5 α	F'/ <i>endA1</i> <i>hsdR17</i> <i>supE44</i> <i>thi-1</i> <i>recA1</i> <i>gyrA</i> <i>relA1</i> Δ (<i>lacZYA-argF</i>)U169 <i>deoR</i> [ϕ 80 <i>dlacZ</i> Δ M15 <i>recA1</i>]	Miller (1972)
TE2680	F ⁻ , λ^- , IN(<i>rrnD-rrnE</i>)1 Δ (<i>lac</i>)X74 <i>rpsL</i> <i>galK2</i> <i>recD1903</i> ::Tn10d-Tet <i>rpDC700</i> :: <i>putPA1303</i> ::[Kan ^S -Cam ^R - <i>lacZ</i>]	Elliott (1992)
TGC000	SA2777 <i>rhlR'</i> - <i>lacZ</i> (nt -500 to +242) wild-type	This study
TGC001	SA2777 <i>rhlR'</i> - <i>lacZ</i> (nt -61: G→T; nt -60: A→T; nt -59: C→G)	This study
TGC002	SA2777 <i>rhlR'</i> - <i>lacZ</i> (nt -114: G→T; nt -113: C→G; nt -112: A→T)	This study
MC4100	<i>araD139</i> Δ (<i>argF-lacU169</i>) <i>rpsL150</i> <i>thiA1</i> <i>relA1</i> <i>fib-5301</i> <i>deoC</i> <i>ptsF25</i> <i>rbsR</i>	Casadaban (1976)
ABI-lasI	MC4100 derivative with a chromosomal Φ (<i>pZlasI'</i> - <i>lacZ</i>)	This study
ABI-rhlA	MC4100 derivative with a chromosomal Φ (<i>pZrhlA'</i> - <i>lacZ</i>)	This study
ABI-rhlR-1	MC4100 derivative with a chromosomal Φ (<i>pZrhlR-1'</i> - <i>lacZ</i>)	This study
<i>P. aeruginosa</i>		
PAO1	Wild-type strain of the MPAO1 subline	Jacobs <i>et al.</i> (2003)
PAO1- <i>rhlA</i>	PAO1 <i>rhlA</i> :: Ω Gm	Rahim <i>et al.</i> (2001)
PAO1V	PAO1 <i>vfr</i> :: Ω Sp/Sm	This study
PAO6301	PAO1 <i>vfr</i> :: Ω Sp/Sm	Fox <i>et al.</i> (2008)
PW6882	PAO1 _{UW} mutant ID:3452 <i>rhlR</i> ::Tn' <i>lacZ</i>	Jacobs <i>et al.</i> (2003)
PW6887	PAO1 _{UW} mutant ID:54908 <i>rhlA</i> ::IS <i>phoA/hah</i>	Jacobs <i>et al.</i> (2003)
PAO1-WV	PW6882 <i>vfr</i> :: Ω Sp/Sm	This study
PAO1-WA	PW6882 <i>rhlA</i> :: Ω Gm	This study
Plasmids		
pLP170	<i>lacZ</i> transcriptional fusion vector that contains an origin of replication for both <i>P. aeruginosa</i> and <i>E. coli</i> , Ap ^R Cb ^R	Preston <i>et al.</i> (1997)
pPCS1002	<i>rhlR</i> promoter region (nt -500 to +242) cloned in pLP170 to create an <i>rhlR'</i> - <i>lacZ</i> transcriptional fusion	Pesci <i>et al.</i> (1997)
pPGC2001	Derived from pPCS1002; <i>rhlR</i> p (nt -115: G→T; nt -114: C→G; nt -113: A→T)-' <i>lacZ</i>	This study
pPGC2002	Derived from pPCS1002; <i>rhlR</i> p (nt -114: G→T; nt -113: C→G; nt -112: A→T)-' <i>lacZ</i>	This study
pPGC2003	Derived from pPCS1002; <i>rhlR</i> p (nt -165: C→T; nt -164: A→T; nt -162: A→T)-' <i>lacZ</i>	This study
pPGC2004	Derived from pPCS1002; <i>rhlR</i> p (nt -209: A→T; nt -208: C→G; nt -207: A→T)-' <i>lacZ</i>	This study
pPGC2005	Derived from pPGC2001; <i>rhlR</i> p (nt -71: C→T)-' <i>lacZ</i>	This study
pPGC2006	Derived from pPGC2002; <i>rhlR</i> p (nt -117: C→T)-' <i>lacZ</i>	This study
pPGC2008	<i>rhlR</i> promoter region from nt -469 to -121 in pLP170; contains promoters 4 and 3	This study
pPGC2008-1	Derived from pPGC2008, with (nt -209: A→T; nt -208: C→G; nt -207: A→T)	This study
pPGC2008-2	Derived from pPGC2008, with (nt -379: C→T; nt -378: T→G)	This study
pRS551	<i>E. coli lacZ</i> reporter gene fusion vector, Ap ^R Km ^R	Simons <i>et al.</i> (1987)
pPGC2010	<i>rhlR</i> promoter (nt -500 to +242) cloned in pRS551 to create an <i>rhlR'</i> - <i>lacZ</i> transcriptional fusion	This study
pPGC2011	<i>rhlR</i> promoter with the following mutations: (nt -61: G→T; nt -60: A→T; nt -59: C→G)-' <i>lacZ</i> on pRS551	This study
pPGC2012	<i>rhlR</i> promoter with the following mutations (nt -114: G→T; nt -113: C→G; nt -112: A→T)-' <i>lacZ</i> on pRS551	This study
pMMB66EH	Broad-host-range <i>tacp</i> expression vector <i>lacI</i> ^q , Ap ^R	Fürste <i>et al.</i> (1986)
pWNP28	<i>tacp-vfr</i> on pMMB66EH	Suh <i>et al.</i> (2002)
pUCP20	Cloning vector able to replicate in <i>P. aeruginosa</i> and <i>E. coli</i> , with <i>plac</i> , Ap ^R Cb ^R	West <i>et al.</i> (1994b)
pMT1	<i>plac-lasR</i> on pUCP20	Medina <i>et al.</i> (2003b)
pGMYC	<i>plac-rhlR</i> on pUCP20	Medina <i>et al.</i> (2003b)

