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Interactions of the quorum sensing regulator QscR: interaction with itself and the other regulators of *Pseudomonas aeruginosa* LasR and RhlR

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Summary

Pseudomonas aeruginosa **controls the production of many exoproteins and secondary metabolites via a hierarchical quorum sensing (QS) regulatory cascade involving the LuxR-like proteins LasR, RhlR and their cognate signal molecules *N*-(3-oxododecanoyl)-L-homoserine lactone (3O-C12-HSL) and *N*-(butanoyl)-L-homoserine lactone (C4-HSL). The finding of a third LuxR-type protein in *P. aeruginosa*, QscR, adds further complexity to this regulatory network. It has been shown previously that QscR represses transcription of three QS-controlled gene clusters, *phz* (phenazine), *hcn* (hydrogen cyanide) and *qsc105* (Chugani, Whiteley, Lee, D'Argenio, Manoil, and Greenberg, 2001, *Proc Natl Acad Sci USA* 98: 2752–2757). In this study, we identify two novel QscR targets these are *lasB*, encoding the extracellular elastase, and the second phenazine gene cluster, both of which are downregulated by QscR. In addition, we show that QscR synthesis is regulated by the two-component response regulator GacA. Taking advantage of the *in vivo* fluorescence anisotropy technology that we have developed, we show that QscR can be found in several different types of association. Indeed, we identify QscR multimers in the absence of any acyl-HSL, lower order QscR oligomers associated either with C4-HSL or 3O-C12-HSL and QscR-containing heterodimers with LasR or RhlR. The formation of heterodimers between QscR and LasR or RhlR, in the absence of acyl-HSLs, is a very exciting, new result that should improve our understanding of the QscR network and its relationship to the production of *P. aeruginosa* virulence factors.**

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Introduction

Pseudomonas aeruginosa is a versatile bacterium that can be found in many different environments. It is also an emerging opportunistic human pathogen. The expression of multiple virulence and survival genes in *P. aeruginosa* is cell density-dependent and relies on the cell-to-cell communication system. Two quorum-sensing (QS) systems that belong to the LuxR/I family have been well characterized in *P. aeruginosa*. The *las* system consists of the transcriptional activator protein LasR, the acyl-HSL synthase LasI and the acyl homoserine lactone (acyl-HSL) 3O-C12-HSL. The *rhl* system consists of the regulator RhlR, the synthase RhlI and C4-HSL. The *las* and *rhl* quorum-sensing circuitries operate in a hierarchical cascade responsible for regulating the expression of many virulence determinants, secondary metabolites, the type-II secretion machinery, stationary phase genes and genes involved in biofilm formation (for review, see Withers *et al.*, 2001).

It is clear that quorum-sensing regulators are almost always integrated into other regulatory networks. Such integration expands the range of environmental signals that influence target gene expression beyond cell density. Among the various examples of QS regulation systems, it is evident that this integration happens through regulation of the genes encoding the LuxR-type regulator and the LuxI-type acyl-HSL synthase (for review, see Miller and Bassler, 2001). In *P. aeruginosa*, there is an interesting co-ordination of the two QS systems, the LasR protein regulating expression of the *rhl*R and the *rhlI* genes (Latifi *et al.*, 1995). The *lasR* gene itself is positively controlled by the GacA two-component response regulator, a common regulator of virulence in *P. aeruginosa* (Reimmann *et al.*, 1997) and also by a CRP homologue called Vfr (Albus *et al.*, 1997). The environmental conditions to which these regulators respond and their influence are not yet fully understood.

The recent identification of a third LuxR-type protein, called QscR (*q*uorum *s*ensing *c*ontrolled *r*epressor) adds further complexity to this regulatory pathway in *P. aeruginosa* (Chugani *et al*., 2001). The analysis of the *Pseudomonas* genome revealed a gene coding for a homologue of LasR and RhlR but no additional genes coding for LasI and RhlI homologues. The predicted ORF consists of 714 nucleotides and codes for a polypeptide of 27 236 Da. A sequence comparison shows that it is 32% identical to RhlR and 29% identical to LasR. The QscR protein presents the two domains characteristic of the LuxR-type regulators: an acyl-HSL binding domain and a DNA binding domain. Chugani *et al*. (2001) showed that QscR represses transcription of three QS-controlled clusters of genes, *phz* (phenazine), *hcn* (hydrogen cyanide) and *qsc105*. This repression appears to be effective in the logarithmic phase of growth. Furthermore, the *qscR* mutant produces the two acyl-HSLs prematurely during the log phase.

To understand in greater detail the mechanisms by which QscR functions, major questions have to be addressed. First, we extended the range of targets regulated by QscR showing that proteases, more precisely elastase, and the second phenazine cluster are also negatively regulated by QscR. We also show that QscR synthesis is regulated by the two-component response regulator GacA. Taking advantage of a novel technique set up for the study of RhlR (Ventre *et al.,* 2003) we have started to address questions about the mechanism of QscR action. We show that QscR can exist as multimers or as heterodimers, depending on the presence of LasR and RhlR, and on the presence of the two acyl-HSLs normally produced by *P. aeruginosa*.

Results

QscR affects protease synthesis and negatively controls lasB *expression at the transcriptional level*

To investigate the pleiotropic role of QscR in the regulation of virulence determinants other than pyocyanine and cyanide, we examined the influence of QscR on protease synthesis. *Pseudomonas aeruginosa* synthesizes several proteases implicated in virulence and infectious process. A *qscR* chromosomal insertion mutant (PAOQ$^-$) was constructed in strain PAO1, as described in *Experimental procedures*, and proteolytic activity was analysed on skimmed milk agar plates. The halo surrounding the colony is larger in case of the *qscR* mutant (PAOQ$^-$) than in the wild-type strain (data not shown). In contrast, PAOQ$^-$ containing the plasmid pMCQ1004 (*qsc*R) developed wild-type haloes on skimmed-milk plates showing a good restoration of the protease phenotype in the PAO1 strain (data not shown). Furthermore, overexpression of *qscR* in strain PAO1 decreased the halo of proteolytic activity (data not shown). These results provide evidence for a globally negative effect of QscR on protease production.

Elastase (LasB) is the major protease synthetized by *P. aeruginosa*. We therefore investigated the effect of QscR more directly on elastase production. For this purpose, the production of extracellular LasB was followed throughout the growth of both PAO1 and PAOQ$^-$ strains in an immunoblot experiment. As shown in Fig. 1C, LasB was produced maximally during the stationary phase of growth when considering the PAO1 strain. This pattern of production is in agreement with the expected pattern of a gene product that is QS regulated. Interestingly, in the case of the *qscR* mutant, LasB was produced earlier in the growth and was much more abundant during the stationary phase than in the parental strain. This experiment thus clearly showed that QscR repressed LasB production in *P. aeruginosa* strain PAO1.

