The *Pseudomonas aeruginosa* *rhlAB* Operon Is Not Expressed during the Logarithmic Phase of Growth Even in the Presence of Its Activator RhlR and the Autoinducer *N*-Butyryl-Homoserine Lactone

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The *Pseudomonas aeruginosa* *rhlAB* operon encodes the enzyme rhamnosyltransferase 1, which produces the biosurfactant mono-rhamnolipid; *rhlAB* induction is dependent on the quorum-sensing transcription activator RhlR complexed with the autoinducer *N*-butyryl-homoserine lactone (C₄-HSL). In this work we studied *rhlAB* induction in a *P. aeruginosa* and *Escherichia coli* background. We found that, in both bacteria, its expression is not induced during the logarithmic phase of growth even in the presence of RhlR and C₄-HSL. Additionally, we found that *rhlAB* expression is partially σ^s dependent.

Pseudomonas aeruginosa is an opportunistic pathogen causing serious nosocomial infections (5). The production of several of the virulence-associated traits created by this bacterium is regulated at the level of transcription by the so-called quorum-sensing response (8, 30).

Rhamnolipids are biosurfactants produced by *P. aeruginosa* (13) that are regulated by the quorum-sensing response dependent on the transcription activator RhlR (16) and the autoinducer (AI) *N*-butyryl-homoserine lactone (C₄-HSL) synthesized by RhlI (17). The *rhlAB* operon encodes rhamnosyltransferase 1, which is responsible for monorhamnolipid production from TDP-L-rhamnose and β -hydroxy-fatty acids (15), and *rhlC* encodes rhamnosyltransferase 2, which produces dirhamnolipid by using monorhamnolipid and TDP-L-rhamnose as substrates (22). The model for the transcription activation of *rhlAB* and *rhlC* is that with increasing bacterial cell density the concentration of C₄-HSL reaches a threshold level and then attaches to the transcription activator RhlR (16). The RhlR–C₄-HSL complex activates the transcription of the *rhlAB* operon (18, 20) and *rhlC* (22).

P. aeruginosa contains a second quorum-sensing regulon, consisting of the transcription regulator LasR, which is activated by the AI *N*-3-oxododecanoyl homoserine lactone (3-o-C₁₂-HSL). The transcription of several genes encoding virulence-associated traits is activated by the Las system (8, 31), and it has a central role in the transcription of *rhlR* (12, 20).

The aim of this work was to determine whether the expression of the *rhlAB* operon is dependent only on the presence of RhlR and C₄-HSL or whether other regulatory elements participate. To do this, we studied *rhlAB* expression along the growth curve of *P. aeruginosa* PAO1/pECP61.5 (*rhlA::lacZ* *ptac-rhlR*) (Table 1) adding C₄-HSL and 0.1 mM isopropyl- β -

D-thiogalactopyranoside (IPTG) from the onset of the culture on the phosphate-limited PPGAS medium used to produce rhamnolipids (32). It was apparent that *rhlAB* promoter is not expressed during the logarithmic phase of growth even in the presence of C₄-HSL and RhlR, as detected by Western immunoblotting (Fig. 1). To ensure that RhlR expression was not a limiting factor, IPTG was added from the beginning of the culture. We detected the presence of RhlR protein as early as 2 h after induction, corresponding to an optical density of 0.1 at 600 nm, while the expression of the *rhlA::lacZ* fusion was apparent until 6 h after induction, when the culture had an optical density of 1.7 (Fig. 1).

We determined that the lack of *rhlA::lacZ* expression during the exponential phase of growth was not due to a limitation of C₄-HSL concentration, since the same results were obtained when a 10-fold concentration of this AI was used (data not shown).

