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Dimerization of the quorum sensing regulator RhlR: development of a method using EGFP fluorescence anisotropy

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Summary

Of considerable interest in the biology of pathogenic bacteria are the mechanisms of intercellular signalling that can lead to the formation of persistent infections. In this article, we have examined the intracellular behaviour of a *Pseudomonas aeruginosa* quorum sensing regulator RhlR believed to be important in this process. We have further examined the modulation of this behaviour in response to various auto-inducers. For these measurements, we have developed an assay based on the fluorescence anisotropy of EGFP fusion proteins that we use to measure protein–protein interactions *in vivo*. We show that the transcriptional regulator, RhlR, expressed as an EGFP fusion protein in *Escherichia coli*, forms a homodimer. This homodimer can be dissociated into monomers by the auto-inducer *N*-(3-oxododecanoyl)-L-homoserine lactone (3O-C12-HSL) whereas *N*-(butanoyl)-L-homoserine lactone (C4-HSL) has little effect. These observations are of particular interest as RhlR modulation of gene expression depends on the presence of C4-HSL, whereas 3O-C12-HSL modulates the expression of genes regulated by LasR. These observations thus provide a framework for understanding the regulatory network that links the various different QS regulators in *P. aeruginosa*. Furthermore, the technique we have developed should permit the study of numerous protein/protein or protein/nucleic acid interactions *in vivo* and so shed light on natural protein function.

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Introduction

Over the last few years, *in vivo* fluorescence measurements have seen a renaissance thanks to the use of autofluorescent proteins since their first cloning 10 years ago (Prasher *et al*., 1992). The genes for these remarkable proteins permit the specific and complete labelling of individual cellular proteins through DNA manipulation and have lead the way to investigating cellular localization, trafficking and are beginning to be used to investigate protein–protein interactions *in vivo* (van Roessel and Brand, 2002). Such measurements have provided a wealth of new information over the last few years, both in the fields of cell biology and protein biochemistry (Cao *et al*., 2001).

A number of different techniques can be used in conjunction with autofluorescent proteins to obtain information on protein biochemistry *in vivo*. First and foremost, proximity between different fluorophores can be estimated from fluorescence energy transfer (FRET) measurements. The application of this method usually requires the engineering of two different autofluorescent fusion proteins with distinct spectral properties, and indeed a number of different variants of GFP have been specifically developed to this end (Mitra *et al*., 1996). Fluorescence energy transfer has proved particularly popular for the examination of protein–protein interactions *in vitro* (Graham *et al*., 2001) and *in vivo* (Chan *et al*., 2001; Sato *et al*., 2002).

In contrast to FRET there is a series of techniques that permit the examination of the hydrodynamic properties of fluorescent molecules, these include fluorescence recovery after photobleaching (FRAP) (Elowitz *et al*., 1999; Reits and Neefjes, 2001), fluorescence correlation spectroscopy (FCS) (Dittrich *et al*., 2001) and fluorescence anisotropy (Swaminathan *et al*., 1997; Mullaney *et al*., 2000). All of these methods have been realised for *in vivo* studies using GFP and its derivatives, in particular the EGFP derivative which has been engineered for enhanced solubility and fluorescence yield compared to the natural protein. An advantage of these techniques is

that they can provide other information beyond simple protein–protein interaction measurements, for example protein–DNA interactions can also be investigated, with the engineering of just one fusion protein. Furthermore, in the case of FCS, sensitivity is pushed to its ultimate limit for a single molecule. Fluorescence recovery after photobleaching and FCS obtain molecular information from measurements of lateral diffusion, which in turn depends on molecular size and shape, whereas fluorescence anisotropy (or polarization) obtains molecular information from the measurement of a rotational correlation time, this is again dependent on molecular size and, to a lesser extent, on shape.

However these techniques, based on autofluorescent fusion proteins have been most used to study eukaryotic cell biology. This situation is the result of a number of different factors notably: size, overexpression and heterogeneity. Measurements of lateral diffusion (FCS or FRAP) depend on making optical measurements with a spatial resolution significantly smaller than the size of a bacterium, however, with most *E. coli* strains having volumes of only a few femtolitres (10^{-15} l) and dimensions barely greater than a μm this is not feasable because of the diffraction limit for visible light. This can in some cases be overcome by perturbing the bacterial physiology, for example causing filamentation (Elowitz *et al*., 1999). Furthermore, protein overexpression in bacteria frequently results in the formation of inclusion bodies, which both perturb bacterial physiology and render the interpretation of results obtained from average measurements difficult. Finally differences in the expression of plasmid-borne genes (Boyd *et al*., 2000; Khlebnikov *et al*., 2000; Leveau and Lindow, 2001) between individual bacteria exacerbate difficulties of interpreting average data, particularly in double hybrid techniques. Consideration of these various problems lead us to examine the possibility of using anisotropy measurements *in vivo* to detect protein–protein interactions in the absence of serious overexpression. Fluorescence anisotropy has indeed been used *in vivo* for measuring protein–protein interactions, although, up until now, attempts in prokaryotic systems have been unsuccessful, often as a result of the formation of inclusion bodies, or aggregates (Mullaney *et al*., 2000).

Fluorescence anisotropy is a measure of the preservation of light polarization during the absorption–fluorescence process. Thus when polarized light is absorbed by the sample the fluorescence anisotropy measures the extent to which this polarization is preserved during the time between absorption and fluorescence. A period typically of the order of the nanosecond. The loss of polarization can occur for a number of different reasons most importantly for our work from molecular rotation or transfer of energy between non-parallel molecules. Fluorescence anisotropy measurements thus provide a spectroscopic method potentially able to measure molecular rotational diffusion coefficients and molecular interactions.

As a test system we have investigated the hydrodynamic behaviour of a bacterial transcriptional regulator. Many of these proteins have proved hard to investigate *in vitro* with little success in the purification or overexpression of active proteins.