To investigate the level at which the modulatory effect of QscR was exerted, we examined the expression of a *lasB-lacZ* transcriptional fusion. Under the same growth conditions as that used for the immunoblot experiment, the expression of the fusion was induced prematurely and β-galactosidase activity had accumulated to a level threefold higher than that of the wild-type by the end of the exponential phase (Fig. 1D). It should be observed that the shift of expression occurred not exactly during the exponential growth phase but rather at the beginning of the transition into the stationary phase. There was a very good correlation between the expression pattern of the *lasB–lacZ* fusion and the production of the LasB protein (Fig. 1C and D). These results confirmed that QscR negatively controls elastase production and demonstrated that this control is exerted at a transcriptional level.

QscR negatively controls the expression of the two phenazine operons

Previously it has been clearly shown that pyocyanine synthesis is regulated both by quorum sensing (Latifi *et al*., 1995) and by the two-component signal transduction system GacA-GacS (Reimmann *et al*., 1997). *Pseudomonas aeruginosa* contains two homologous clusters of seven genes *phzA1-G1* and *phzA2-G2*. The two *phz* operons are 98% identical at the DNA level, but they differ markedly in their promoter regions. Recently, Whiteley *et al*. (1999) identified the *phzA1-G1* operon among the genes controlled by quorum sensing in *P. aeruginosa* and, according to Chugani *et al*. (2001), QscR negatively regulates expression of *phzA1-G1.* Recent studies by Mavrodi *et al*. (2001) showed clearly that both operons are functional when under the control of the *lac* promoter and expressed in a heterologous host *E. coli*. Moreover, the *qscR* gene is located just upstream to the cluster *phzA2-G2*. We

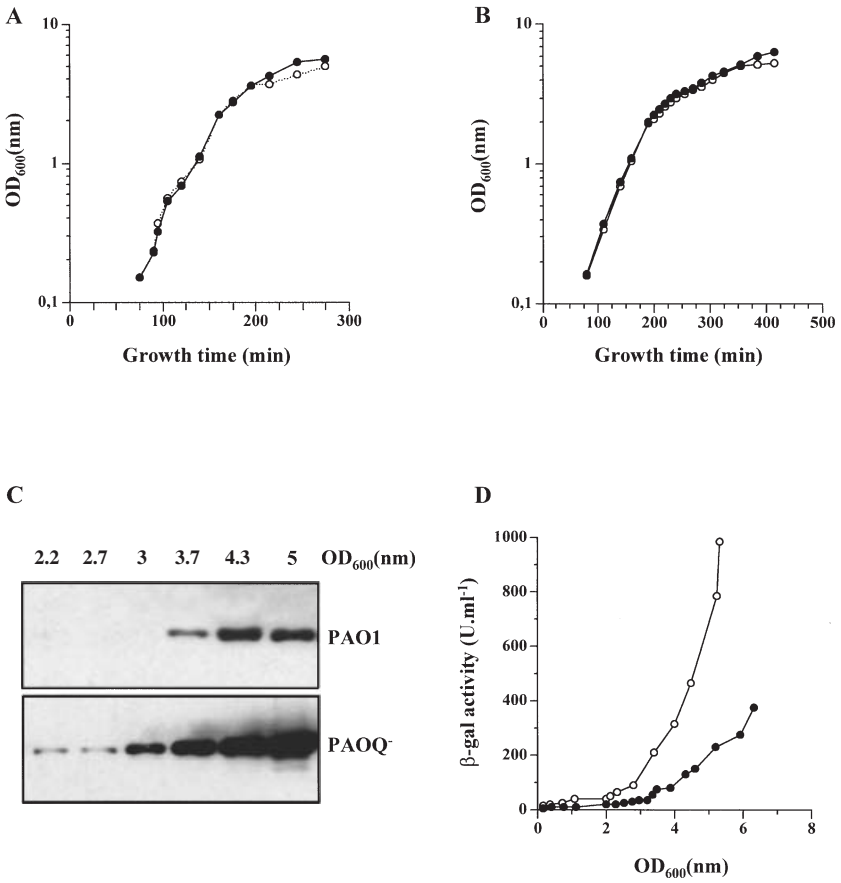

Fig. 1. Effect of *qscR* mutation on elastase production.
A. Growth curves of PAO1 (solid circles) and PAOQ⁻ (open circles) in LB medium. Samples of cell-free supernatant taken during the growth were used for immunoblot experiments (C).
B. Growth curves of PAO1 (solid circles) and PAOQ⁻ (open circles) containing *lasB-lacZ* reporter plasmid pMAL.B, in LB medium. Samples taken during the growth were used to determine β-galactosidase activity (D).
C. Growth-phase dependency of the LasB protein level in PAO1 and PAOQ⁻ strains. Supernatants corresponding to a constant mass of cells were analysed by an immunoblot against LasB elastase.
D. Expression of *lasB-lacZ* fusion into PAO1 (solid circles) and PAOQ⁻ (open circles).

therefore addressed the question of the regulation by QscR of this second operon *phzA2-G2* in strain PAO1.

PAO1 and its *qscR* derivative mutant were grown in glycerol-alanine medium, a specific medium which facilitates pyocyanin detection at 520 nm. The induction of pyocyanin synthesis appeared at an $OD_{600} = 4.5$ in the PAO1 wild-type strain, which correspond to the stationary phase (Fig. 2A and C). In the *qscR* mutant, induction of pyocyanin is advanced and pyocyanin appeared at an $OD_{600} = 3$ which corresponds to the transition between the exponential phase and the stationary phase. Complementation of the *qscR* mutation almost completely restored the level and timing of pyocyanin production. Furthermore, overexpression of *qscR* in PAO1 further delayed pyocyanin synthesis ($OD_{600} = 5$).

To understand the functioning of the two *phz* operons, two transcriptional fusions were constructed, *phzA1-lacZ* and *phzA2-lacZ*. Each fusion was introduced into PAO1 strain and into the *qscR* mutant strain and expression of the fusions was studied in each strain. As observed previously (Chugani *et al*., 2001), we found that the *phzA1-lacZ* fusion was negatively regulated by QscR (data not shown). Interesting new results were observed for the *phzA2-lacZ* fusion. First of all, it is important to point out that the level of expression of the *phzA2-lacZ* fusion is 10 times lower than that of the *phzA1-lacZ* fusion (data not shown). As can be seen in Fig. 2D, the *phz*A2 operon was not significantly expressed in PAO1 strain when cells were grown in glycerol-alanine medium. In the *qsc*R mutant, expression of this operon was greatly enhanced, by a factor of 13 (Fig. 2D). The same results were observed when cells were grown in LB medium up to an $OD_{600} = 6$ and, in this case, the enhancement factor observed was of 50 (data not shown). It is clear that the *phz2* operon is repressed by QscR and can reach a significant level of expression when *qscR* is inactivated. Therefore, the two *phz* operons are both under the negative control of QscR, this regulation appears to be stronger for *phz2* than *phz1*.