fragments amplified. A reverse transcription reaction in which reverse transcriptase was omitted served as a negative control.

Construction of plasmids. For construction of plasmid pPGC2008, an *rhlR* promoter containing a DNA fragment corresponding to nucleotides -468 to -120 relative to the *rhlR* translational start site was amplified by PCR from PAO1 chromosomal DNA using primers

designed to contain *EcoRI* and *BamHI* restriction sites (Table S1). The PCR product was digested with *EcoRI* and *BamHI* and the resulting DNA fragment was ligated into *EcoRI*- and *BamHI*-digested plasmid pLP170 (Preston *et al.*, 1997). To generate plasmids pPGC2001, pPGC2002, pPGC2003, pPGC2004, pPGC2005 and pPGC2006, containing point mutations in the different *vfr* boxes and *las* boxes, pPCS1002 was used as a template and the QuikChange XL

site-directed mutagenesis kit (Stratagene) was used according to the manufacturer's instructions; the sequences of the plasmids were corroborated. The oligonucleotides used in the construction of *rhIR* transcriptional fusions are provided in Table S1.

To construct plasmids pPGC2010, pPGC2011 and pPGC2012, the corresponding *rhIR'*-*lacZ* transcriptional fusions were released from plasmid pLP170 (Preston *et al.*, 1997) by digestion with *EcoRI* and *BamHI*, and inserted into plasmid pRS551 (Elliott, 1992), previously digested with the same enzymes.

Construction of *E. coli* strains. Wild-type and mutant *rhIR* promoter fragments were removed from plasmid pPCS1002 (Pesci *et al.*, 1997; Table 1) and derivatives with an *EcoRI* and *BamHI* double digest. Promoter fragments were cloned in pRS551 (Elliott, 1992) previously digested with the same enzymes. Plasmids pPGC2010–12 carrying the *rhIR'*-*lacZ* fusions were introduced into the *E. coli* TE2680 chromosome by the method described previously (Elliott, 1992; Simons *et al.*, 1987). P1_{vir} transduction was used to move promoter fusions from the chromosome of TE2680 to the chromosome of SA2777 (Miller, 1972; Nam *et al.*, 2005).

Determination of LasR and RhIR binding to *las* boxes. To measure *in vivo* RhIR and LasR binding to different DNA sequences (*las* boxes) we constructed hybrid promoters with consensus -10 and -35 RNA polymerase recognition sequences separated by 19 bp with the sequence corresponding to the *rhIA* *las* box, the *rhIR*-1 *las* box or the *lasI* *las* box. Autoinducers were added at 5 µM (3O-C12-HSL) or 10 µM (C4-HSL) in the presence of LasR (encoded in plasmid pMT1) or RhIR (encoded in plasmid pGMYC), respectively.

