Direct Evidence for the Modulation of the Activity of the 
Erwinia chrysanthemi Quorum-sensing Regulator ExpR by Acylhomoserine Lactone Pheromone*

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In Erwinia chrysanthemi production of pectic enzymes is controlled by a complex network involving several regulators. Among them is ExpR, the quorum-sensing regulatory protein. ExpR is a member of the LuxR family of transcriptional regulators, the activity of which is modulated by the binding of diffusionable N-acylhomoserine lactone pheromones to the N-terminal receptor site of the proteins. Previous in vitro DNA-ExpR binding studies suggested that ExpR might activate pectic enzyme production and repress its cognate gene expression. This report presents genetic evidence that ExpR represses its own gene expression in the absence of pheromone and that the addition of pheromone promotes concentration-dependent de-repression. In vitro experiments show that (i) ExpR binds target DNA in the absence of pheromone and that the pheromone dissociates ExpR-DNA complexes, (ii) ExpR binds target DNA in a non-cooperative fashion, and (iii) two molecules of pheromone are bound per molecule of ExpR dimer. In the absence of N-(3-oxo-hexanoyl)-homoserine lactone, ExpR prevents RNA polymerase access to the expR promoter, thereby directly repressing transcription initiation. The presence of pheromone renders the expR promoter accessible to RNA polymerase and results in the de-repression of transcription initiation. Overall we have established that there is a direct modulation of the repressive activity of a LuxR family regulator by a pheromone. Furthermore, site-directed mutagenesis experiments strongly suggest that the ExpR residues Leu-19, Tyr-31, and Ser-125 are involved in the transduction of conformational changes induced by ligand binding, and this provides new insights into the structure-function relationship of this bacterial regulator family.

Many proteobacteria appear to use chemical signals to regulate various physiological processes, including virulence, secondary metabolism, and bioluminescence, in response to fluctuations in cell population density (1, 2). This phenomenon, called quorum sensing, was first described in the LuxI/LuxR system of the bioluminescent marine bacteria Vibrio fischeri. Quorum sensing requires the release of diffusible signal molecules, mainly N-acyl homoserine lactones (acyl-HSLs), produced by proteins that resemble LuxI. When the signal molecules reach a certain threshold level, they are detected by regulators that resemble LuxR. Although a few LuxR-type proteins act as repressors, most characterized members of this family are transcriptional activators, including LuxR (3, 4). In pathogenic bacteria, it is assumed that quorum sensing is used as a strategy to mount a sustained attack on the host only when their numbers are high enough to ensure that they have a reasonable chance of success.

The enterobacteria Erwinia chrysanthemi and other soft-rot Erwinia species can infect a wide range of economically important crops causing soft-rot diseases. Plant tissue maceration by the soft rot Erwinia spp. is because of the disintegration of the plant cell wall by extracellular enzymes, among which pectinases play a predominant role (5, 6). In E. chrysanthemi, the production of pectic enzymes is modulated by a complex network involving several regulatory proteins (7–11). Among them is the ExpR protein, a member of the LuxR family of transcriptional regulators. The expR gene is transcribed convergently to expL, which is responsible for the synthesis of N-(3-oxo-hexanoyl)-homoserine lactone (OHHL) and N-hexanoyl-homoserine lactone (12). Of these two pheromones, OHHL is the most abundant and was, thus, postulated to be the most physiologically important quorum sensing signal (12). Disruption of the signal synthase gene, expL, results in a decrease of some pectinase gene expression. Although no clear phenotype was associated with a mutation in expL, in vitro gel shift and DNase I footprinting experiments have revealed that ExpR binds specifically to the promoter region of the genes encoding the main pectinases and that the addition of OHHL modified the ExpR-DNA band-shift profiles. These results suggest an involvement of ExpR in the regulation of the virulence gene expression dependent on the OHHL concentration (12). ExpR also specifically interacts with the regulatory region of expL and with that of its own gene. Of all the genes on which it binds,