QscR expression is modulated by the two-component response regulator GacA

As seen in the previous experiments, QscR regulates several important virulence factors. These observations lead to the idea that QscR synthesis might be tightly regulated. We therefore examined for a regulation of *qscR* expression. Two transcriptional fusions were constructed. The upstream DNA region (from −216) and the first 14

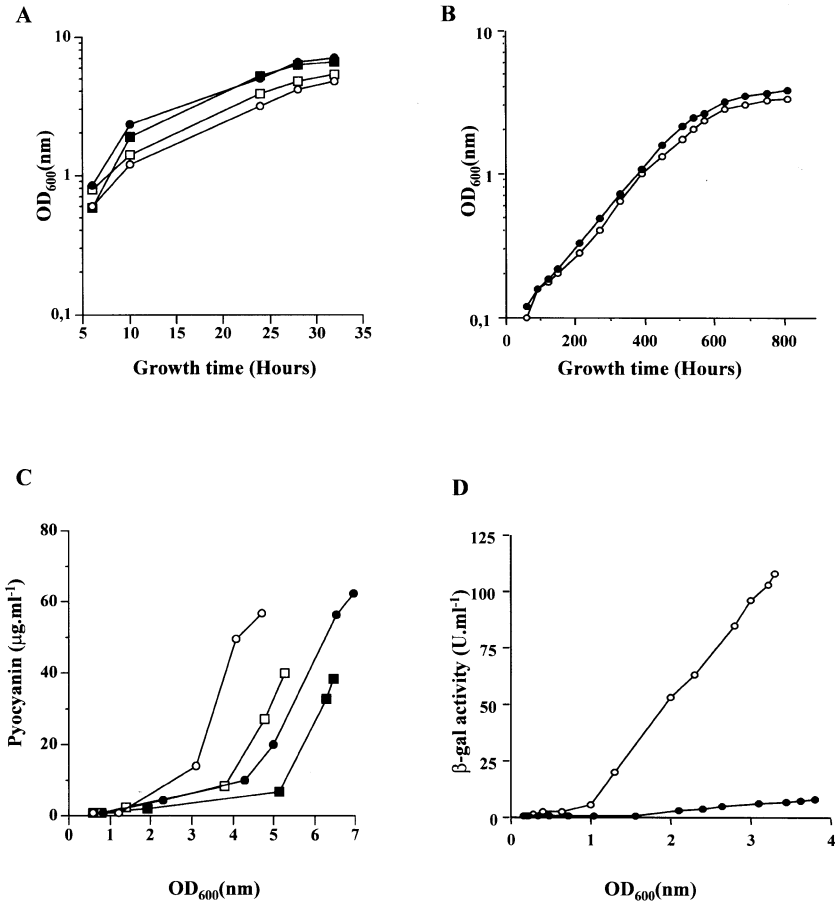

Fig. 2. Effect of *qscR* mutation on pyocyanin production.
A. Growth curves of PAO1 (pBBR1MCS2) (solid circles), PAO1 (pMCQ1004) (solid squares), PAOQ⁻ (pBBR1MCS2) (open circles) and PAOQ⁻ (pMCQ1004) (open squares), in glycerol-alanine medium. Samples taken during the growth were tested for pyocyanin synthesis (C).
B. Growth curves of PAO1 (solid circles) and PAOQ⁻ (open circles) containing *phzA2-lacZ* reporter plasmid, pMAL.P2, in glycerol-alanine medium. Samples taken during the growth were used to determine β-galactosidase activity (D).
C. Pyocyanin production in PAO1 (pBBR1MCS2) (solid circles), PAO1 (pMCQ1004) (solid squares), PAOQ⁻ (pBBR1MCS2) (open circles) and PAOQ⁻ (pMCQ1004) (open squares).
D. Expression of *phzA2-lacZ* fusion into PAO1 (solid circles) and PAOQ⁻ (open circles).

nucleotides of the *qscR* gene were introduced into plasmid pMP220 to give plasmid pMAL.Q1. In the second fusion, the DNA region from −724 and the first 14 codons were cloned into plasmid pMP220 to give plasmid pMAL.Q2. When introduced into strain PAO1, these two fusions had different levels of expression (Fig. 3). The expression of fusion pMAL.Q1 was not significantly different from that of the control vector, showing that the DNA region present in the fusion is not sufficient to allow a significant expression of *qscR*. The expression of the fusion from pMAL.Q2 was higher and was subsequently tested in different genetic backgrounds. When introduced in a *lasR* mutant strain, the expression of the fusion was not significantly changed showing that the expression of *qscR* was not under the control of the quorum-sensing regulator LasR, at least in our experimental conditions

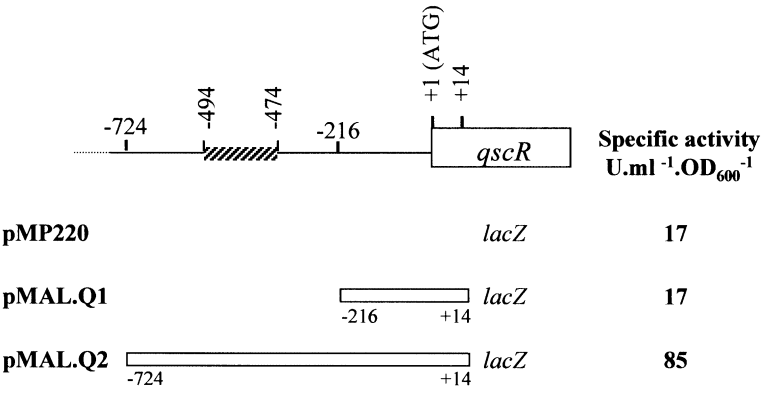

Fig. 3. *qscR* transcriptional expression in PAO1 wild-type strain. Genetic organization of the regulatory region of *qscR* gene. The fragment trancriptionally fused to the *lacZ* gene in pMAL.Q1 and pMAL.Q2 plasmids is shown. PAO1 harbouring pMP220, pMAL.Q1 or pMAL.Q2 were grown in LB medium at 37°C. The specific activity of β-galactosidase (U $ml^{-1}.OD_{600}$) for each reporter fusion is reported.The box between −494 and −474pb represents the putative '*lux-box*'.

(data not shown). This result was obtained despite the presence of a very well conserved '*lux-box*' between −494 and −474.