Many bacteria perceive and respond to their population density, usually relying on the production and subsequent response to diffusible signal molecules. This regulatory mechanism is designated quorum sensing (QS). A significant number of Gram-negative bacteria produce acylated homoserine lactones (acyl-HSLs) as signal molecules that function in quorum sensing (Withers *et al*., 2001). Depending upon the bacterial species, the physiological processes regulated by QS are extremely diverse. Acyl-HSLs are synthesized at a low level by acyl-HSL synthases (LuxI-type proteins). Newly synthesized acyl-HSLs are rapidly removed from the cell by diffusion down their concentration gradient. These synthesis and accumulation continues until the cell density (and therefore the acyl-HSL concentration) exceeds a critical value. At this threshold level, the signal interacts with a transcriptional factor (homologous to the LuxR protein of *Vibrio fisheri*), and in turn the transcriptional factor modulates expression of quorum sensing-regulated genes (for reviews, see Fuqua *et al*., 2001; Miller and Bassler, 2001).

Pseudomonas aeruginosa is a versatile bacterium that can be found in many different environnments. It is also an emerging opportunistic pathogen of humans. Two complete quorum sensing systems, the LasR-LasI and the RhlR-RhlI systems have been identified in *P. aeruginosa*. A large number of genes including numerous virulence factors are regulated by quorum sensing in this bacteria. The Las system consists of the transcriptional activator LasR and of the LasI synthase, which directs the synthesis of the auto-inducer *N*-(3-oxododecanoyl)-L-homoserine lactone (3O-C12-HSL). Similarly, the Rhl system consists of the transcriptional activator RhlR and the RhlI synthase, which directs the synthesis of *N*-(butanoyl)-L-homoserine lactone (C4-HSL). Each system modulates a regulon comprising an overlapping set of genes. However, the Las and the Rhl systems are not independent of each other and form a regulatory hierarchy in which LasR/3O-C12-HSL activates the expression of *rhlR* (Latifi *et al*., 1996; Pesci *et al*., 1997). Moreover, it was shown that the Las system also controls RhlR at a post-translational level (Pesci *et al*., 1997). In addition, a third regulatory protein, QscR, homologous to the transcriptional activators RhlR and LasR, was recently identified and appeared to play an important role in quorum sensing modulation (Chugani *et al*., 2001; Ledgham *et al.,* 2003).

Despite a wealth of genetic and physiological information, there is little direct biochemical evidence or structural

information shedding light on the mechanism of action of the transcriptional activators and the modulation of their activity by their cognate inducers in *P. aeruginosa*. There is also no information at the molecular level of the various interactions between the three different regulatory proteins of *P. aeruginosa*, RhlR, LasR and QscR. This lack of molecular information is due at least in part to the difficulty observed in overexpressing or purifying the different activator proteins, either from *Pseudomonas aeruginosa* or from the heterologous host *Escherichia coli*.

In this article we develop a new technique to allow the investigation of protein–protein interactions *in vivo.* We show the application of this technique to the analysis of bacteria expressing very low levels of fluorescent proteins. Furthermore we use an EGFP-RhlR fusion protein to investigate the oligomeric state of the quorum sensing regulator RhlR, and the modulation of this by the QS inducers 3O-C12-HSL and C4-HSL. In the accompanying article (Ledgham *et al*., 2003) we have used a variety of different techniques to investigate the interactions of QscR with the other quorum sensing regulators at the molecular level.

Results

Measurement of EGFP steady state fluorescence anisotropy in vivo

Induction of the expression of EGFP in *E. coli*, results in the accumulation of fluorescence in the cytoplasm. As mentioned in the introduction, heterologous expression of EGFP or EGFP-fusion proteins can lead to the accumulation of fluorescent inclusion bodies. In order to verify that the EGFP was not present in inclusion bodies we routinely examined expressing cells under a fluorescence microscope (Fig. 1A). We found no evidence for the formation of EGFP inclusion bodies under our expression conditions, usually visible by phase-contrast or differential interference-contrast microscopy. Nevertheless, under our expression conditions, the level of expression appear to be slightly heterogeneous. This phenomenon has been reported before (Khlebnikov *et al*., 2000; Leveau and Lindow, 2001), and indeed can be quantified by measuring the mean pixel intensity variation (Leveau and Lindow, 2001). We find from micrographs such as that shown in Fig. 1A and B a standard deviation of 11.5% in the expression of EGFP using this method. This relative homogeneity is important for our interpretation of average anisotropy measurements, and is strongly dependent on the growth and induction conditions.

Because it is important for anisotropy measurements that the produced protein is soluble in the cytoplasm and is neither in inclusion bodies nor associated with membranes, we confirmed the microscopic observations by cell fractionation measurements. These results are shown in Fig. 2. As is apparent, the expressed EGFP (lanes 1–4) is preferentially associated with the soluble fraction (lane 4) and much reduced in the insoluble fraction (lane 3). Indeed the low levels that are found to be insoluble appear to result from incomplete lysis or entrapment, as can be appreciated from the lower panel in which a blot of a duplicate gel is revealed with antibodies against the