To carry out the immunodetection of RhlR, New Zealand rabbits were immunized with a protein fusion, ThioR-RhlR, in order to produce polyclonal antibodies. To construct a ThioR-RhlR protein fusion, the *rhlR* gene was PCR amplified. The product was digested with *Kpn*I and *Sal*I and cloned into plasmid pThioC (Invitrogen) digested with the same enzymes. *Escherichia coli* cultures were grown until they reached the indicated optical density at 600 nm. The cells were collected and lysed by boiling for 5 min in loading buffer (23). An equal amount of protein from each lysate was separated by sodium dodecyl sulfate–14% polyacrylamide gel electrophoresis. The proteins were transferred by electroblotting from the gel to Hybond-C nitrocellulose membranes (Amersham Life Science Corp.). RhlR was detected by using the rabbit polyclonal antiserum raised against ThioR-RhlR.

Different *P. aeruginosa* genes regulated by the quorum-sensing response have been classified depending on their response to 3-o-C₁₂-HSL and C₄-HSL and the time course of their induction (28). Several of the genes identified were not ex-

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TABLE 1. Strains and plasmid used in this study

Strain or plasmid	Relevant characteristics	Reference
<i>P. aeruginosa</i> strains		
PAO1	Wild-type strain	10
PAS1	PAO1 <i>rpoS</i> ::Gm mutant	This study
<i>E. coli</i> strains		
DH5 α	<i>supE44</i> Δ <i>lacU169</i> <i>hsdR17</i> <i>recA1</i> <i>endA1</i> <i>avrA96</i> <i>thi1</i> <i>relA1</i>	23
MC4100	F ⁻ Δ (<i>argF-lac</i>) <i>thiA</i>	24
JV1065	MC4100 derivative λ pir <i>rpoS13</i> ::Tn10	27
Plasmid		
pECP61.5	Contains an <i>rhlA</i> :: <i>lacZ</i> translation fusion and <i>rhlR</i> under <i>ptac</i> , Ap ^r	18

pressed during the exponential phase of growth (classified as types 2 and 4). According to this study (28), the *rhlAB* promoter was classified as a type 3 promoter. This type of promoters responds only in the presence of both AIs and is expressed from the onset of the culture (28). All genes regulated by RhlR and C₄-HSL are expected to be dependent on both AIs in the experimental conditions promulgated by Whiteley et al. (28), since the expression of *rhlR* is dependent on 3-o-C₁₂-HSL (12, 20). The results presented here are in contradiction with this observation, since we found that *rhlAB* is not expressed in the logarithmic phase of growth even in the presence of RhlR and C₄-HSL. To determine whether the differences in our results with those previously reported (28) could be due to the lack of expression of a factor different from RhlR, depending on the presence of 3-o-C₁₂-HSL, we studied *rhlAB* expression on PPGAS medium supplemented with both AIs. Our data show that in the PAO1 background *rhlAB* is not expressed during the logarithmic phase of growth even when C₄-HSL and 3-o-C₁₂-HSL are present (Fig. 1).

Another possibility to explain the discrepancy between our results and the previously reported data (28) was the use of different culture media to study *rhlAB* expression. To explore this possibility we did the experiments on Luria-Bertani (LB) medium (14) used by Whiteley et al. (28). We found that *rhlAB* was not expressed on the logarithmic phase of growth of PAO1 cells grown on LB medium even when it was supplemented with C₄-HSL or both C₄-HSL and 3-o-C₁₂-HSL (Fig. 1). It is apparent that *rhlAB* is expressed at a lower level on LB medium; we obtained considerably lower β -galactosidase activities than those obtained when this bacterium is grown on PPGAS medium (Fig. 1). It can be concluded from our results that the *rhlAB* operon belongs to the type 4 group of quorum-sensing-regulated genes according to the classification of Whiteley et al. (28). It is possible that *rhlAB* expression during the exponential phase of growth reported (28) was due to a particular status of the quorum-sensing response in the *lasI*, *rhlI*-derived PAO1 mutant, supplemented with both AIs, that was used in that study.