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3 The abbreviations used are: acyl-HSL, N-acyl homoserine lactone; OHHL, N-(3-oxo-hexanoyl)-homoserine lactone; MES, 4-morpholineethanesulfonic acid; EDC, N-ethyl-3-(3-dimethylaminopropyl)carbodiimide; RNAP, RNA polymerase.
ExpR displays the greatest affinity for the expR regulatory region, which encompasses a well-conserved lux box-like sequence. Moreover, the position of the binding site on the expR promoter corresponds with repressor activity (11, 12), whereas the position of the binding site of the regulator on the pectinase and expI genes is in accordance with an activator function.

LuxR members belong to the large UhpA/FixJ/LuxR family of transcriptional regulators in which the activity of C-terminal, DNA binding domains is modulated through modifications of their N-terminal domains. The events that trigger the regulatory switch at the receiver domain take many forms, including covalent modification (e.g. phosphorylation of NarL and FixJ) or binding of a signaling molecule (e.g. OHHL to LuxR) (13, 14). These signaling events usually lead to multimerization of the regulator essentially mediated by the N-terminal domain. Extensive studies, mainly conducted on LuxR from V. fischeri and TraR from Agrobacterium tumefaciens, CarR from Erwinia carotovora subsp. carotovora, and on LasR and RhlR from Pseudomonas aeruginosa, have resulted in the accumulation of a large set of data on the structure-function relationship of the activators of the LuxR protein family (4, 15–19). In the case of LuxR, TraR, LasR, and RhlR, autoinducer was shown to be required for multimerization and for activation of target gene expression. TraR was recently crystallized as a complex with its cognate autoinducer and its DNA binding site (20, 21). These crystal structures are the first obtained for a quorum-sensing regulator, and they revealed TraR as a dimer with the N-terminal part of each monomer binding to its pheromone and the C-terminal domain of each monomer binding to DNA. Although the N-terminal domains are sufficient for dimer formation, the C-terminal parts also have a dimerization interface although it is less extensive. CarR can exist as a stable preformed dimer able to specifically interact with its DNA binding site in the absence of pheromone. Pheromone binding causes the dimers to form higher order multimers, and this results in activation of target gene expression (22). Thus, the structural modifications induced by ligand binding on the activators of the LuxR family appear to be varied. Finally, site-directed mutagenesis experiments led to the identification of residues involved in ligand binding and in dimerization in the regulators LuxR, TraR, LasR, and RhlR as well as in the interaction with RNA polymerase for TraR and LuxR (15, 23–25). It is, however, important to note that the precise mechanism used by most of the activators of the LuxR family to regulate target gene expression still needs to be elucidated. Indeed, except for LuxR and for TraR (26, 27), no direct evidence of the effect of the activators on RNA polymerase activity has been shown.

Among the repressor members of the LuxR family, apart from the studies mentioned above performed with the E. chrysanthemi ExpR protein, only EsaR from Pantoaea stewartii ssp. Stewartii has been biochemically characterized. EsaR exists as a dimer and binds the target promoter in the absence of its ligand, whereas the presence of the ligand alters the binding characteristics of EsaR and results in a de-repression of target gene expression (28). However, as for most of the quorum sensing activators, no direct evidence of EsaR action on the RNAP activity was found.

The present study focuses on a well-conserved lux box-like sequence, the expR box associated with the expR gene, to provide a fuller understanding of the nature of the molecular mechanism used by ExpR to direct the control of gene expression in E. chrysanthemi. We used in vivo gene fusion quantification and in vitro experiments, including DNase I and potassium permanganate footprinting as well as transcription, to explore whether ExpR is a direct repressor of quorum-sensing. Moreover, we characterized the influence of selected amino acid substitutions on the transcriptional activity of ExpR. Based on this, we propose a model for the initiation of structural modifications induced by the ligand binding in the N-terminal domain.