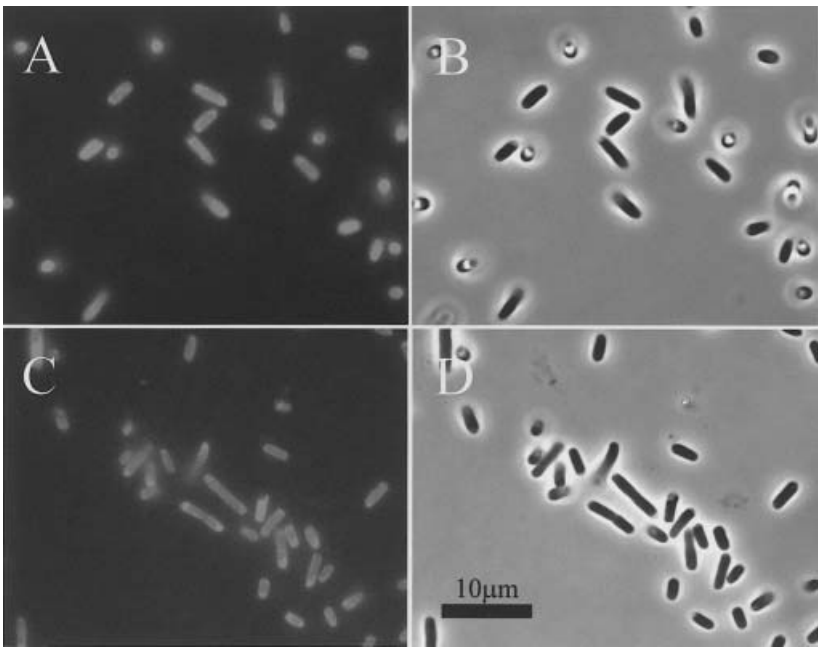

Fig. 1. Expression and distribution of EGFP and EGFP-RhlR fusion proteins. TG1 cells expressing the EGFP (A and B) or the EGFP-RhlR (C and D) proteins are visualized by fluorescence microscopy with an integration time of 3 s (A and C) or by phase-contrast of the same field (B and D). The scale bar in D represents 10 μm and applies to all images.

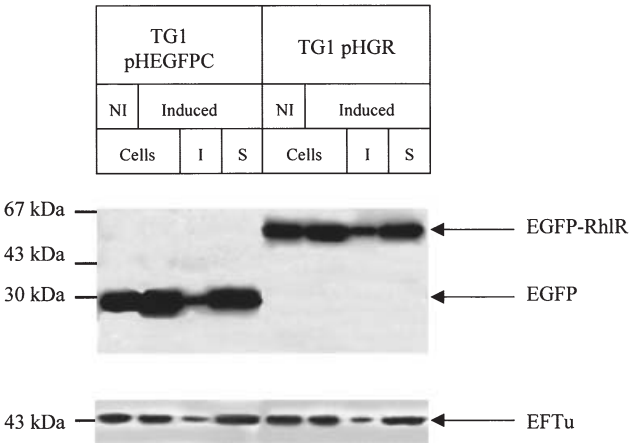

Fig. 2. Intracellular location of the fluorescent proteins EGFP and EGFP-RhlR (upper panel) and the cytoplasmic marker EFTu (lower panel). The Western blot in the upper panel was revealed with an antibody against GFP. In the lower panel, the revelation was with an antibody against EFTu. TG1 pHEGFPC expressed EGFP and TG1 pHGR expressed EGFP-RhlR. Cells were induced with 50 μM of IPTG. NI, non-induced cells; I, insoluble fraction; S, soluble fraction.

cytoplasmic marker EFTu. It is also important to note that there is no evidence for degradation of EGFP in the samples examined, in no cases are lower molecular weight bands detected. Thus, in conclusion the fluorescent protein can be expressed in *E. coli* and does not form inclusion bodies under the expression conditions used and can thus be used to examine protein–protein interactions *in vivo*.

The two major challenges to realising fluorescence anisotropy measurements in bacterial suspensions are the strong scattering of such samples, and bacterial autofluorescence. In order to minimize these problems we attempted to reduce both the detection of scattered light, and increase our ability to accurately discriminate between bacterial autofluorescence and EGFP fluorescence. To these ends we optimized our choice of excitation wavelength and filters on the one hand and our methods of data analysis on the other. Both these aspects are described in *Experimental procedures*. Briefly, we opted for excitation with a filter to reject rayleigh scattered light at a wavelength where the water Raman signal was not recorded and analysed the fluorescence emission spectrum by multiple regression.

Multiple regression deconvolution analysis required three basis components, corresponding to a cell autofluorescence background, a variable contaminating autofluorescence signal and the EGFP fluorescence. A typical deconvolution is illustrated in Fig. 3 which shows the contribution of each of the basis components (Fig. 3A, broken lines) to an experimental spectrum (Fig. 3A, solid line) and the resulting residuals spectrum, amplified five times (Fig. 3B). The multiple deconvolution procedure appears to be robust and rapid allowing the accurate estimation of low levels of EGFP fluorescence in scattering and autofluorescent bacterial samples. Typically it was possible to obtain reproducible measurements of expression at levels greater than about 1 nM, at a cell density of 10^{12} l^{-1}. This corresponds to about 600 molecules per cell, and assuming a cellular volume of 3.5 fl, based on micrographic measurements, an internal concentration of about 300 nM.

To calculate EGFP fluorescence anisotropy spectra obtained with parallel and perpendicular excitation and emission polarizers were independently deconvoluted and the anisotropy calculated from the contribution of the EGFP reference spectrum to the two experimental spectra. Estimation of the fluorescence anisotropy of EGFP expressed in *E. coli* cells gave a value of 0.292 ± 0.009 (Table 1), this value should be compared to the value of 0.4 for EGFP in a rigid system as has previously been published (Hink *et al.*, 2000; Volkmer *et al.*, 2000). In order to obtain information on the intracellular environment we compared the anisotropy of EGFP within cells to that measured for purified EGFP in solution. Measurements of purified EGFP in aqueous solution containing or not unlabelled cells gave very similar values of 0.197 ± 0.005, or 0.195 ± 0.01 respectively. The fact that we obtain similar