Complementary oligonucleotides with the sequence of the artificial promoters with *rhIA*, *rhIR*-1 or *lasI* *las* boxes between the -10 and -35 RNA polymerase recognition sites, flanked by *EcoRI* and *BamHI* restriction sites, were hybridized to form a double-stranded DNA fragment and cloned into plasmid pRS551 (Elliott, 1992; Simons *et al.*, 1987) digested with the two endonucleases, to produce transcriptional *lacZ* fusions. These plasmid-encoded *lacZ* fusions were introduced into the *E. coli* MC4100 chromosome by the method described above (Elliott, 1992; Simons *et al.*, 1987). Plasmids coding for RhIR (pGMYC, Table 1; Medina *et al.*, 2003b) or LasR (pMT1, Table 1; Medina *et al.*, 2003b) were introduced into the *E. coli* strains (ABI-*rhIA*, ABI-*rhIR*-1, and ABI-*lasI*, Table 1) harbouring the chromosomally encoded *lacZ* fusions with the artificial promoters. The extent of *lacZ* repression in the presence of RhIR, RhIR/C₄-HSL or LasR/3O-C12-HSL corresponds to the binding of these proteins with or without their corresponding autoinducers.

RESULTS AND DISCUSSION

Vfr directly activates *rhIR* transcription

The *rhIR* gene was proposed to be under Vfr control because the *rhIR'*-*lacZ* fusion encoded in plasmid pPCS1002, which contains the four reported promoters (Fig. 1), showed reduced expression when transferred to a *vfr* mutant derivative of the PAO1 strain (Medina *et al.*, 2003a). However, it was not determined whether this effect was a direct consequence of Vfr binding to the *rhIR* promoter region or an indirect effect, since Vfr activates *lasR* transcription (Fuchs *et al.*, 2010) and in turn LasR/3O-C12-HSL directly activates *rhIR* transcription (Latifi *et al.*, 1996; Medina *et al.*, 2003a).

To determine whether Vfr directly activates *rhIR* transcription, we measured Vfr-dependent activation of an *rhIR'*-*lacZ* fusion inserted into the chromosome of an *E. coli* *crp* mutant strain (TGC000, Table 1, Fig. 2). We used an *E. coli* *crp* mutant since *vfr* has been shown to complement a *crp* mutation (West *et al.*, 1994a). Vfr is indeed able to directly activate *rhIR* transcription in the *E. coli* genetic background, since a significant increase in β-galactosidase activity from 73 ± 11 to 225 ± 32 Miller units was obtained when Vfr was induced; the uninduced level was similar to that obtained with the vector alone: 83 ± 43 Miller units in the presence of IPTG (Fig. 2a). The use of *E. coli* as heterologous host for Vfr-dependent expression of *rhIR* shows that this protein directly activates *rhIR* transcription, but cannot be used to infer the strength of *rhIR* Vfr-dependent promoter in *P. aeruginosa* background since there are several factors that can modify the level of expression of Vfr-dependent promoters in *E. coli*, such as the expression of Vfr itself, the concentration of cAMP that is suitable for Vfr activity (in the case that the binding of this cyclic nucleotide is required for the activation by Vfr), and the interaction of Vfr with its RNA polymerase, for example. It is also important to bear in mind that *lacZ* expression is driven by a single copy of this reporter gene encoded in the chromosome and that it cannot be compared to the expression of this gene when gene fusions

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-499 GCAGCGCGCTACGCGCCACTGGGAGCCTTGCTGCCATCGTGCGCCGGGCTGGTCCATCCGGGCGGTATCGGCGCCATGA
                                     las box 2
-419 GCCTAGCCTTGCGCGCGGGGTGCCGAGGTGCTGCTGCCCTGTGCCCCAGCAGTTCGACAATGCCGAACGGCTGGTC
                                     T4 → VBS 4
-339 CGGCTCGGCTGCGGGATGCGCCTGGGCGTGGCGTTCGCGAGCAGGAGTTGCGCGGGGCGCTGTGGCGCTTGCTCGAGG
-259 ACCCGGCCATGGCGCGCGCCTGTGCGGCTTTCATGGAATTGTCAACAACCGCACAGTATCGCTTGGGTAAGCGGCCACGG
      VBS 3      T3 →      VBS 2      las box 3
-179 TGGTCAACGTTGTCATAGGGAGGGGGATGCTCGATGGCTGAAGGCTGCGTCTCTGAACGGTGTGGCATACAGATAGGG
      VBS 1      T2 →      T1 →
-99  TTGCCATGATTTGCCGTATCGGCAGGGCTGCGCTTGACAGCTCATACCCGGGCCAATTCTGCTGTGATGCAATTTTATC
                                     las box 1
-16  GATCAGGGCTACTGCAATGAGGATGACGAGGCTTTTGTGTGGTGGGACGTTTGTGTAAGCGAGATGC

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Fig. 1. Nucleotide sequence of the *rhIR* promoter region, showing relevant DNA motifs. The *rhIR* start and *rhIB* stop codons are indicated in grey. The *rhIR* mRNA start sites are indicated in bold italic and with a bent arrow. The *las* box nucleotide sequences are underlined. Putative Vfr-binding sites (VBSs) are boxed.