**Experimental Procedures**

_Bacterial Strains, Media, and Chemicals—_Bacterial strains and plasmids used in this study are listed in Table 1. _Escherichia coli_ cells were grown at 30 or 37°C in LB medium (29) and, when required, with antibiotics at the following concentrations: 50 μg/ml−1 kanamycin and chloramphenicol; 100 μg/ml−1 ampicillin. Synthetic preparation of OHHL was provided by the Laboratoire de Chimie Organique (Institut National des Sciences Appliquées de Lyon, France) and was produced as previously described (30).

_Plasmid Constructions—_The coding region of expR was amplified by PCR using primers ExpR-Deb and ExpR-fin (Table 2) containing unique restriction sites, so that the resulting fragment contained a NdeI site at the ATG initiation codon and an EcoRI site after the stop codon of ExpR. The resulting 800-bp NdeI–EcoRI restriction fragment PCR product was cloned into the pET20b(+) vector (Novagen) to generate pSC3008. In the resulting plasmid pSC3008, the expR gene was placed under the control of the T7 RNA promoter.

The 366-bp EcoRI–BamHI PCR fragment of the expR regulatory region (RRexpR) was amplified with the oligonucleotides RRexpR(EcoRI) and RRexpR(BamHI) (Table 2), digested by the enzymes EcoRI and BamHI and then inserted into the low copy number plasmid pFZY1 (31) digested by the same enzymes. The resulting plasmid pSC2908 contains the RRexpR::lacZ reporter fusion.

Plasmid pSC2912, which contains the expR coding sequence under the control of the l-(−)-arabinose-inducible P_BAD promoter, was constructed as follows: The XbaI–HindIII expR cloning sequence fragment from pSC3008 was cloned into pBAD33 that contains the P_BAD promoter of the araBAD (arabinose) operon and the gene encoding the regulator of this promoter, araC (32). Then, the 2.1-kilobase ClaI–HindIII araC-P_BAD::expR fragment of the resulting plasmid was cloned into the low copy number plasmid pWSK29 (33). The 2.1-kilobase Apal–PstI araC-P_BAD::expR fragment of the resulting plasmid was further cloned into the kanamycin resistant low copy number plasmid pWSK129 (33). Plasmids pSC3207, pSC3209, pSC3211, pSC3216, and pSC3239 containing, respectively, expR-L19A, expR-Y31A, expR-Y31W, expR-S125A, and expR-S125T genes were generated in the same way.

_Site-directed Mutagenesis—_The single mutations L19A, Y31A, Y31W, S125A, and S125T were introduced into ExpR by site-directed mutagenesis with the QuikChange kit (Strat-
ed gene) to create the plasmids pSC3119, pSC3120, pSC3121, pSC3124, and pSC3125, respectively. The primers ExpRLeu1919Ala and ExpRLeu191Alarev, ExpRTyr31Ala and ExpRTyr31Alarev, ExpRTyr31Trp and ExpRTyr31TrpRev, ExpRSer125Ala and ExpRSer125Alarev, and ExpRSer125Thr and ExpRSer125ThrRevev were used to generate L19A, Y31A, Y31W, S125A, and S125T, respectively. Plasmid pSC3008 was used as the template for the PCR. Sequences of oligonucleotides are given in Table 2 (mutated codons are in bold). The five amino acid substitutions L19A, Y31A, Y31W, S125A, and S125T lead to the creation of a new restriction site Ndel, StyI, BamHI, Nhel, and Hpal, respectively. These restriction sites were used to select the plasmids containing the mutations, which were further confirmed by DNA sequencing (Genome Express, Grenoble, France).