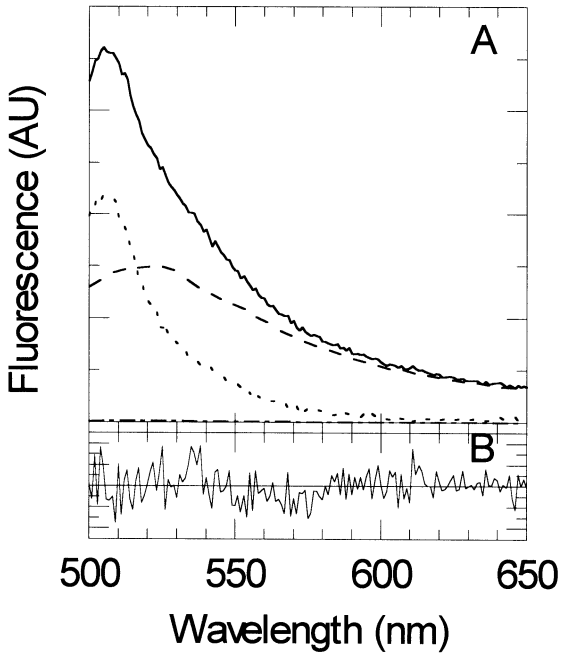

Fig. 3. Deconvolution of a polarized fluorescence emission spectrum.
A. Shows the emission spectrum of the EGFP-RhlR fusion protein in TG1 after induction observed with parallel polarizers (solid line), and the three components observed corresponding to the EGFP fluorescence (dotted line) and two different cell autofluorescence signals (dashed and dot-dashed lines).
B. Shows the residual spectrum (the *y*-scale is amplified by 5 relative to A).

Table 1. Static anisotropy values and expression levels for EGFP and EGFP-RhlR fusion protein under various conditions.

Strains	Additions	Typical expression level[a]	Average fluorescence anisotropy[b]
TG1 pHEGFPC	None	150	0.292 ± 0.009
	3O-C12-HSL		0.280 ± 0.020
	C4-HSL		0.260 ± 0.021
TG1 pHGR	None	20.1	0.200 ± 0.019
$pBBR1MCS_2$	3O-C12-HSL		0.267 ± 0.018
	C4-HSL		0.200 ± 0.015
TG1 pHGR pMCR	None	7.8	0.288 ± 0.024

a. Expression levels are quoted as nM EGFP detected after 30 min induction with 15 μM IPTG at 15°C, see *Experimental procedures* for details. Auto-inducers (added at a final concentration of 1 μM) had no significant effect on the induction.
b. The estimated errors were obtained from both the errors associated with the spectral deconvolution and those observed between replicate experiments.

value for the anisotropy in scattering (with cells) and non-scattering (without cells) samples provides an internal control of our measurement system and suggests that it is relatively free from systematic errors because of the presence of scattering and cell derived fluorescence.

The measurements that we describe above show that we are able to measure fluorescence anisotropy in bacteria. We wished to extend these measurements to show that we could use the technique to obtain new insights to biological problems. The problem we have addressed is the molecular characterization of the *P. aeruginosa* transcriptional activator RhlR and its modulation by the auto-inducers C4-HSL and 3O-C12-HSL.

The transcriptional activator RhlR forms a dimer

As the homogeneous cytoplasmic distribution of the EGFP-RhlR fusion protein is important for the interpretation of our results, we again examined expressing cells by fluorescence microscopy (Fig. 1C and D) and subcellular fractionation (Fig. 2A, lanes 5–8). These examinations allowed us to find conditions in which production of the fluorescent fusion protein EGFP-RhlR could be induced and the fusion protein was not aggregated (Fig. 1C) and was located in the soluble fraction (Fig. 2, lane 8), but nearly absent from the insoluble protein fraction (Fig. 2, lane 7). As with EGFP, the fusion protein present in the insoluble fraction appears to arise from entrapment or incomplete cell breakage as the level of contamination is similar for the marker EFTu (Fig. 2, lower panel). In the case of fusion protein production, an additional important criterion is that the entire protein is produced and that it is not unduly degraded by proteolytic enzymes. The stability of the protein is also visible in Fig. 2, in which a single band at the expected molecular mass (55 kDa) is observed with no evidence for the production of proteolytic fragments. In the presence of significant proteolysis the band detected by the antibodies – which recognise the N-terminal of the fusion protein – shifts to lower apparent molecular weight, this has been observed after longer inductions at higher temperatures (not shown).

We first tested the activity of the EGFP-RhlR hybrid protein. A *rhlR* deletion mutant was constructed in strain PAO1. This mutant showed a decreased protease production as compared to that of the wild-type strain PAO1. This phenotype could be visualized on skimmed-milk agar plates where a halo is observed around the *P. aeruginosa* colonies secreting proteases due to the degradation of proteins present in the medium. When EGFP-RhlR is produced *in trans* from a plasmid, the wild-type phenotype was restored (data not shown), showing that the EGFP-RhlR protein is active.

Estimations of fluorescence anisotropy in cells expressing the EGFP-RhlR fusion protein gave values of 0.200 ± 0.019 (see Table 1). This is interesting as the anisotropy of the fusion protein is less than that of EGFP alone, unfused to another protein, whereas the expected effect of increasing the molecular size is to reduce the rate of rotation and thus increase the anisotropy. The most probable explanation for this effect is a depolarization due to energy transfer between different fluorophores within an oligomer or aggregate. Such 'Homo-FRET 'has indeed been previously reported both in dimers (Gautier *et al.*, 2001) and in larger aggregates (Mullaney *et al.*, 2000). However other factors could give rise to such a phenomenon, for example an artefactually high anisotropy in EGFP caused by interactions with other cellular components involving parts of the molecule masked in the fusion protein. We therefore wished to verify that the fusion protein was indeed oligomeric and try to determine the size of the oligomer.

We used two different approaches to verify the existence of EGFP-RhlR oligomers, *in vivo* chemical cross-linking with dithio-bis(succinimidylpropionate) (DSP), and modification of both anisotropy and cross-linking by the co-expression of untagged RhlR.