Fig. 2. Expression of different *rhIR'*-*lacZ* fusions inserted in the chromosome of an *E. coli* *crp* mutant promoted by Vfr (encoded in plasmid pWNP28) or LasR (encoded in plasmid pMT1) supplemented with 5 μ M 3O-C12-HSL. TGC000 corresponds to the wild-type *rhIR* promoter region, TGC001 to the same region carrying mutations that inactivate the VBS1 motif, and TGC002 to the region with mutations that inactivate the VBS2 sequence. The statistical significance of the results was determined by Student's one-tailed *t*-test. The results obtained have a level of significance of $P < 0.001$ when compared with TGC000 induced by Vfr (*); and of $P < 0.05$ (**) and $P < 0.001$ (***) when compared with the results obtained with TGC000 induced by LasR.

encoded in plasmids are used. In general, the study of a *P. aeruginosa* regulatory mechanism in the *E. coli* background enables a complex phenomenon to be dissected and analysed, but this approach has the drawback of missing some parts of the regulatory network that are not present, or are different in *E. coli*.

Activation of the *rhIR'*-*lacZ* fusion was also observed when a plasmid encoding CRP, instead of Vfr, was used. These findings confirm that there is a direct interaction of Vfr with the *rhIR* promoter region, and suggest that it presumably occurs through its binding to a VBS, which has a similar sequence to *crp* boxes (Kanack *et al.*, 2006).

Identification of VBSs in the *rhIR* upstream region

Since the effect of Vfr on the expression of *rhIR* is positive, we performed the identification of VBS in the upstream region of this gene using the reported consensus sequence

for Vfr binding (Kanack *et al.*, 2006). This search revealed the presence of four imperfect putative Vfr-binding sequences (VBS1–VBS4) (Fig. 1, Table 2) that were not detected in a previous analysis (Kanack *et al.*, 2006), possibly because VBS2, VBS3 and VBS4 are encoded within the *rhIB* open reading frame and only intergenic regions were analysed, and due to differences of VBS1 sequence from the consensus sequence used to do the search (Table 2).

To test the functionality of these sequences we constructed mutations that eliminate each of the putative VBSs detected that are present in plasmid pPCS1002 (mutations are encoded in plasmids pPGC2001, pPGC2002, pPGC2003 and pPGC2004, described in Table 1) and tested their expression in *P. aeruginosa* PAO1 (Table 2). These experiments show that three of the putative VBSs (VBS1, VBS2 and VBS3) are functional for Vfr binding (data corresponding to VBS4 are discussed below), having a positive effect on *rhIR* expression, and that the greatest positive effect is shown by VBS1 (Table 2). To determine whether the results seen in *P. aeruginosa* as to VBS1 being the most important VBS for activation of *rhIR* were also seen in the *E. coli* model, we introduced the *rhIR* promoter sequences, carrying the same point mutations that were present in plasmids pPGC2001 and pPGC2002, into the *E. coli* chromosome, in the presence of plasmid pWNP28, which codes for Vfr. The results showed that VBS1 mutation has a significant effect on *rhIR* promoter expression in the *E. coli* background, while the effect of VBS2 inactivation was not significant (Fig. 2a). These results reinforce those obtained in the PAO1 background, showing that Vfr directly activates *rhIR* transcription and that the greatest effect of gene activation by this protein is seen by its interaction with VBS1 (Fig. 2a).

Since we found that VBS4 was not involved in activation of *rhIR* gene expression and this VBS is present downstream of promoter 4 (Fig. 1; Medina *et al.*, 2003a), we explored the possibility that this site indeed bound Vfr, but that its effect was negative on *rhIR* expression dependent on promoter 4. To study the effect of VBS4 on *rhIR* regulation we constructed a plasmid containing promoter 4 (pPGC2008, Fig. 3a, Table 1) and a derivative of this plasmid with three point mutations in VBS4 changing nucleotides described as necessary for Vfr DNA-recognition and binding (pPGC2008-1, Fig. 1, Table 1). Fig. 3(b) shows that *rhIR* expression from plasmid pPGC2008 is enhanced in the *vfr* mutant and that inactivation of VBS4 in plasmid pPGC2008-1 resulted in increased expression of *rhIR* in the wild-type strain. These results show that Vfr binding to VBS4 resulted in the repression of *rhIR* promoter 4, as was expected by the position of this sequence with respect to the transcription start site corresponding to promoter 4 (Figs 1 and 3a). However, the level of expression of promoter 4 is so low that the reduction of *rhIR* expression due to its repression by Vfr binding to VBS4 cannot be detected in the context of the entire *rhIR* upstream regulatory region encoded in plasmid pPCS1002 (Fig. 3, Table 2).