It was previously shown that the GacA response regulator has a positive effect on *lasR* and *rhlR* expression (Reimmann *et al*., 1997). GacA is part of a two-component system GacS/GacA strictly required for the production of several virulence factors (Laville *et al*., 1992). The fusion pMAL.Q2 was introduced into strain PAO6281, a *gacA* mutant derived from PAO1 strain and the expression of *qscR* was followed. When the strains were cultivated in LB medium, the expression of the fusion decreased by a factor of three in the *gacA* mutant compared to the wild type (Fig. 4). We can conclude that, as for *lasR* and *rhlR*, the two-component response regulator GacA has a positive effect on *qscR* expression. So, in response to some still unknown environmental signals, the amount of QscR present in the cell can be modulated.

QscR is a multimer in the absence of acyl-HSL and can form heterodimers with LasR and RhlR

In the accompanying article (Ventre *et al*., 2003), we used fluorescence anisotropy measurements and *in vivo* cross-linking to assess the oligomeric state of the quorum-sensing regulator RhlR. Furthermore, we extended these measurements to assess the effects of the two acyl-HSLs produced by *P. aeruginosa* on the oligomeric state of RhlR to gain insight into the molecular mechanisms behind the quorum-sensing response. Therefore, we decided to use the same approach to examine how QscR exerts a negative effect on virulence gene expression.

We fused the fluorescent protein EGFP with QscR to obtain a hybrid protein. We examined the cytoplasmic distribution of EGFP-QscR by fluorescence microscopy and its solubility by subcellular fractionation (data not shown). The results obtained were the same as for the EGFP-RhlR hybrid protein indicating production of a soluble cytoplasmic protein as expected (Ventre *et al*., 2003). We also tested the activity of the EGFP-QscR fusion protein. When EGFP-QscR was produced *in trans* from a plasmid introduced in the *qscR* mutant, the wild-type level of protease production was restored showing complementation of the mutation (data not shown). This experiment provides evidence that the EGFP-QscR fusion protein is in an active conformation.

As we had previously observed for the EGFP-RhlR fusion protein (Ventre *et al*., 2003), the anisotropy of the EGFP-QscR fusion protein fluorescence is much lower (0.184 ± 0.031, Table 1) than that of EGFP expressed in the same conditions (0.292 ± 0.009, Table 1) (Ventre *et al*., 2003). This low value can be attributed to fluorescence energy tranfer between transfer in an oligomer or aggregate, and indicates that QscR is present in cells as an oligomer or aggregate rather than a monomer. In order to better characterize this oligomeric state, we performed *in vivo* cross-linking with dithio-bis(succinimidylpropionate) (DSP), as shown in Fig. 5. In lane 1, a single strong band is visible at an apparent molecular mass of 54 kDa in the absence of DSP. In addition, several proteolysis products can be detected at lower molecular mass suggesting a cleavage of the linker between the EGFP and QscR portions of the fusion protein followed by a rapid degradation (data not shown). These breakdown products cannot be detected after the brief induction used for the fluorescence measurements but become visible after the stronger induction used for cross-linking experiments. Upon cross-linking with 0.12 mM of DSP, two major bands are observed (lane 2) at relative molecular masses of approximately 120 kDa and 150 kDa. The 120 kDa band is observed at approximately the same molecular mass as the EGFP-RhlR dimer (lane 5 and accompanying article). However, we suspected that this band is composed of higher order oligomers in which some degradation of one or more monomers has occurred, see below. The highest order oligomer at 150 kDa apparent molecular

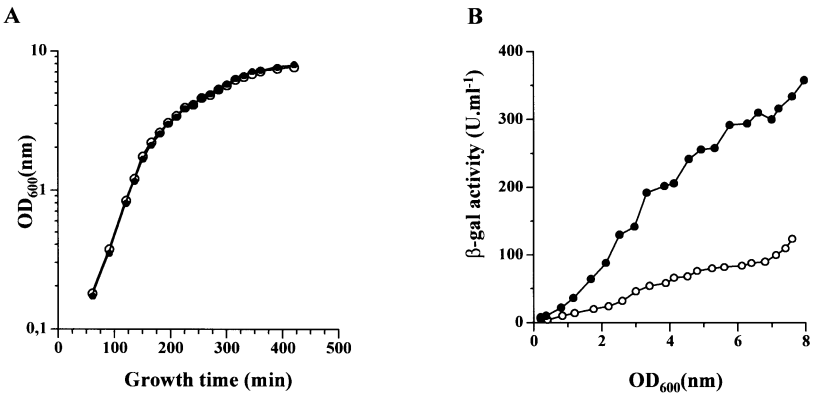

Fig. 4. GacA-dependent expression of the *qscR* promoter in pMAL.Q2.
A. Growth curves of PAO1 (solid circles) and PAO6281 (open circles) in LB medium.
B. Expression of the *qscR2-lacZ* transcriptional fusion in PAO1 (solid circles) and PAO6281 (open circles) during growth in LB medium.

Table 1. Static anisotropy values of EGFP, EGFP-QscR and EGFP-RhlR fusion proteins under various conditions.

Proteins[a]	Acyl-HSL added[b]	Average fluorescence anisotropy[c]
EGFP	–	0.292 ± 0.009
EGFP-QscR	–	0.184 ± 0.031
	C4-HSL	0.289 ± 0.040
	3O-C12-HSL	0.261 ± 0.020
EGFP-QscR + QscR	–	0.267 ± 0.014
	C4-HSL	0.265 ± 0.041
	3O-C12-HSL	0.267 ± 0.029
EGFP-QscR + RhlR	–	0.209 ± 0.014
	C4-HSL	0.221 ± 0.046
	3O-C12-HSL	0.241 ± 0.030
EGFP-QscR + LasR	–	0.333 ± 0.063
	C4-HSL	0.315 ± 0.061
	3O-C12-HSL	0.257 ± 0.020
EGFP-RhlR[d]	–	0.200 ± 0.019
EGFP-RhlR + QscR	–	0.248 ± 0.020

a. Fusion proteins were produced from pHEGFPC plasmid derivatives and untagged regulators were produced from pBBR$_1$MCS2 plasmid derivatives. See Table 2 and Fig. 5 for more details.
b. Auto-inducers were added at a final concentration of 1 μM.
c. The estimated errors were obtained from both the errors associated with spectral deconvolution and those observed between replicate experiments.
d. See accompanying article for more explanations.

mass might be a trimer or a tetramer. This result is entirely consistent with our fluorescence anisotropy observations and we can conclude that QscR is in a multimeric state.