In Fig. 4, we show Western blots obtained after *in vivo* cross-linking of cells expressing EGFP or the EGFP-RhlR

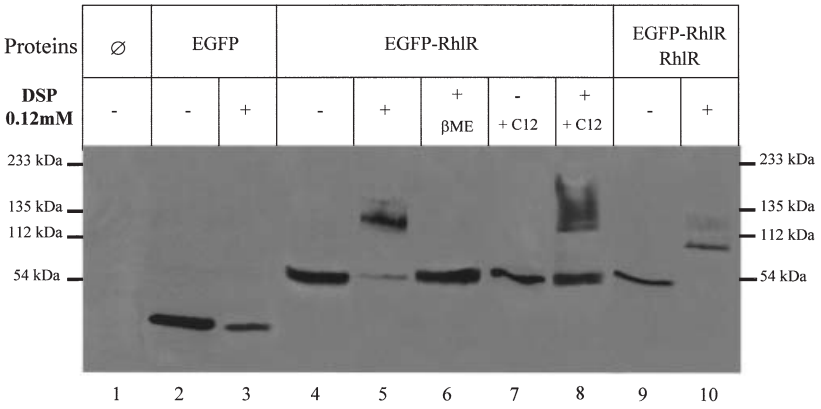

Fig. 4. *In vivo* cross-linking with DSP shows RhlR dimers. TG1 cells expressing EGFP (lanes 2 and 3), or EGFP-RhlR (lanes 4–10) with (lanes 9 and 10) or without (lanes 4–8) untagged RhlR co-expressed *in trans* were treated with the cross-linking reagent DSP and as indicated with β mercaptoethanol (βME) (see *Experimental procedures*). Samples loaded on lane 7 and 8 were extracted from cells grown with 1 μM of 3O-C12-HSL and buffers used for cross-linking were supplemented with 1 μM of 3O-C12-HSL.

fusion protein. In the case of EGFP we did not observe cross-linking by DSP, in the presence (lane 3) and absence (lane 2) of DSP, a single band was visible in the Western blots corresponding to the monomeric EGFP protein. In cells expressing the EGFP-RhlR fusion protein, addition of DSP (lane 5) resulted in the near quantitative appearance of a band with an apparent molecular mass near 130 kDa at the expense of the monomeric fusion protein visible at 55 kDa (lane 4). Furthermore addition of β-mercaptoethanol to reduce the DSP generated cross-link resulted in the disappearance of the cross-linked band and re-appearance of the monomeric fusion protein band (lane 6). The 130 kDa apparent molecular mass of the cross-linked protein lies between that expected for a dimer (110 kDa) and a trimer (165 kDa), the fact that the appearance of this band is near quantitative and the absence of evidence for intermediate bands, even in the presence of lower concentrations of cross-linker, both argue for this product being a cross-linked dimer. When the EGFP-RhlR fusion is co-expressed with the RhlR protein (lanes 9 and 10), DSP cross-linking results in the appearance of two different bands at apparent molecular masses of 100 kDa and 130 kDa. These two bands corresponding to a heterodimer containing one fusion protein and one RhlR protein at 100 kDa and the second band corresponds to the fusion protein homodimer already observed. It is interesting to note that in these conditions all the EGFP-RhlR fusion protein appears to be cross-linked by DSP, the majority forming heterodimers. The different quantities of hetero and homodimers in lane 10 probably reflect the different expression levels of the two proteins. Furthermore the absence of an EGFP dimer band in lane 3 implies that this dimerization is driven by the RhlR part of the fusion protein.

Measurement of anisotropy in cells co-expressing RhlR and EGFP-RhlR (Table 1), showed that the co-expression increased the average anisotropy of the EGFP-RhlR fusion protein. This is entirely consistent with the cross-linking results above. The formation of EGFP-RhlR/RhlR heterodimers breaks the EGFP-RhlR homodimers and thus stops the energy transfer between different EGFP domains. The reduction in 'Homo-FRET' increases the observed anisotropy from 0.200 to 0.288. One would expect if there is sufficient untagged RhlR produced to break all the dimers, the anisotropy might approach a value of about 0.36, this value is calculated from the EGFP anisotropy (Table 1), and the difference in molecular mass between a EGFP-RhlR/RhlR heterodimer (80 kDa) and EGFP (27 kDa) ignoring shape effects and linker flexibility both of which might be expected to reduce this limiting anisotropy.

It should be noted that the anisotropy measurements alone do not permit the unequivocal demonstration of homodimer or heterodimer formation, as we have access only to average values and are unable to observe individually the various different molecular species present within the cells. This might be possible by extending the currently reported technique to time resolved anisotropy measurements or by using fluorescence correlation spectroscopy. Nevertheless, the effect of untagged RhlR on the EGFP-RhlR anisotropy coupled with the observations of dimers and heterodimers upon *in vivo* DSP cross-linking together provide strong evidence for the existence of dimers of EGFP-RhlR and EGFP-RhlR/RhlR heterodimers. Furthermore the absence of evidence for oligomerization of EGFP, either from cross-linking or anisotropy measurements indicates that this interaction is mediated by the transcriptional activator domain of the fusion protein.

It should also be remarked that the fluorescence measurements were made under conditions of very weak induction where the levels of heterologous protein accumulation were low, thus approaching the physiological levels, whereas the cross-linking measurements had to be made with stronger and longer induction, thus under much less physiological conditions.

Modulation of dimerization by 3O-C12-HSL and C4-HSL

Of considerable biological interest is the interaction of acyl-HSL receptors such as RhlR with their auto-inducers, and the molecular mechanism of signal transduction. *In vivo*, C4-HSL activates RhlR mediated gene expression whereas 3O-C12-HSL activates LasR mediated gene expression. We therefore investigated the effects of the two *P. aeruginosa* quorum sensing inducers, C4-HSL and 3O-C12-HSL, on the anisotropy of the EGFP-RhlR fusion protein. Our results are shown in Table 1. First, the auto-inducers have no effect on the anisotropy observed for EGFP alone, indicating that those effects that we observe are due to specific interactions between the auto-inducers and the RhlR domain of the fusion protein. Second, C4-HSL has little, if any, effect on EGFP-RhlR dimerization, the anisotropy remains low. In contrast, 3O-C12-HSL causes a dramatic rise in anisotropy suggesting that it is able to induce monomerization of the EGFP-RhlR homodimers. This pair of results is interesting because we did not observe any effect of the cognate inducer (C4-HSL) on RhlR dimerization but we did observe an effect of the LasR inducer (3O-C12-HSL) on RhlR dimerization. It thus appears that C4-HSL has no significant effect on RhlR dimerization whereas 3O-C12-HSL is able to disrupt RhlR homodimers. To confirm these results we examined the effect of 3O-C12-HSL on *in vivo* cross-linking, this is shown in Fig. 4 (lanes 7 and 8) where addition of DSP in the presence of 3O-C12-HSL causes only a partial cross-linking of the fusion protein. Quantitative analysis of lanes 5 and 8 suggests that the proportion of the fusion protein present as crosslinked dimers is reduced from 81% to 37% by the presence of the auto-inducer, equivalent to a 24-fold increase in the apparent equilibrium constant.