Table 2. Role of *vfr* and *las* boxes in *rhIR* expression in a *P. aeruginosa* PAO1 background

Sequences corresponding to the putative conserved half-sites are indicated in bold, and the invariant nucleotides of *las* boxes are shown in italics and underlined.

Plasmid	Vfr box	Vfr putative DNA-binding sequence	β -Galactosidase activity (Miller units) [†]
–	Vfr consensus sequence	WW TGNGA WNY:AGWT CACAT	
pPCS1002	VBS1	AAG GCTG CGC:GCT TGACAG	25 427 $\pm$ 3955
	VBS2	TC CTGA ACGG:TG CTGGCA T	
	VBS3	TG GTCGA ACG:TTG TCATAG	
	VBS4	AT TGTCACA –:ACC GCACAG	
pPGC2001	VBS1 [–]	-----:----TTG--	5600 $\pm$ 2080*
pPGC2002	VBS2 [–]	-----:----TGT–	16 745 $\pm$ 2963*
pPGC2003	VBS3 [–]	-----:----TT–T–	14 300 $\pm$ 2178*
pPGC2004	VBS4 [–]	-----:----TGT–	25 181 $\pm$ 2588
pPGC2005	VBS1 [–] / <i>las</i> box 1 [–] ‡	----T-----:----TTG--	2836 $\pm$ 783**
pPGC2006	VBS2 [–] / <i>las</i> box 3 [–] ‡	-----:--T--TGT–	5969 $\pm$ 1506***

[†]The statistical significance of the results was determined by Student's one-tailed *t*-test. The results obtained have a level of significance of $P < 0.001$ when compared with the results obtained with pPCS1002 (*); or when compared with pPGC2001 (**); or with pPGC2002 (***), respectively.

‡Sequences are shown in Fig. 1.

LasR-binding sequences overlap VBS1 and VBS2

Transcription of *rhIR* has been shown to depend on its activation by LasR/3O-C12-HSL (Latifi *et al.*, 1996; Medina *et al.*, 2003a). Two *las* boxes were originally described in the *rhIR* promoter region by analysing the expression of *rhIR*–*lacZ* fusions in *E. coli* (Medina *et al.*, 2003a). One of these *las* boxes, located at –42 nt from the promoter 1 transcription start site (*rhIR*-1 *las* box, Fig. 1), was proposed to be responsible for activation of *rhIR* transcription by LasR, while the putative *las* box located between –364 and –379 nt from the translation start site (*rhIR*-2 *las* box, Fig. 1) was proposed to have a negative effect on *rhIR* transcription. We constructed point mutations that eliminate the *rhIR*-2 *las* box in pPGC2008 and did not find any significant change in *rhIR* expression from this mutated plasmid (pPGC2008-2, Table 1) (data not shown).

When LasR/3O-C12-HSL binding to DNA was studied *in vivo* by immunoprecipitation of chromatin, a third *las* box was described (Gilbert *et al.*, 2009). This *las* box (*rhIR*-3 *las* box, Fig. 1) was proposed to be the site for *rhIR* activation, but this has not been experimentally validated.

To determine the functionality of the *rhIR*-1 *las* box for LasR/3O-C12-HSL binding *in vivo* we constructed an artificial *lacZ* fusion inserted into the *E. coli* MC4100 chromosome. This fusion was constructed by inserting the 19 nt sequence of this putative LasR-binding site (*rhIR*-1 *las* box, Table 3) between the –10 and –35 RNA polymerase consensus binding sequences. Thus, using this construct we can measure the binding of a protein to the DNA sequence present between the RNA polymerase –10 and –35 binding sequences, by the reduction of *lacZ* expression. In this experiment (Table 3) we used as positive control the DNA sequence of the *lasI* *las* box that is

specifically bound by LasR/3O-C12-HSL (Schuster *et al.*, 2004), and as negative control for LasR/3O-C12-HSL binding and control of binding specificity we used the *las* box of *rhIA* that is only bound by RhlR in both the presence and absence of C4-HSL (in the first case it activates transcription, while in the second one it acts as a repressor: Medina *et al.*, 2003b). It has been recently reported that some structural characteristics of LasR that affect its capacity to reversibly bind 3-O-C12-HSL, and ultimately to bind *las* boxes, are not maintained in the purified protein (Sappington *et al.*, 2011). Thus it is preferable to use *in vivo* systems to determine LasR DNA-binding specificity rather than *in vitro* systems such as electrophoretic mobility shift assays.