\[ \text{β-Galactosidase Determinations—β-Galactosidase activity was measured on toluene-treated cell extracts by following the degradation of } o\text{-nitrophenyl-β-D-galactoside in } o\text{-nitrophenol that absorbs at } 420 \text{ nm (29). Specific activity is expressed as nmol of products liberated min }^{-1} \text{ mg }^{-1} (\text{dry weight bacteria). Repetition of the experiments is indicated.} \]

\[ \text{Overexpression and Purification of the ExpR Protein—Over-production of the native ExpR and its derivative mutants was carried out in } E. coli\text{. Purification of the ExpR protein was achieved from cells grown at } 25 °C \text{ in LB medium containing ampicillin and chloramphenicol to maintain both pSC3008 and pLysS. When the optical density at 600 nm reached 0.6, isopropyl 1-thio-β-D-galactopyranoside was added to a final concentration of 200 } \mu\text{M to induce T7 RNA polymerase synthesis. Then the cells were grown for an additional 2 h at } 25 °C \text{. The overproduced proteins were purified as described previously (12).} \]

\[ \text{In Vitro DNA-Protein Interaction—The } E. chrysanthemi \text{ expR regulatory region (RRExpR) was recovered from plasmid pSR2211 (11) by XbaI-HincII double digestion. The RRExpR DNA fragment obtained was further end labeled by filling up the XbaI extremity in the presence of } [α^{32}P]\text{ dCTP (3000 Ci/mmol, Amersham Biosciences) and the klenow fragment of DNA polymerase. The labeled DNA fragment was purified after electrophoresis on agarose gel using the Qiagen gel extraction kit (Qiagen, Chatsworth, CA). 10,000 (0.0075 pmol) to 100,000 cpm (0.075 pmol) of the labeled probes were used for band-shift assays, as described previously (12), and apparent dissociation constants } (K_d) \text{ were determined, also as described earlier (8). The signals obtained were detected by autoradiography on Amersham Biosciences Mp film and quantified using ImageMaster TotalLab version 2.01 software (Amersham Biosciences). DNase I footprinting experiments were conducted as previously described except that Nonidet P-40 (Roche Applied Science) was added to a final concentration of 0.1% (v/v) in the binding buffer (12).} \]
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RESULTS

ExpR Regulatory Activity Is Modulated in Vivo by the OHHL Pheromone—In a previous paper we showed that in the presence of the quorum-sensing pheromone OHHL the purified E. chrysanthemi ExpR protein specifically interacts in vitro with the regulatory region of various genes, including the pelA-D, expI, and expR genes. By contrast, in the absence of the pheromone, only a specific interaction with expR was observed in DNase I footprinting experiments (11, 12). This last observation was correlated with the fact that ExpR displays the strongest affinity for its cognate gene. Because one of the main objectives of this study is to elucidate the modulation of ExpR activity by OHHL, we retained the expR gene as a model target DNA.