Co-expression of untagged QscR with the EGFP-QscR fusion protein resulted in increased fluorescence anisotopy (Table 1). The increase in fluorescence anisotropy can be attributed to a reduction in the degree of fluorescence energy transfer in oligomers due to dilution of the chromophore (EGFP) within the QscR oligomers. But addition *in trans* of untagged QscR did not apparently qualitatively modify the bands visible after cross-linking, though the lower band became much stronger (Fig. 5, lane 3). It is important to note the absence of heterodimers EGFP-QscR/QscR in the presence of DSP, which are expected to migrate near 80 kDa, by analogy with what we observed with RhlR and LasR (see below) and with the EGFP-RhlR fusion protein (accompanying article and below). This reinforced the conclusion that the lower band (120 kDa) is derived from multimers in which some monomers have lost their EGFP tag.

We also investigated the effects of co-expression of the other unlabelled transcriptional regulators, RhlR and LasR, on the oligomeric state of the EGFP-QscR fusion protein. In both cases, this co-expression resulted in a significant increase in the anisotropy (Table 1). After *in vivo* cross-linking in cells containing EGFP-QscR with untagged RhlR (Fig. 5 lane 4), or with untagged LasR (Fig. 5, lane 5), a band at 80 kDa appeared and corresponds to the size of a heterodimer. These two methods both indicate that QscR is able to associate with the two other quorum-sensing regulators, RhlR and LasR, to form heterodimers. In order to confirm this observation we examined the effect of co-expression of the EGFP-RhlR fusion protein and the untagged QscR. Fluorescence anisotropy measurements gave a value of 0.248 ± 0.02 with EGFP-RhlR with QscR compared to 0.200 ± 0.019 with EGFP-RhlR alone (Table 1). Moreover, cross-linking experiments indicate the formation of a complex corresponding to a dimer EGFP-RhlR/QscR (Fig. 5, lanes 6 and 7). All these experiments gave entirely coherent results and we can conclude that QscR is able to interact with LasR and with RhlR to form heterodimers.

The *in vivo* activation of the transcriptional regulators RhlR and LasR depends on the two acyl-HSLs, C4-HSL and 3O-C12-HSL respectively. In the accompanying article, we have shown that the RhlR regulator is able to interact with 3O-C12-HSL, that induces RhlR dimer dissociation. We therefore investigated if acyl-HSLs were able to modulate QscR oligomerization, or its interactions with the other QS regulators. In the second two lines of Table 1, we show the effects of C4-HSL and 3O-C12-HSL on the anisotropy measurements. In all cases, a relatively high level of anisotropy was observed (between 0.221 and 0.315, Table 1). Thus, both C4-HSL and 3O-C12-HSL are able to dissociate the QscR oligomers and generated less aggregated forms, probably dimers or monomers. The anisotropy measurements in the presence of autoinducers and unlabelled quorum-sensing regulators are also relatively high though it is unclear whether one effect

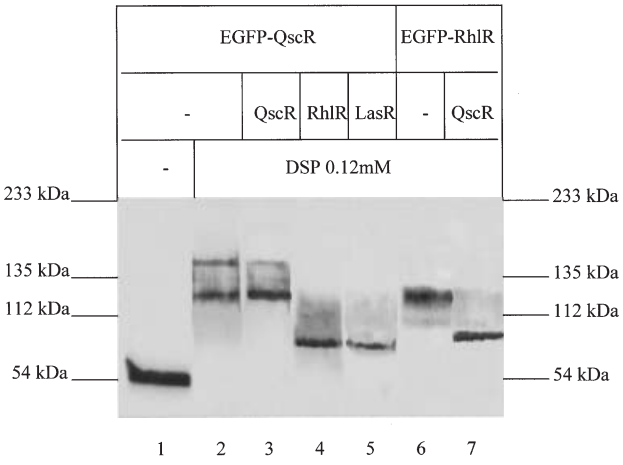

Fig. 5. *In vivo* cross-link of EGFP-QscR and EGFP-RhlR hybrid proteins.TG1 cells expressed EGFP-QscR from pHGQ (lanes 1–5), EGFP-RhlR from pHGR (lanes 6 and 7) and untagged quorum sensing regulators LasR, RhlR and QscR from pMCL (lane 5), pMCR (lane 4) and pMCQ1004 (lane 3) respectively. Cells were treated as described in *Experimental procedures*. Western blot was developed with a primary antibody against GFP and a secondary HRP-conjugated antibody before revelation using chemiluminescence.

(auto-inducers or untagged regulators) predominates over the other.

From these data, we can conclude that QscR can interact with both C4-HSL and 3O-C12-HSL, and this interaction induces a dissociation of the QscR oligomer.

Discussion

The existence of three homologous LuxR-type proteins in *P. aeruginosa* is very exciting and offers a challenge for understanding the fine tuning of the regulatory mechanism exerted in concert by these three regulatory proteins, LasR, RhlR and QscR.

What do we know precisely at the moment? It was established, several years ago, that there is a hierarchical regulatory cascade in which LasR positively regulates the expression of the *rhlR* gene (Latifi *et al*., 1995; Pesci *et al*., 1997). These results are based on studies of transcriptional fusions and on heterologous expression studies in *E. coli*. This does not give much information on the precise amounts of each regulatory protein present in the cell at a given time during the growth. In the absence of kinetic studies, based on immunodetection of each of the R proteins, which might give more direct information with respect to this problem, it is difficult to have precise data on this point. Meanwhile, using a technique based on fluorescence anisotropy measurements (Ventre *et al.,* 2003), we have attempted to determine which complexes could be formed *in vivo* between the three R-proteins and how QscR might be integrated into the QS cascade.

In the first part of this study, we identified novel targets for QscR regulation. Interestingly, we found evidence for a negative regulatory effect of QscR on protease synthesis in general and on elastase more particularly. The *lasB* gene is negatively regulated by QscR at a transcriptional level. We also extended the results obtained by Chugani *et al*. (2001) concerning pyocyanin synthesis. We confirmed the negative modulation exerted by QscR on the *phzA1-G1* operon expression. We extended this observation to the second *phzA2-G2* operon. By the study of the expression of a fusion *phzA2-lacZ* fusion, we have shown that, in the standard laboratory conditions used, this *phz2* operon is not expressed in PAO1 strain. QscR is a strong repressor of this operon because when QscR is inactive, a significant expression of this operon is observed. One has to bear in mind that the second *phz2* cluster is fully functional (Mavrodi *et al*., 2001). Therefore, there are probably natural conditions in which it can be expressed.