It has been reported that SdiA, an *E. coli*-encoded protein of the LuxR family, can interfere with RhlR-based biosensors designed to measure C4-HSL (Lindsay & Ahmer, 2005); however, we detected no interference of SdiA in the assays used to measure RhlR binding to *las* boxes as judged by the lack of *lacZ* repression in the absence of RhlR upon the addition of C4-HSL to strain MC4100 containing the chromosomally encoded *lacZ* fusions with different *las* boxes (data not shown).

The results in Table 3 clearly show that only LasR/3O-C12-HSL and not RhlR/C4-HSL was able to specifically bind to the *rhIR*-1 *las* box DNA sequence. It is not clear why the LasR/3O-C12-HSL complex was not found bound to the *rhIR*-1 *las* box when chromatin immunoprecipitation was studied (Gilbert *et al.*, 2009), but it may be that this complex is more unstable than the one formed between LasR/3O-C12-HSL and the *rhIR*-3 *las* box DNA sequence.

When the sequence of the *rhIR* promoter region was analysed, it was apparent that VBS1 overlaps the *rhIR*-1 *las* box, and

Fig. 3. Vfr represses the expression of *rhlR* promoter 4 by binding to VBS 4. (a) Diagram, not to scale, of the *rhlR* promoter 4 putative Vfr-binding site VBS4. (b) Expression of a *lacZ* fusion to *rhlR* promoters 3 and 4 (plasmid pPGC2008) in wild-type strain PAO1 and its *vfr* isogenic mutant, and the expression of plasmid pPGC2008-1, which contains the same DNA region, but carrying mutations that inactivate the VBS4 sequence, in wild-type strain PAO1. The statistical significance of the results was determined by Student's one-tailed *t*-test. The results obtained have a level of significance of $P < 0.001$ when compared with plasmid pPGC2008 expressed on PAO1 (*) and of $P < 0.001$ when compared with the expression in the PAO1 background (**).

that VBS2 also has some bases in common with the *rhlR*-3 *las* box (Fig. 1). To determine whether the same DNA sequences were involved in the binding of both Vfr and LasR transcriptional regulators, the point mutations that eliminate *vfr* boxes were used to determine the LasR/3O-C12-HSL activation of *rhlR*-*lacZ* fusions inserted into the *E. coli* chromosome (strains TGC001 and TGC002, eliminating VBS1 and VBS2, respectively, Fig. 2b). It is important to point out that these mutations were not located in positions reported to be essential for the binding of LasR (Soberón-Chávez *et al.*, 2005).

Fig. 2 shows that in the *E. coli* background the same DNA sequences as found to be involved in Vfr binding (VBS1

Table 3. Percentage repression of the chromosome-encoded artificial *lacZ* fusions containing *las* box sequences between the -10 and -35 promoter sequences, through binding of a *P. aeruginosa* quorum-sensing regulatory protein

<i>las</i> box	LasR		RhIR	
	3O-C12-HSL (5 μM)	No addition	C4 (10 μM)	No addition
<i>rhlR</i> -1	40	0	0	0
<i>lasI</i>	70	0	0	0
<i>rhlA</i>	0	0	50	50

and VBS2, Table 2) were involved in LasR/3O-C12-HSL activation of *rhlR* expression. These results also show that both *las* box *rhlR*-1 and *las* box *rhlR*-3 are functional for LasR-dependent *rhlR* activation (Fig. 2b, Table 2).

We have interpreted the results in Table 2 on the basis of the interaction of Vfr with VBS1 and VBS2, but we cannot rule out that the observed effect was due, at least in part, to the inactivation of *rhlR*-1 and *rhlR*-3 *las* boxes since, as has been shown, the binding sites for these two regulatory proteins overlap. To determine whether complete inactivation of *las* box sequences by mutation of the indispensable C nucleotide at position 3 had any effect on *rhlR* expression, we constructed this mutation in the plasmids carrying inactive VBS1 and VBS2 (plasmids pPGC2005 and pPGC2006, Table 2). The results in Table 2 indicate that the effect of inactivation of both Vfr- and LasR-binding sequences is additive, suggesting that interaction with both transcriptional regulators is important for the activation of *rhlR*.

In summary, our results show that there are four functional binding sites for Vfr in the *rhlR* promoter region (Fig. 2, Table 2). Two of them (VBS1 and VBS2), located in the *rhlB*-*rhlR* intergenic region, have a positive effect on *rhlR* expression, and partially overlap the LasR/3O-C12-HSL-binding sites (*rhl*-1 and *rhl*-3 *las* boxes) (Fig. 1). Our results show that these sequences are involved in Vfr- and LasR-dependent regulation, and that there is a synergistic effect of the two types of regulation (Table 2).