Binding of ExpR on its cognate gene results in the protection of a 36-bp region that includes the 18-bp predicted expR box. Because the protected region overlaps the expR promoters, it was suggested that ExpR negatively regulates its own gene transcription (11). However, this assertion had not been supported by experimental results. To begin to investigate the control mechanism of ExpR on the expR gene, we constructed a low copy number plasmid-based reporter system. In this system the regulatory region of expR (RRexpR) was inserted into the plasmid pFZY1 (31) upstream of the lacZ gene to generate an RRexpR:lacZ transcriptional fusion in the resulting plasmid pSC2908. In addition, the expR coding sequence was cloned in a second compatible low copy number plasmid, pWSK129, under the control of the l- (+)-arabinose-inducible PBAD promoter to generate plasmid pSC2912. The AraC protein directing the PBAD promoter regulation by arabinose is also encoded on pSC2912. The l- (+)-arabinose-inducible production of ExpR by pSC2912 was assessed by Western blot analysis, which revealed a high level synthesis of the protein only in the presence of the inducer (Fig. 1A). The E. coli strain NM522 containing either pSC2908 alone or both pSC2908 and pSC2912 was grown overnight at 30 °C in LB medium supplemented with or without the PBAD inducer l- (+)-arabinose. In this system, E. coli transformed with pSC2908 constitutes the negative control and indicates the basal level of expression of the reporter in the absence of any specific regulators. The addition of l- (+)-arabinose, OHHL, or both had no impact on the level of β-galactosidase produced by this strain (data not shown). The β-galactosidase activity measured without inducer in the strain containing the two plasmids pSC2908 and pSC2912 reached the same level as that produced by the negative control (Fig. 1B, black and white bars, respectively), indicating that the basal production of ExpR by pSC2912 in the absence of l- (+)-arabinose (Fig. 1A) was not sufficient to modulate the expression of the reporter system. As expected, the addition of 1 μM OHHL did not modify the measured β-galactosidase activity (Fig. 1B, black striped bars). On the other hand, the addition of l- (+)-arabinose resulted in a decrease of the β-galactosidase activity, and remarkably, the level of the reduction is proportional to the amount of inducer provided (Fig. 1B, gray bars). These results showed that ExpR represses its own gene expression and that this repression is dependent on the cellular concentration of the protein. The addition of 1 μM OHHL partially restores the β-galactosidase activity, indicating
a neutralization of ExpR action on the reporter system by the pheromone (Fig. 1B, gray striped bars). In similar experiments, titration of OHHL showed that neutralization of ExpR by the pheromone is dose-dependent and that 1/9262 M is the concentration needed to fully neutralize ExpR repression at the retained arabinose concentration of 0.005% (Fig. 1C). Thus, it clearly appears that ExpR represses its own gene expression and that this action is relieved by the OHHL pheromone in a concentration-dependent manner. Consistently, in E. chrysanthemi, AHL species linearly accumulated with growth to reach 1/9262 M at the end of the exponential phase depending on the culture medium used (LB or M63 minimal medium supplemented with glycerol or glucose) (10). This value is in a same order of magnitude to that obtained in P. stewartii (37).

ExpR Binds OHHL with a Stoichiometry of Two Molecules of Ligand per Dimer of Protein—A previous gel-shift study has shown that ExpR specifically interacts with its own gene regulatory region and that this interaction is relieved by OHHL. However, the affinity of ExpR for the operator and the stoichiometry with which the regulator binds the ligand have not been investigated (11). Therefore, a titration experiment of ExpR against expR was undertaken. In the absence of OHHL, ExpR bound to the expR probe with an affinity (K_d) of 6 nM and a Hill coefficient ~1 (Fig. 2A), indicating that ExpR binds this promoter probe with high affinity and without any cooperativity. Furthermore, with increasing ExpR concentrations, highly retarded complexes appeared at the expense of the lower complexes (Fig. 2C, lanes 6, 7, and 13). This suggests the existence in the expR operator of several ExpR binding sites, including two high (C1 and C2) and several low affinity (C3–C6) sites. Competition and chase experiments conducted in the presence of a large excess of cold-competitor DNA (Fig. 2C) revealed that ExpR displays a higher affinity to its binding sites involved in C1 and C2 complexes than those involved in C3–C6 complexes and that complexes C1 and C2 are more stable than complexes C3–C6. Next, we performed a titration of OHHL against the dissociation of the ExpR-DNA complex. In this experiment we used 7 nM ExpR, a concentration at which at least 70% of the DNA probe is engaged in a complex. The addition of a gradually increasing concentration of OHHL resulted in dissociation of the ExpR-DNA complexes, and although this dissociation was never complete, it increased with the concentration of OHHL supplied (Fig. 2B). The OHHL concentration required to dissociate half of the DNA-ExpR complexes was estimated to be 9.5 nM. Because 7 nM dimeric ExpR were used in the experiment, these data suggest that ~2 molecules of OHHL were bound per molecule of ExpR dimer. Although obtained by an indirect method, this result is similar to those obtained with other LuxR family quorum-sensing regulators by using fluorescence quenching studies (CarR and EsaR) and by quantification of the ligand bound to the regulator (TraR and LasR) (22, 27, 28, 38).
FIGURE 2. Binding of purified ExpR to the expR promoter and the effect of the OHHL pheromone. A, band-shift assay is shown on the left. Reactions were performed in the presence of the end-labeled XbaI-HincII fragment of the expR regulatory region (0.0075 pmol, ~10,000 cpm) and the indicated concentrations of purified ExpR. A Hill plot of the data obtained on the left is shown on the right. B, band-shift assay with 7 nM purified ExpR (lanes + ) and increasing amounts of OHHL (0–30 nM). A plot of the ExpR-DNA dissociation is shown on the right. F, free DNA probe; DNA-ExpR complexes (C1–C3); quantification was performed with the three complexes. C, competitive band-shifts were conducted on the expR operator (0.015 pmol, ~20,000 cpm) with two ExpR concentrations (15 and 150 nM) and with variable excesses of the cold competitor expR operator; in chase experiments, the cold-competitor DNA was added 10 min after incubation of ExpR and labeled expR operator followed by an additional 10-min incubation, whereas in competition experiments cold and labeled probes were added together in the reaction mixture. F, free DNA probe; DNA-ExpR complexes (C1–C6).
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These findings, thus, support a 1:1 molar ratio for the ExpR-OHHL interaction.