QscR regulator modulates expression of important virulence factors such as hydrogen cyanide (Chugani *et al*., 2001) pyocyanine (Chugani *et al*., 2001 and this study) and elastase (this work). *Pseudomonas aeruginosa* must have a tight control of the essential functions needed in its infectious process. We have observed on pyocyanin and elastase synthesis the importance of the relative amount of the QscR protein on the amplitude of the regulation it exerts on target genes. Therefore, we thought that *qscR* expression could be regulated by global regulatory network such as quorum sensing and/or two-component regulatory systems. As we observed a well-conserved '*lux-box*' located from −494 to −474 upstream the initiation codon, we investigated if *qscR* expression was regulated by LasR. Despite the presence of this consensus sequence which matches perfectly that described by Whiteley and Greenberg (2001), we did not observe a regulation of the *qscR* gene by LasR, at least in our experimental conditions. It was previously shown that *lasR* and *rhlR* were regulated by the GacA response regulator (Reimmann *et al*., 1997). In this study, we showed that, like *lasR* and *rhlR*, *qscR* expression is modulated *via* the two-component response regulator GacA, in response to still undefined environmental conditions. This result is important because it allows the integration of QscR into the complex network of quorum-sensing activation in *P. aeruginosa*.

In the second part of this study, based on results obtained by fluorescence anisotropy measurements, we have shown that QscR can form various complexes depending on the conditions tested: multimers in the absence of any acyl-HSL, lower order oligomers complexed either to C4-HSL or to 3O-C12-HSL and heterodimers with LasR or with RhlR.

The formation of heterodimers between QscR and LasR or RhlR, in the absence of acyl-HSLs, is a very interesting result. This closely resembles what is found in *Agrobacterium tumefaciens* where two different proteins modulate TraR-dependent transcriptional activation by direct protein–protein interaction. Both of these mechanisms function through formation of inactive heterocomplexes with TraR. The TraM protein prevents TraR from activating target genes under non-inducing conditions (Fuqua *et al*., 1995). TraM forms inactive heteromultimers with TraR by interaction with its C-terminal domain, although TraM shares no clear sequence similarity with TraR (Hwang *et al*., 1999; Luo *et al*., 2000). In contrast to TraM, TrlR (TraS) is close to TraR but encodes a truncated protein that resembles amino-acids 1–181 of TraR (88% identity) (Oger *et al*., 1998; Zhu and Winans, 1998). After this codon, the reading frame of TrlR shifts and the rest of this protein bears no resemblance to TraR. TrlR-mediated inhibition of TraR function requires the formation of inactive heterodimers containing one TraR monomer and one TrlR monomer. These heterodimers are incapable of high-affinity binding to *tra-box* (Chai *et al*., 2001). Inhibition is really due to the lack of a DNA-binding module as this protein can be converted to a functional activator by restoring translation of this module (Zhu and Winans, 1998).

In this study, we clearly show that heterodimers can be formed between QscR and LasR or QscR and RhlR by using two experimental approaches: fluorescence anisotropy and *in vivo* cross-linking. Up to now, there was only genetic evidence that QscR acts to inhibit expression of LasR and RhlR regulated promoters (this work, Chugani *et al*., 2001). These novel experiments allow us to suggest that QscR-mediated formation of heterodimers may be responsible of the negative effect observed on the LasR and RhlR target genes. Nevertheless, even if there are some similarity between these two cases TrlR and QscR, some major differences exist. QscR is a full-length R-type protein. Unlike TrlR, it presents a carboxyl domain with the characteristic DNA binding domain. Therefore, as it is easy to understand why a heterodimer TraR/TrlR shows a low affinity for DNA, it is more difficult to explain why a heterodimer LasR/QscR should be inactive though the consensus recognition sequence might be expected to change.

Another important finding from the fluorescence anisotropy measurements is that QscR multimers can be dissociated by the presence of either acyl-HSL. This observation opens up a large field of speculation. First, this is the proof that QscR can bind both acyl-HSLs and therefore can respond to a signal dependent on cell density. This was previously an open question because no '*I*-gene' associated to the *qscR* gene was found in the genomic sequence. Second, we can conclude that both acyl-HSLs dissociate QscR multimers but we cannot discriminate if the resulting complex is a homodimer or a monomer. One should keep in mind that the role of QscR in the cell might not be limited to interacting with LasR and RhlR, but that it may exist under active monomeric or homodimeric forms. Third, in the absence of acyl-HSL, QscR forms multimers as has been found for the QS repressor CarR in *Erwinia carotovora* (Welch *et al*., 2000) and, like CarR, QscR multimers are dissociated by acyl-HSLs, in contrast to TraR which dimerizes in the presence of acyl-HSL (Zhu and Winans, 2001). Very recently, it was shown, using molecular and genetic approaches, that LasR forms multimers only when 3O-C12-HSL is present, and that the ability of LasR to multimerize correlates with its ability to function as a transcriptional activator. Nevertheless, the authors did not discriminate between a multimer or a dimer form of LasR (Kiratisin *et al*., 2002). Therefore, one has to be cautious before considering one particular QS regulator as a paradigm for the R-regulator mode of action.

What is the physiological significance of these forms observed in *E. coli*? Do they form, and when, during the various phases of the cellular growth in *P. aeruginosa*? It is important to consider that the effective apparition of one or the other complex is dependent on the relative amounts of regulatory proteins present at the same time inside the cell and is also dependent on the relative concentrations of the various acyl-HSLs. This point has also to be considered very carefully, as some recent studies have shown that the various acyl-HSLs may be synthesized with a complex temporal pattern in *Rhizobium leguminosarum* and that important differences are observed between results established on isolated systems and results obtained with more integrated systems (Blosser-Middleton and Gray, 2001). It was also shown recently that a temporal and spatial regulation was observed in the expression of *lasI* and *rhlI* during biofilm maturation in *Pseudomonas* (de Kievit *et al*., 2001). Therefore, kinetic studies of the apparition of the numerous acyl-HSLs synthesized in *P. aeruginosa* in various growth conditions would be also very helpful to understand more precisely the molecular mechanism of regulation by quorum sensing in the bacterium.

Taken together, all our experimental data are consistent with the hypothesis that, at the beginning of the exponential phase of growth, when the relative concentrations of acyl-HSLs are low, QscR could form inactive heterodimers with LasR and/or RhlR, therefore inhibiting the expression of some quorum-sensing regulated genes. When the concentration of the respective acyl-HSLs increases, the equilibrium would be displaced towards the formation of LasR homodimers bound to 3O-C12-HSL and/or RhlR homodimers bound to C4-HSL at the onset of the stationary phase, leading to the regulated expression of target genes. This explanation relies also on the hypothesis that the R proteins are present at equivalent concentrations in the cell at the moment the control is exerted. Indeed, the relative protein concentration is very important in this type of regulation relying on protein–protein interaction. It has been shown in *A. tumefaciens*, assuming that TraR–TrlR dimers have an affinity similar to TraR–TraR dimers, that a slight overexpression of TrlR relative to TraR could inhibit the formation of TraR homodimers and thereby virtually blocks *tra* gene expression (Chai *et al*., 2001). For example, the expression of *trlR* is regulated by a subset of plant tumour-released opines, thereby influencing TraR activity in response to the specific opine composition of the environment (Oger *et al*., 1998; Zhu and Winans, 1998). In this context, the finding that QscR synthesis could be modulated in response to environmental signals *via* the two-component signal transduction system GacS-GacA is very significant.