RhIR positively autoregulates its expression from the *rhlA* promoter

Analysis of the *rhlR* upstream sequence (Fig. 1) shows that several of the regulatory elements involved in the regulation of this gene are encoded inside the *rhlB* coding region, and that there is no apparent transcriptional terminator upstream of *rhlR*. In addition, *rhlB* is transcribed from the *rhlA* promoter, which is specifically activated by RhIR/C4-HSL (Medina *et al.*, 2003b). This arrangement led us to propose the hypothesis that *rhlR* could be transcribed either from the four promoters described above that we and others have studied using plasmids (Latifi *et al.*, 1996; Medina *et al.*, 2003a) or from the upstream *rhlA* promoter, producing a positive loop of autoregulation. To test this hypothesis we used an *rhlR* mutant that has an in-frame *lacZ* fusion (PW6882) caused by insertion of a transposon, and expressed *rhlR* *in trans* from plasmid pGMYC (Fig. 4a). We found that β-galactosidase levels almost doubled in the presence of pGMYC, showing that RhIR positively autoregulates its expression. Overexpression of LasR or Vfr also had a positive effect on *rhlR* expression in the PW6882 background, but this effect was not as high as that seen with RhIR (Fig. 4), suggesting that the positive-feedback autoregulation of RhIR plays a major role in *rhlR* expression.

It is also interesting to point out that the introduction of a *vfr* mutation into strain PW6882 (PAO1-WV, Table 1, Fig. 4a) reduces the expression of *rhlR*, but does not cause its

Fig. 4. *rhIR* is expressed from the *rhIA* promoter, forming a positive autoregulatory loop. (a) Expression of the *rhIR* promoter in the PAO1 chromosome background, using the transposon-insertion *rhIR* mutant (PW6882, Table 1), which has an in-frame *lacZ* fusion, in the presence of plasmids pGMYC, pMT1 and pWNP28, which express the RhlR, LasR and Vfr proteins, respectively, *in trans*. A PW6882 *vfr* mutant derivative (PAO1-WV, Table 1) was used as a control for the amount of *rhIR* expression that depends on Vfr and is independent of the RhlR positive autoregulatory loop. (b) The polarity of an *rhIA* mutation was determined by the expression of the same chromosomally encoded *rhIR-lacZ* fusion (PW6882) with an *rhIA::Gm* insertion (PAO-WA, Table 1) in the presence of plasmid pGMYC and the vector pUCP20. The statistical significance of the results was determined by Student's one-tailed *t*-test. The results obtained have a level of significance of $P < 0.001$ when compared with plasmid pUCP20 (*), of $P < 0.001$ when compared with plasmid pMMB66EH (**), and of $P < 0.001$ when compared with strain PW6882 in the case of panel (a); and of $P < 0.001$ when compared to PW6882/pGMYC (*) in the case of panel (b). –, No plasmid.

complete silencing, showing that there is a significant *rhIR* expression that is both Vfr-independent and LasR-independent (*lasR* is not expressed in a *vfr* mutant). The contribution to *rhIR* transcription in the *vfr* mutant of promoter 4 (repressed by Vfr) and the constitutive promoter 2 remains to be determined.

To further support the existence of the *rhIABR* operon we determined by RT-PCR the presence of a transcript extending from *rhIA* to *rhIR* (Fig. 5). The detection of this transcript further supports the existence of a positive

Fig. 5. Identification of the mRNA extending from *rhIA* to *rhIR* using RT-PCR. (a) Presence of the *rhIABR* transcript in wild-type strain PAO1 and its absence in the *rhIA* mutant PW6887. Lanes: 1, PCR product using PAO1 chromosomal DNA as template; 2, negative control consisting of RNA purified from PAO1 cells cultured in PPGAS medium to an OD₆₀₀ of 1.5, incubated in conditions used to perform the RT-PCR, but lacking reverse transcriptase; 3, RT-PCR product from RNA purified from PAO1 cells cultured in PPGAS medium to an OD₆₀₀ of 1.5 using primers F-*rhIA* and R-*rhIABR*; 4, RT-PCR product from the same RNA sample as used for lane 3, but using primers F-*rpoD* and R-*rpoD*; 5, RT-PCR product from RNA purified from PW6887 (*rhIA::ISphoA/hah*) cultured in PPGAS medium to an OD₆₀₀ of 1.5 using primers F-*rhIA* and R-*rhIABR*; 6, RT-PCR product from the same RNA sample as used for lane 5, but using primers F-*rpoD* and R-*rpoD*; 7, DNA size standard. (b) Amplification by PCR of the RT-PCR product shown in lane 3 of (a) using primers R-*rhIABR* and F'-*rhIA*. Lanes: 1, PCR product using RT-PCR product of lane 3 as template; 2, reverse transcription reaction, where reverse transcriptase was omitted, as a negative control; 3, PCR product using PAO1 chromosomal DNA as template; 4, DNA size standard.