Discussion

In this study, we set up a new experimental approach based on fluorescence anisotropy measurements to monitor protein–protein interactions *in vivo* and for this purpose, we studied the multimerization state of the quorum sensing regulator RhlR in the heterologous host *E. coli*.

Comparison of the anisotropy values of the EGFP observed in dilute solution (0.195) and *in vivo* (0.292) is instructive. This difference in static anisotropy derives from differences in the molecular dynamics caused by the change in environment in passing from a dilute aqueous solution to the interior of the cell. If this difference is attributed to a change in viscocity then an intracellular viscocity of 2.1 cp can be calculated. This assumes the difference in anisotropy arises entirely from an increase in rotational correlation time as a result of viscocity differences. Clearly this difference could come from other sources notably from molecular crowding and in particular attractive interactions with other macromolecular cellular solutes. The globular form of the EGFP protein would suggest that the effects of molecular crowding on rotational diffusion should be small, though it is hard to estimate the possible effects of attractive interactions.

Measurements of intracellular EGFP diffusion in *E. coli* cells have previously been published using FRAP (Elowitz *et al.*, 1999), can be used to give an estimate of the viscocity for lateral diffusion of about 11 cp. The difference between these two values is to be expected and can be attributed to the much greater effect of molecular crowding on translational diffusion than on rotational diffusion. Indeed in principal the difference between the two values gives information on the co-solute size distribution, though to obtain useful information on this parameter the two values should be obtained for tracers of different size.

Assuming that the observed rotational correlation time is largely controlled by viscocity, the obtained value of 2.1 cp provides an estimate of the cytoplasmic solvent viscocity. This value is comparable to that of a 20% wt/vol solution of a small organic molecule at 20°C (Wheast, 1973). However, estimates of cytoplasmic composition in *E. coli* cells (Cayley *et al.*, 1991) suggest that the osmolyte concentrations are much lower than this, closer to 2% w/v. It would therefore seem likely that the observed slow rotational correlation time reflects, at least in part, a contribution from non-specific interactions with co-solutes. This is interesting as it suggests that molecular crowding within the cell will modify the behaviour of proteins and nucleic acids not only through excluded volume effects but also through non-specific interactions, equivalent to a positive second virial coefficient in pure solution studies.

In this study, based on fluorescence anisotropy measurements of autofluorescent protein, EGFP-RhlR, we provide evidence that RhlR exists as a homodimer, when synthesized in the absence of acyl-HSL in the cytoplasm of *E. coli*. Chemical cross-linking analysis strongly support this interpretation. For technical reasons, this experiment was far more feasible in *E. coli* than in *P. aeruginosa*. However, it seems probable that this finding applies to both organisms. The RhlR dimer was observed not only in cross-linking experiments where expression level is quite high, but also in anisotropy measurements where very little protein is produced. Our finding that RhlR forms homodimer in the absence of acyl-HSL is similar to the situation encountered with CarR, the QS regulator in *Erwinia carotovora*, but different to that with TraR, the QS regulator of *Agrobacterium tumefaciens*.

Ligand-induced multimerization is common among regulatory proteins. There is now ample evidence that R proteins form dimers and multimers even though relatively few biochemical studies have been done on LuxR-type proteins. The *A. tumefaciens* TraR protein was purified and it was shown that, whereas apo-TraR is a monomer,

the active, ligand-bound form of TraR is a dimer (Qin *et al*., 2000). Ligand binding is necessary to induce dimerization of TraR and auto-inducer binding to TraR increases affinity for target promoters *in vitro* (Zhu and Winans, 1999). Similarly, LasR has been shown to form an active multimer in the presence of its auto-inducer using a genetic test (Kiratisin *et al*., 2002). In contrast, the CarR protein of *E. carotovora* pre-exists as a dimer in the absence of ligand and is shifted to a higher-order multimer in response to 3O-C6-HSL addition (Welch *et al*., 2000). Despite the differences between TraR and CarR, in both cases, DNA binding is mediated by protein dimers (see Fuqua *et al*., 2001). To our knowledge, all full-length R type proteins so far examined must dimerize as a prerequisite for DNA binding. Such a model is consistent with the dyad symmetry of the DNA binding sites.

It appears clear from our experiments *in vivo* that the behaviour of RhlR is similar to that of CarR in the absence of any acyl-HSL. These facts have strong implications at the level of synthesis of these proteins as the sensitivity of TraR to auto-inducer binding was related to the instability of the unliganded protein (Zhu and Winans, 2001).

From genetic and physiological experiments, it is well established that RhlR is specifically activated by binding with C4-HSL (Latifi *et al*., 1996; Pesci *et al*., 1997). The binding of C4-HSL to RhlR in *E. coli* induces an activation of the regulatory protein as it was previously shown that, in *E. coli,* the presence of C4-HSL was necessary for the expression of RhlR-regulated genes (Latifi *et al*., 1996). In our experimental conditions, the EGFP-RhlR fusion protein forms a dimer with or without C4-HSL. To account for the genetic experiments, we can hypothesize that preformed RhlR dimers bound C4-HSL, and that this leads to a conformational change of the dimer which activates it. This is slightly different from what was observed with CarR. When Welch *et al*. (2000) addressed the question of consequences of acyl–HSL interactions on multimerization of CarR, they found that 3O-C6-HSL binding to His6-CarR lead to a change in the aggregation state of the protein.