Several gene products that exert a negative effect on *P. aeruginosa* quorum-sensing response have been reported (2, 4, 7, 21, 25, 29). The products of these genes have recently been postulated to participate in preventing the early expression of type 2 and 4 quorum-sensing-regulated genes (19). Most of these negative regulators, such as RpoS (25, 29), RsmA (21), DksA (2), and QscR (4), exert their negative regulatory effect through the repression of one or both genes encoding AI

synthetase *lasI* or *rhlI* and are thus not expected to have any repressing activity when AIs are supplemented to the culture medium, as in the case that we are studying. It has very recently been shown that *P. aeruginosa* MvaT has a negative effect on the expression of different quorum-sensing-regulated traits and could have a minor role in preventing the expression of the *lecA* gene during the exponential phase of growth in the presence of AIs (7, 19). The mechanism of MvaT control of quorum-sensing-regulated gene expression is unknown, but it is known that this regulator is present only in *Pseudomonas* (7).

To determine whether the regulatory elements (a repressor or the lack of an activator) that prevent *rhlAB* expression during logarithmic phase of growth were present only in the *P. aeruginosa* genetic background, we studied the kinetics of *rhlAB* expression along the growth curve of *E. coli* DH5 α /pECP61.5. It was apparent that this operon was not expressed during the exponential phase of growth, as was found in *P. aeruginosa*, even in the presence of C₄-HSL and RhlR (Fig. 2). These results show that the presence of RhlR and C₄-HSL is a necessary condition for *rhlAB* expression but that there is a regulatory element that prevents the expression of this operon during the exponential phase of growth.

FIG. 1. (A) Expression of the *rhlA*::*lacZ* translational fusion encoded in plasmid pECP61.5 in *P. aeruginosa* strain PAO1 (rhombus), as a function of the optical density at 600 nm (OD_{600}) of a culture grown on PPGAS (open symbols) and LB (closed symbols) media at 37°C and on these media supplemented with 10 μ M C₄-HSL and 0.1 mM IPTG (circles) or 10 μ M concentrations of both C₄-HSL and 3-o-C₁₂-HSL and 0.1 mM IPTG (triangles). β -Galactosidase activity is expressed in Miller units (14). (B) Immunoblotting of the RhlR protein along the growth curve of strain PAO1 on PPGAS medium (32) shown in panel A. Lanes 1 to 3 show RhlR expressed in cells grown on this medium at optical densities at 600 nm of approximately 0.1, 0.7, and 2.4, respectively. Lanes 4 to 6 present RhlR expressed in cells grown on PPGAS medium supplemented with 10 μ M C₄-HSL and 0.1 mM IPTG at the same cell densities. The size of the molecular markers is shown at the left side of the lane (M_r).

FIG. 2. (A) Expression of the *rhlA::lacZ* translational fusion encoded in plasmid pECP61.5 in *E. coli* strain DH5 α as a function of the optical density at 600 nm (OD_{600}) of a culture grown at 37°C on LB medium (14) (rhombi) and on this medium supplemented with 10 μ M C₄-HSL and 0.1 mM IPTG (circles). β -Galactosidase activity is expressed in Miller units (14). (B) Immunoblotting of the RhlR protein along the growth curve of strain DH5 α shown in panel A. Lanes 1 to 3 show RhlR expressed in cells grown on LB medium at an optical density at 600 nm of approximately 0.4, 1.6, and 3.4, respectively. Lanes 4 to 6 present RhlR expressed in cells grown on LB supplemented with 10 μ M C₄-HSL and 0.1 mM IPTG at the same optical densities. The size of the molecular markers is shown at the left side of the lane (M_r).

We further characterize the exponential-phase silencing of the *rhlAB* promoter in an *E. coli rpoS* mutant, and we found that it also occurs but that the level of *rhlA::lacZ* expression on the stationary phase of growth was considerably lower (Fig. 3A), suggesting that in *E. coli* this sigma factor is involved, directly or indirectly, in *rhlAB* expression during the stationary phase of growth.