In conclusion, it is more and more evident that the quorum-sensing cascade and onset of QS dependent gene expression are tightly regulated processes. Our present study together with that of Chugani *et al*. (2001) clearly established that QscR represses expression of several QS regulated genes during the exponential phase of growth. A model was postulated by Chugani and colleagues in which the primary role of QscR was repression

of *lasI* because a *qscR* mutant produced 3O-C12-HSL prematurely. This overproduction would result in premature transcription of QS-regulated genes. This model is attractive but probably too simple and should be integrated into a more global regulatory circuitry. Particularly, one should take into account recent results showing that some QS-regulated genes in *P. aeruginosa* are super-regulated in a growth phase-dependent manner by a novel regulator, MvaT, which modulates the expression of several virulence genes and is part of a growth phase-dependent control system (Diggle *et al*., 2002). In this study, it was shown that the induction of *lecA* and *lasB* (two QS-regulated genes) which are normally expressed during the transition into stationary phase, could not be advanced only by addition of exogenous C4-HSL and 3O-C12-HSL, indicating that a critical auto-inducer concentration is required but is not sufficient for their expression. Similarly, expression of *rhlR* could not be advanced under these experimental conditions. Therefore, it seems obvious that QscR is not the only regulator involved in a growth phase-dependent control and that a critical auto-inducer concentration is required but is not sufficient for expression of some QS-regulated genes. It seems likely that growth phase-dependent control of quorum sensing-dependent genes is multifactorial.

Experimental procedures

Bacterial strains, plasmids and culture conditions

The bacterial strains and plasmids used in this study are listed in Table 2. *Escherichia coli* and *P. aeruginosa* were routinely grown at 37°C in Luria–Bertani broth or Luria–Bertani agar. For pyocyanin assays *P. aeruginosa* were grown with aeration in glycerol-alanine medium (Frank and deMoss, 1959). *Escherichia coli* TG1 was used as host for cloning experiments. Plasmids were introduced into *P. aeruginosa* by electroporation or by conjugation using pRK2013 as a helper plasmid in triparental matings. Media were supplemented with antibiotics for selection as follows: for *E. coli*, ampicillin(Ap) at 50 μg ml^{-1}, kanamycin (Km) at 50 μg ml^{-1} and tetracyclin (Tc) at 15 μg ml^{-1}; for *P. aeruginosa*, carbenicillin (Cb) at 300 μg ml^{-1}, Km at 750 μg ml^{-1} and Tc at 100 μg ml^{-1}. For experiments with *tac* promoter-controlled genes, media were supplemented with 1 mM of isopropyl-β-D-thiogalactopyranoside (IPTG). To create the *EGFP-qscR* translational fusion, we amplified a 733pb DNA fragment with the oligonucleotides 5′-TAAACTATCAATTGATGCATGAT GAGAGAGAGGGA-3′ and 5′-TTTTATATTTGCGGCCGCTC GATTCAGTTGAGCAGGC-3′ from PAO1 genome. This PCR generated fragment was purified by PCR purification kit (Qiagen), digested with *Mfe*I-*Not*I and cloned in frame with EGFP into *Mfe*I/*Not*I-digested pHEGFPC to give pHGQ. A 1550pb PCR fragment carrying the *EGFP-qscR* translational fusion was generated using as oligonucleotides 5′-TAATTTGGGCCCCACAGGAAACAGCTATGA-3′ and 5′-TTTTATATTTGCGGCCGCTCGATTCAGTTGAGCAGGC-3′ and as template the plasmid pHGQ, digested with *Apa*I and *Not*I, and cloned into *Apa*I/*Not*I-digested pBBR1MCS$_2$ to generate pMCEGFP-Q. To construct pMCL plasmid, a 839-pb PCR fragment corresponding to *lasR* gene was amplified with the oligonucleotides 5′-AGGAAGCCGGGATTCTCGGAC-3′ and 5′-CGCCGACCTGAGAGGCAAGAT-3′ from PAO1 genomic DNA. The PCR product was T/A cloned using the original TA cloning kit (Invitrogen). *lasR* was then digested from the resulting plasmid by *EcoRI* and

Table 2. Bacterial strains and plasmids used in this work.

	Relevant characteristics	Origin reference
Strains		
P. aeruginosa		
PAO1	Wild type	Holloway
PAOQ$^-$	*qscR* mutant of PAO1, Cb^R.	This study
PAO6281	*gacA* mutant of PAO1	Reimmann *et al*., 1997)
E. coli		
TG1	*supE hsdΔ (lac, proAB) F′(traΔ36, proAB, lacI^q, lacZΔM15)*	Maniatis *et al*. (1982)
Plasmids		
pUC19	Broad-host-range cloning vector, *ColE1*, *bla*	Yanisch-Perron *et al*., 1985)
pUCQΔ	Gene exchange construct: pUC19 carrying a 412pb internal fragment of *qscR* gene	This study
pBBR1MCS$_2$	Broad-host-range cloning vector, Km^R*lacZα,* mob$^+$, P*T3*	Kovach *et al*. (1994)
pMCQ1004	pBBR1MCS2 carrying a 1kbp SmaI fragment containing the *qscR* gene, Km^R	This study
pMCEGFP-Q	pBBR1MCS$_2$ carrying a *EGFP-qscR* translational fusion, Km^R	This study
pMCL	pBBR1MCS$_2$ carrying a 839-bp *EcoRI* fragment containing the *lasR* gene, Km^R	This study
pMCR	pBBR1MCS$_2$ carrying a 1.5-kb *EcoRI* fragment containing the *rhlR* gene, Km^R	Ventre *et al*. (2003)
pMP220	*IncP* replicon for *lacZ* transcriptional fusions, Tc^R	Spaink *et al*. (1987)
pMAL.B	*IncP* replicon carrying a *lasB-lacZ* transcriptional fusion, Tc^R	This study
pMAL.P2	*IncP* replicon carrying a *phzA2-lacZ* transcriptional fusion, Tc^R	This study
pMAL.Q1	*IncP* replicon carrying a *qscR1-lacZ* transcriptional fusion, Tc^R	This study
pMAL.Q2	*IncP* replicon carrying a *qscR2-lacZ* transcriptional fusion, Tc^R	This study
pHEGFPC	Derivative of pEGFP with C-terminal cloning sites, ApR	Ventre *et al*. (2003)
pHGQ	pHEGFPC carrying a EGFP-qscR translationnal fusion, ApR	This study
pHGR	pHEGFPC carrying a EGFP-rhlR translationnal fusion, Ap^R	Ventre *et al*. (2003)

cloned into pBBR1MC$_2$ digested by the same restriction enzyme.