We also tested the effect of the presence of 3O-C12-HSL on RhlR homodimers. Unexpectedly, we found that the binding of 3O-C12-HSL induced monomerization of the RhlR homodimers. This finding is very exciting especially in the light of previous results obtained by Pesci *et al*. (1997) and Winzer *et al*. (2000). It is known that LuxR-type proteins weakly recognize non-cognate acyl-HSLs. Indeed such non-cognate acyl-HSLs can function as effective competitors of the correct signal, in some circumstances. Experiments on the interchangeability of the Las and Rhl system components showed that they were not compatible, in that C4-HSL does not activate LasR and 3O-C12-HSL does not activate RhlR in *E. coli*. However it was apparent that these two systems were not completely independent of one another. Indeed it was shown, in *E. coli*, that the LasR-dependent auto-inducer, 3O-C12-HSL, prevents the binding of the RhlR-dependent auto-inducer, C4-HSL, to its cognate regulator RhlR (Pesci *et al*., 1997). By developing a radioactive binding assay, the authors showed that 3O-C12-HSL could block the C4-HSL binding site(s) of RhlR and cause the inhibition of a RhlR-controlled gene. This indicated that 3O-C12-HSL controls RhlR activity at a post-translational level. It should be remarked however, that these effects have not yet been observed in *P. aeruginosa* (Winzer *et al*., 2000) presumably because of the more complex molecular context. In the light of these previous experiments, our finding that 3O-C12-HSL binding can induce dissociation of RhlR homodimers is particularly interesting, as we show that there is not only competition for the acyl-HSL binding site but that also dissociation of the RhlR homodimers can be induced. These results may have a significant relevance in the physiology of the cells. If, in some conditions, the concentration of 3O-C12-HSL is higher than the concentration of C4-HSL, this would allow *P. aeruginosa* to delay the induction of genes controlled by Rhl quorum sensing and provide this organism with yet another mechanism to temporally control the activation of important factors.

We have therefore shown, through the study of this biological system, the utility of fluorescence anisotropy measurements for the monitoring of protein–protein interactions *in vivo*. The variations in anisotropy that we observe arise from the interplay of hydrodynamic and energy transfer dynamics, have allowed us to demonstrate the formation of dimers by the quorum sensing regulator RhlR from *P. aeruginosa*. This type of measurement has a number of possible developments, first measurements are easilly extendable to protein–DNA or protein–membrane interactions, in each case interactions should immobilize the fusion protein. Second, unlike FRET-based methods this use of anisotropy allows information to be obtained using a single hybrid technique. Finally and perhaps most importantly, fluorescence anisotropy measurements can be made in cells containing relatively few marked molecules in which physiological modifications due to the overexpression of heterologous proteins can thus be minimized. Nevertheless, it should be emphasized that the measurements obtained are average measurements of a population. As such, the measurements are rather sensitive to cell to cell heterogeneity, on the one hand, and are difficult to interpret in the face of complex mixtures of components.

Possible extensions of this method can be suggested that would paliate these shortcomings. For example, measuring the time-resolved anisotropy and thus perhaps more thoroughly analyse the complex mixtures that can be obtained, rather than relying entirely on average mea-

surements. Also, using a microscope equiped to measure anisotropy on a cell by cell basis would reduce the undoubted sensitivity of cell to cell heterogeneity.

Experimental procedures

Bacterial strain and media

All the experiments were done with the *Escherichia coli* TG1 strain. Plasmids used in this work are described in Table 2. The antibiotic concentrations in culture media were as follows: ampicillin, 100 μg ml^{-1}; kanamycin, 50 μg ml^{-1}. The C4-HSL and 3O-C12-HSL auto-inducers were added at a final concentration of 1 μM. TG1 cells were grown in Luria–Bertani rich medium (LB) or in a minimal medium composed of M9 minimal medium supplemented with 0.1 mM $CaCl_2$, 1 mM $MgSO_4$, 0.6% glucose and 25 mg l^{-1} vitamin B1.

DNA manipulations and plasmid constructions

Digestion of plasmids and inserts with restriction enzymes and PCR reactions were performed as described by Sambrook *et al*. (1989). Plasmids were prepared with the Qiagen kit.

The plasmid pHEGFPC was constructed from the commercially available plasmid pEGFP (Clontech), to introduce a hexahistidine tag to the N-terminal of EGFP and to introduce cloning sites in C-terminal coding region for the construction of fusion proteins. The modified plasmid was constructed in three steps. First, pEGFP was digested with *PstI* and ligated with the oligonucleotides $UpHis_6$ and $DwHis_6$ to introduce the hexahistidine tag and give pHEGFP. This plasmid was then digested, to remove some of the unique N-terminal restriction sites, with *MfeI* and *XmaI*, the sticky ends removed with the Klenow fragment of DNA polymerase I and then religated giving pHEGFPm1. Finally, this plasmid was digested with *NotI* and *BsrGI* and religated with the oligonucleotides UpGFP-C and DwGFP-C which introduced some unique restriction sites and gave pHEGFPC. This plasmid codes for a N-terminal hexahistidine tagged EGFP variant under control of the *lac* promoter and importantly contains three C terminal cloning sites within the coding region (*MscI*, *NaeI* and *SalI*) for the construction of fusion proteins with a N-terminal fluorescent domain. The intermediate plasmid pHEGFP contains *MscI* and *SalI* restriction sites in the same reading frame to allow construction of equivalent fusion proteins with a C-terminal fluorescent domain.

For the construction of pHGR, *rhlR* gene was amplified with U-GFP*rhlR* and L-GFP*rhlR* from PAO1 genomic DNA. The 746 pb fragment obtained was digested by *MfeI* and *NotI* and cloned in frame with the EGFP between the same restriction sites of the pHEGFPC. The resulting fusion protein was composed of an N-terminal hexahistidine tagged EGFP domain and a C-terminal RhlR regulator domain. The construction of the pMCR was described in Table 2.