autoregulatory loop in *rhIR* expression through activation of the *rhIA* promoter. This observation also predicts that an *rhIA* mutant should have a reduction of RhlR concentration and could explain the reduced pyocyanin production of the *rhIA::Gm* mutant reported previously (Rahim *et al.*, 2001). To support this hypothesis, we confirmed that indeed another *rhIA* mutant (PW6887, *rhIA::ISphoA/hah* with the transposon inserted in the opposite direction to *rhIA* transcription; Table 1) produced reduced amounts of pyocyanin: strain PAO1 produced $0.814 \pm 0.043 \mu\text{g ml}^{-1}$ while its *rhIA* mutant derivative produced $0.017 \pm 0 \mu\text{g ml}^{-1}$. Furthermore, introduction of plasmid pGMYC expressing *rhIR* from the *lac* promoter to strain PW6887 (*rhIA* mutant) fully restored its ability to produce pyocyanin ($1.11 \pm 0.017 \mu\text{g ml}^{-1}$).

Rhamnolipids have been reported to participate in the solubility of the *Pseudomonas* quinolone signal (PQS) that is involved in the regulation of pyocyanin (Calfee *et al.*,

2005), so the reduced pyocyanin production by *rhIA* mutants could have been due to the lack of rhamnolipid production. However, the fact that pyocyanin production is restored in the *rhIA rhIR* double mutant by the expression *in trans* of *rhIR* shows that rhamnolipid production is not essential for the production of this phenazine.

These results suggest that the lack of pyocyanin production in the *rhIA* mutant is due to polarity of the mutation, which results in reduced levels of RhIR, and that expression of *rhIR* from a plasmid compensates for this reduction. The polarity of *rhIA* mutation on *rhIR* expression was shown by determining that in strain PAO-WA (PW6882 *rhIR* mutant with a Gm cassette inserted in *rhIA*, Table 1) the introduction of plasmid pGMYC expressing RhIR has no effect on *rhIR* expression (Fig. 4b). The polarity of the *rhIA::Gm* mutation on *rhIR* expression was confirmed by the absence of the transcript comprising the *rhIABR* operon in mutant PW6887 as determined by RT-PCR (Fig. 5).

Integrative model of *rhIR* transcriptional regulation

In this work we have shown that Vfr, LasR and RhIR have a role in the regulation of *rhIR* transcription and we propose a

kinetic model of *rhIR* expression that integrates all these elements throughout the *P. aeruginosa* growth curve (Fig. 6).

This working model is as follows. When *P. aeruginosa* is growing at an exponential rate, Vfr is produced at low levels (Heurlier *et al.*, 2003) and *rhIR* is transcribed at a reduced rate from promoter 2, which is constitutive, and from promoter 4 (Vfr is not bound to VBS4 and there is no repression of transcription of this promoter); there are low levels of RhIR and the *rhIAB* operon is not transcribed. During the transition from exponential to stationary phase, Vfr concentration increases (Heurlier *et al.*, 2003) and expression of both *lasR* and *rhIR* is activated through direct binding of this protein to VBSs. The increased LasR concentration also leads to increased expression of *lasI*, *rhII* and *rhIR*; when threshold RhIR and C4-HSL concentrations are achieved, the *rhIA* promoter is activated by RhIR/C4-HSL, which in turns leads to increased expression of *rhIR* by a positive-feedback autoregulatory loop, as shown in Fig. 6.

It remains to be determined whether the activation of *rhIR* transcription by Vfr is dependent on the interaction of this protein with cAMP or is independent of the binding of this cyclic nucleotide as has been shown for Vfr-dependent *lasR* activation (Fuchs *et al.*, 2010).

Fig. 6. Schematic model of the mechanism of *rhIR* expression in *P. aeruginosa*.

It has been shown recently that the PqsE enzyme has a stabilizing effect on RhlR. The mechanism of this activation is still unknown since the substrate of this hydrolase has not been identified, but its effect can be seen in the *E. coli* background (Farrow *et al.*, 2008). We did not address the role of PqsE transcriptional activation of *rhlR* in this work, since it has been reported that the effect of PqsE on RhlR activity is not at the level of transcription. However, we would expect that if the stabilization of RhlR by PqsE activity increases its concentration, this increment of RhlR concentration would result in the increased autoregulatory activation of *rhlR* caused by increased expression of the *rhlA* promoter.

The results presented in this work enhance our understanding of the very complex regulatory mechanisms involved in *rhlR* transcription and provide an integrative picture of the *P. aeruginosa* QS response. The understanding of this regulatory network is of key importance for the development of strategies to control *P. aeruginosa* acute infections.

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