Construction of the P. aeruginosa qscR *mutant*

To generate a null allele of *P. aeruginosa qscR* gene, we amplified by PCR, using the PAO1 genome as a template and the two oligonucleotides 5′-CGCCTAAATATCATTTCCT GTCCA-3′ and 5′-CCACTTGAGCATCTCGGTTTCCCT-3′, a 420-pb internal fragment of the *qscR* coding sequence corresponding to amino acids 48–186 of the *P. aeruginosa* QscR protein (237 residues). The PCR product was blunt ended and cloned into the *Sma*I site of pUC19. The resulting plasmid (pUCQΔ) was electroporated into PAO1, and potential *qscR* mutants were selected for carbenicillin resistance (encoded by the suicide plasmid pUC19). Insertional inactivation of *qscR*, resulting from homologous recombination between pUCQΔ and the chromosomal *qscR* locus, was analysed by PCR using oligonucleotides 5′-TATGTACTTC CAGGTCCGGCTTGA-3′ and 5′-ACTAGCGTCCGGACAA CATGAGGA-3′ that hybridized *to P. aeruginosa qscR* flanking sequences in combination with primers that hybridized to vector sequence. We identified a mutant, PAOQ^{-} containing pUCQΔ inserted in *qscR* gene.

The *qscR* mutation was complemented by introduction of pMCQ1004 harbouring *qscR* under the control of the *tac* promoter. A1kb DNA fragment carrying *qscR* was amplified by PCR using oligonucleotides 5′-TATGTACTTCCAGGTCCG GCTTGA-3′ and 5′-ACTAGCGTCCGGACAACATGAGGA-3′ from PAO1 genome, blunt ended and then cloned into *Sma*I-digested pBBR1MCS$_2$ to generate pMCQ1004.

Exoproduct assays

Total protease production was qualitatively assayed on tryptic soy agar plates containing 1.5% skimmed milk. Pyocyanin was extracted and assayed spectrophotometrically at 520 nm from cell-free supernatant of *P. aeruginosa* grown in glycerol-alanine medium (Essar *et al*., 1990). Overnight cultures of *P. aeruginosa* harbouring *lacZ* transcriptional fusion plasmids were diluted to a turbidity of 0.01 at 600 nm in fresh medium containing appropriate antibiotics. Samples were harvested at time intervals for determination of turbidity at 600 nm β-galactosidase (β-gal) assays and specific activities were performed by the Miller method (Sambrook *et al*., 1989). One β-galactosidase unit (U) corresponds to the enzyme activity liberating 10^{-9} mol of ONP per minute at 28°C (using $\varepsilon = 4.5 \times 10^3$ cm^{-1} M^{-1}). Typical experiments from a series of at least three similar experiments are shown.

Construction of reporter gene fusions

To construct the *lasB-lacZ* reporter plasmid, pMAL.B, a 385pb PCR fragment beginning 329 pb upstream of the *lasB* translational start site was T/A cloned using the original TA cloning kit to form pCR.B. *lasB* promoter was then digested from pCR.B by *Eco*RI and cloned in pMP220 to generate pMAL.B. As for pMAL.B, all the following reporter gene fusions were constructed by using the TA cloning kit. The *phzA2-lacZ* reporter plasmid was constructed by using oligonucleotides which annealed with *qscR* coding region and with *phz2* regulatory region, 5′-CGCCTAAATATCATTTCCT GTCCA-3′ and 5′-GGTGCGAATCTCCGCCAGTTCGAAT-3′ respectively. The 1050 pb PCR fragment was purified by gel extraction and used as template to amplify a 449-bp DNA fragment flanking −410– 39 pb relative to the *phzA2* translational start. This 449 bp DNA fragment was cloned into *Eco*RI-digested pMP220 to generate pMAL.P2. The *qscR1-lacZ* reporter plasmid, pMAL.Q1, carrying a 230-bp PCR generated fragment flanking base pairs −216 to +14 relative to *qscR* translational start site. To construct pMAL.Q2, a 738 pb PCR fragment flanking −724 to +14 bp relative to the *qscR* translational start of was cloned into *Eco*RI-digested pMP220. All the constructs were verified by DNA sequencing.

SDS-PAGE and immunoblot analysis

Cells were harvested during growth in LB medium. Proteins were precipitated from the supernatant with 17% (wt/vol) trichloroacetic acid. Extracellular proteins were solubilized by heating for 5 min at 95°C in sample buffer (2% sodium dodecyl sulphate (SDS), 0.75 M β-mercaptoethanol, 10% glycerol, 62 mM Tris-HCl (pH 6.8), 0.01% bromophenol blue) prior to SDS-polyacrylamide gel electrophoresis (PAGE) on 11% acrylamide gels. Proteins were transferred onto nitrocellulose membranes and probed with 1:5000 dilution of antisera directed against elastase (LasB). Immunoblots were developed by chemiluminescence (Pierce) using a 1:20000 dilution of secondary antibodies conjugated to horseradish peroxidase.

Fluorescence anisotropy measurements and in vivo *cross-linking*

Cells for anisotropy measurements were grown overnight in LB medium supplemented with 0.6% glucose and appropriate antibiotics. The overnight culture was diluted 100 fold into fresh LB media, and growth continued at 28°C until an OD_{600} of 0.6 was reached. Cells were collected and resuspended in minimal medium supplemented with 5% LB and 15 μM IPTG. Induction was allowed to continue for 30 min at 15°C before the cells were harvested and resuspended to an OD_{600} of 2.0 in minimal media and kept on ice until measurement of the anisotropy. Fluorescence anisotropy measurements were made as described in the accompanying article (Ventre *et al*., 2003), briefly front face fluorescence emission spectra, between 500 and 650 nm, were measured for perpendicular and parallel components excited at 450 nm. During the measurements the samples, in a 1-mm path length cuvette, was thermostated at 15°C to minimize evolution. The recorded spectra were deconvoluted into four components and the anisotropy of the EGFP component calculated. Fluorescence anisotropy measurements allow an estimation of the exciton mobility between absorption and fluorescence events, this is sensitive in particular to molecular diffusion and energy transfer.

For *in vivo* cross-linking, cells were grown as for fluorescence measurements except that induction was performed in 150 ml of LB medium supplemented with 50 μM IPTG at 28°C for 90 min. Cells were harvested by centrifugation and

washed three times in 10 ml of 0.01 M phosphate-buffered saline, (PBS) pH 7.4 (Sigma). The membrane-permeable homo-bifunctional cross-linker DSP was added (0.12 μM final concentration) and the mixture was incubated at room temperature for 30 min The reaction was stopped by the addition of Tris-HCl pH 8 (25 mM final concentration) and incubated at room temperature during 15 min. Cells were collected by centrifugation and treated as previously described (Ventre *et al*., 2003).

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