Purification of EGFP

The EGFP was purified using the hexahistidine tagged protein expressed from the plasmid pHEGFPC. Cells were grown in LB medium at 37°C to a cell density of 0.6 before induction with 0.1 mM IPTG, growth was continued for 2 h and cells were allowed to accumulate EGFP. Cells were harvested by centrifugation, washed, lysed and the proteins bound to a Talon Co-resin (Clontech). The resin was washed and then EGFP eluted with an imidazole gradient. Electrophoretic analysis and coomassie blue staining showed the

Table 2. Strains, plasmids and oligonucleotides used in this work.

	Relevant characteristics	Origin reference
Strains		
E. coli		
TG1	*supE, hsdΔ5 (lac, proAB) F′ (traD36, proAB, lacI^q, lacZM15)*	Maniatis *et al*. (1982)
P. aeruginosa		
PAO1	Wild type	Holloway *et al*. (1979)
PAO1*rhlR*::Tc	PAO1 with Tc cartridge inserted into unique *Bam*HI site of *rhlR*	Beatson *et al*. (2002)
Plasmids		
pEGFP	EGFP expression plasmid	Clontech
pHEGFP	pEGFP with N-terminal His_6 tag	This work
pHEGFPC	pHEGFP with C-terminal cloning sites	This work
pHGR	pHEGFPC with *rhlR* cloned at the 3′ end of the EGFP gene	This work
pMW47.1	pUCP18 with a 2kb PstI PAO1 DNA insert containing *rhlR and rhlI*	Latifi *et al*. (1996)
$pBBR1MCS_2$	Broad-host-range cloning vector, $Km^{R.}$ *lacZα*, mob^+, P*T3*	Kovach *et al*. (1994)
pMCR	$pBBR1MCS_2$ carrying a 1,5kb *Eco*RI of pMW47.1 containing rhlR under Plac	This work
Oligonucleotides		
$UpHis_6$	GTCATCATCATCATCATCACCAATTGCTTAGACTCGAGTGCA	
$DwHis_6$	CTCGAGTCTAAGCAATTGGTGATGATGATGATGATGACTGCA	
UpGFP-C	GTACCTTCAATTGGCCGGCGTCGACTAGC	
DwGFP-C	GGCCGCTAGTCGACGCCGGCCAATTGAAG	
U-GFP*rhlR*	TAAACTATCAATTGATGAGGAATGACGGAGGCTTT	
L-GFP*rhlR*	TTTTATATTTGCGGCCGCTGCGCTTCAGATGAGACCC	

protein to be reasonably pure (better than 98% based on Coomassie staining), and intact.

Fluorescence anisotropy measurements

Anisotropy measurements were made on a Spex fluorolog III spectrofluorimeter (Jobin-Yvon/Spex, Longjumeau, France) equiped with a double excitation monochromator, single emission monochromator and a peltier cooled photomultiplier tube. The sample compartment contained accessories for measuring front face fluorescence, polarizers (either Glan-Thompson prisms or film polarizers) to polarize the excitation light and measure independantly the parallel and perpendicular components of the emitted light. In addition a GG475 glass filter was included in the emission path to reduce interference from scattered excitation light.

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 measuring the front face fluorescence in a 1 mm cuvette. The sample compartment was thermostated at 15°C to reduce the accumulation of a fluorescent compound in some cells. The sample was excited with polarized light at 450 nm and emission spectra of both the parallel and perpendicular components recorded between 500 and 650 nm.

Fluorescence microscopy

Cells for fluorescence microscopy were fixed to a poly lysine film and observed with a 100 × oil immersion objective. Routinely fluorescence images were recorded, with an integration time between 40 ms and 8 s as appropriate, and a phase-contrast image of the same field recorded. Mean pixel intensity was measured as described previously (Leveau and Lindow, 2001)

In vivo *cross-linking*

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 for 15 min. These conditions were chosen because we observed that longer treatments with higher concentrations of cross-linker or treatments after more extensive induction tended to show many higher order and non-specific aggregates with both the RhlR-EGFP fusion protein and EGFP. Treated cells were collected by centrifugation and washed three times with PBS. Bacterial pellets were resuspended in the lysis buffer [50 mM Tris-HCl pH 8, 1 mM EDTA, 250 mM sucrose, 50 μg ml^{-1} DNase, 400 μg ml^{-1} lysosyme, proteases inhibitor cocktail (Roche)]. The lysis was performed during 1 h at 4°C, the lysates was diluted twice in distilled water and incubated 5 min at −180°C and 5 min at 37°C. The samples were centrifuged at 45 000 *g* for 20 min to separate the soluble and particulate fractions. Then, 250 μl of 4 ×-concentrated disaggregation buffer (2% SDS, 10% glycerol, 62.5 mM Tris-HCl pH 6.8) was added to 1 ml of the soluble fraction to obtain a final concentration of $5U_{OD}$ per ml. The DSP cross-linking was broken by the addition of 5% of β-mercaptoethanol in the disaggragation buffer and incubation 5 min at 100°C. Then, $0.5U_{OD}$ of each sample were loaded on a 9% denaturing polyacrylamide gel (SDS-PAGE), electrotranferred onto a nitrocellulose membrane. The Western blot was developed with a primary antibody against GFP and a secondary HRP-conjugated antibody before revelation using chemiluminescence.

Fractionation experiments

Cells were cultivated as for the cross-linking experiments. They were collected by centrifugation and resuspended in TBP buffer: 50 mM Tris-HCl pH 7.2; 10 mM β-mercaptoethanol; 10 μM PMSF. The cells were broken in the French press and the lysates were separated into soluble and particulate fractions as described above. The fractions were loaded on a 11% SDS-PAGE ($0.2U_{OD}$ of each sample), electrotransferred onto a nitrocellulose membrane and probed with the appropriate antibody.

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