OHHL Modifies the Interaction Properties with DNA of the ExpR-RNAP Complex—To elucidate whether or not ExpR and σ70RNAP from E. coli directly compete for the occupation of the same DNA portion on the promoter region, we carried out in vitro experiments in the presence of both proteins. Although no comparative studies have been performed between σ70RNA polymerase from E. coli and E. chrysanthemi, it appeared us to relevant to perform in vitro studies with RNA polymerase from E. coli. Indeed there is more than 90% identity between the different subunits of RNA polymerase from the two species, and E. chrysanthemi genes (e.g. expR) are usually correctly expressed in E. coli. The mutual influence of ExpR and RNAP on their binding ability was estimated by using control reactions containing only one of these two proteins. Initially we used band-shift assays for these investigations. In the absence of OHHL, as previously mentioned, the addition of increasing concentrations of ExpR to the mixture containing the DNA probe resulted in the formation of two high affinity complexes (C1 and C2) (Fig. 3A, lanes 2 and 3) and several low affinity complexes (C3–C6) (Fig. 3A, lanes 4–6). On the other hand, in the presence of RNAP, only a highly retarded complex is observed (Fig. 3A, lane 7). The addition of the two proteins in combination showed that only a slight over-shift, corresponding to the binding of both ExpR and RNAP, was observed and that most of the probe gives bands migrating at the positions of the ExpR-DNA and RNAP-DNA complexes (Fig. 3A, lanes 8–10). Moreover, a co-binding of ExpR and RNAP resulted in the disappearance of most of the highly retarded ExpR-DNA complexes at the expense of the RNAP-DNA complex. Thus, it appears that, in the absence of OHHL, ExpR and RNAP could simultaneously bind to the expR operator, mostly in an exclusive fashion, and that the low affinity binding sites of ExpR and the binding site of RNAP are either superimposed or at least overlapping. In the presence of OHHL, the ExpR-DNA complexes, particularly those that are highly retarded, were dissociated (Fig. 3B, lanes 2–4). This observation is in accordance with our previous results (11). The addition of RNAP showed similar results to those obtained in the absence of OHHL (Fig. 3B, lane 5). In the presence of RNAP, ExpR, and OHHL, the binding of RNAP (arrowhead) is more pronounced, as observed in the reactions with the separate proteins, and no ternary complex involving ExpR-RNAP-DNA could be observed (Fig. 3B, lanes 6 and 7). Thus, based on the results of these band-shift experiments, it appears that OHHL dissociates ExpR-DNA complexes without significantly affecting the fixation of RNAP on the expR operator.