It has been reported that in *P. aeruginosa* an *rpoS* mutation causes increased pyocyanin production (25, 29), presumably due to increased production of C₄-HSL by this mutant (29). However some quorum-sensing-regulated traits, such as exotoxin A, are produced at a lower level (25), and the production of lectins, for example, is completely abolished, since the *lecA* gene has a σ^s -dependent promoter (30).

We constructed a PAO1 *rpoS::Gm* mutant called PAS1 (Table 1) by the following procedure: a PCR product containing the *rpoS* gene was digested with *Pst*I and *Sma*I and was subsequently ligated into pBluescript II KS (Stratagene). A 1.1-kb Gm cassette from pBSL141 (1) was inserted into the unique *Hinc*II site of pCOC1 within *rpoS*. The resulting knockout construct was transformed into *P. aeruginosa*, and mutants

were selected as gentamicin resistant and carbenicillin sensitive. PCR and Southern blot analysis was performed to confirm the presence of the Gm cassette within the chromosome of the *P. aeruginosa rpoS* mutant (data not shown).

Strain PAS1 produces, as expected, high levels of pyocyanin measured as described previously (6) (PAO1 produces 0.46 μ g/ml, while PAS1 produces 0.71 μ g/ml at 8 h of growth in PPGAS medium at 37°C). PAS1 mutant produces a rhamnolipid level similar to that produced by PAO1, determined by the orcinol method (3) (the wild-type strain produces 172 μ g/ml, while the *rpoS* mutant produces 161 μ g/ml under the same conditions used to measure pyocyanin). We found that in PAS1, as in the *E. coli rpoS* mutant, the *rhlAB* operon is not

FIG. 3. Effect of an *rpoS* mutation on the expression of the *rhlA::lacZ* fusion encoded in plasmid pECP61.5 in *E. coli* (A) and *P. aeruginosa* (B) as a function of the optical density at 600 nm (OD_{600}) of cultures grown on LB (14) and PPGAS (32) media, respectively. Symbols correspond to *E. coli* strain MC4100 grown on LB medium (rhombi) and on this medium supplemented with 10 μ M C₄-HSL and 0.1 mM IPTG (circles); the *rpoS*-derived mutant JV1065 was grown on LB medium (squares) and on the same medium supplemented with 10 μ M C₄-HSL and 0.1 mM IPTG (triangles). *P. aeruginosa* PAO1 was grown on PPGAS medium (rhombi) and on the same medium supplemented with 10 μ M C₄-HSL and 0.1 mM IPTG (circles); its *rpoS*-derived mutant PAS1 was grown on PPGAS medium (squares) and on the same medium supplemented with 10 μ M C₄-HSL and 0.1 mM IPTG (triangles). β -Galactosidase activity is expressed in Miller units (14).

expressed in the exponential phase of growth and that its expression is significantly lower in stationary phase (Fig. 3B). These results show that *rhlAB* expression is partially σ^S dependent, even in the presence of an increased C_4 -HSL concentration (31).

It was reported that the expression of the *E. coli relA* gene, encoding the ppGpp synthase, during the exponential growth of *P. aeruginosa* PAO1 causes an early expression of different traits involved in the quorum-sensing response, including AIs (26). One possible explanation for the exponential-phase silencing of the *rhlAB* promoter is that it is dependent on the stringent response and that, hence, this operon will not be expressed when there is no limitation of bacterial growth. This would explain the dependence of its expression on σ^S , since it has been shown in *E. coli* that gene expression by this alternative sigma factor is tightly linked to the stringent response (9, 11). We have preliminary evidence suggesting that, in *P. aeruginosa* as in *E. coli*, the *rhlAB* promoter is not silenced during exponential-phase growth in minimal media (data not shown). These results suggest that *P. aeruginosa* nutritional status, maybe sensed through the stringent response, is involved in the regulation of *rhlAB* expression.

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