DNase I footprinting experiments were conducted next to establish the precise location of the RNAP binding site on the regulatory region of the expR gene and to analyze more accurately the possible functional interaction/competition between these two proteins. As previously reported (11), ExpR protects a 46-bp region (Fig. 4) on the expR promoter either in the absence or in the presence of OHHL, the protection being, however, more pronounced in the presence of the pheromone. This protected area extended from nucleotides +6 to −40 with respect to the main expR transcription start site P2. In addition to the protected site, ExpR binding induces the appearance of several nuclease-hypersensitive sites (+6, −2, −12, −24, and −34). Because the increase in ExpR concentration to saturation point does not reveal any additional protected region in the absence of OHHL (Fig. 4 compare lane 1 to lanes 2 and 3), it seems that the stability of the highly retarded complexes observed in band-shift experiments is not sufficient for their identification by DNase I footprinting experiments. Alternatively, a partial aggregation of the protein at a relatively high concentration may also result in the formation of the highly retarded complexes. The addition of RNAP protected several positions in the upstream region (e.g. −64, −52/−57, −44, and −34) from DNase I cleavage and increased cleavage around positions +6, +19, −18, −27/−29, and −37/−40 with respect to the start point of transcription P2. Because only DNase I hypersensitivity was observed, indicative of DNA distortion, but no extensive protection region was apparent, it appears that RNAP does not

![FIGURE 3. Binding of ExpR and RNAP to the expR promoter and the effect of OHHL. A, band-shift assay performed in the absence of OHHL. B, band-shift assay performed in the absence of OHHL. All lanes contain the end-labeled XbaI-HincII fragment of the expR regulatory region (0.075 pmol, ~100,000 cpm). The concentration used for ExpR is indicated. RNAP was added to a final concentration of 200 nM. F, free DNA probe; DNA-ExpR complexes (C1–C6), C1, and C2 correspond to the complexes with the high affinity binding sites, whereas C3 to C6 correspond to complexes obtained by binding to the high affinity sites and additional lower affinity site(s); DNA-RNAP (arrowhead) and ternary DNA-ExpR-RNAP complex (open arrowhead) are indicated.](Image 313x26 to 388x38)
FIGURE 4. A, DNase I footprinting of ExpR and RNAP binding at the expR promoter. The purified ExpR was added to the final concentration indicated for each lane in the presence (+) or absence (−) of 200 nM E. coli α70 RNAP and with (+) or without (−) 100 μM OHHL; the concentration of the expR regulatory region was as for the band-shift assay. The sequence is numbered from the start site (+1) of the main expR P2 promoter (11). The regions binding ExpR, RNAP, and ExpR-RNAP complex are indicated by thick, dotted, and open bars, respectively; the region protected by RNAP when the two proteins are both added (lanes 5, 6, 11, and 12) is shown by a dotted line. The hypersensitive sites induced by the binding of ExpR in the presence or absence of OHHL are shown by the closed arrowheads; the new hypersensitive sites observed in the presence of ExpR, RNAP, and OHHL are indicated by open arrowheads. A position with an asterisk represents a DNase I hypersensitive site induced by the binding of both RNAP and ExpR (lanes 5 and 6), the intensity of which is decreased in the presence of ExpR, RNAP, and OHHL (lanes 11 and 12). B, sequence of the expR promoter. The binding sites for proteins are indicated as in A. The −10 and −35 hexamers of the two expR promoters P1 and P2 are boxed, and the start points of transcription are shown by arrows. The two KMnO4-sensitive thymines at position −6 and −7 are indicated by open hexagons; the 22-bp expR box consensus is boxed.
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FIGURE 5. ExpR prevents transcription initiation at the ExpR promoter. A, KMnO₄ reactivity of the expR promoter on supercoiled templates. The expR regulatory region from pSR2681 was incubated with 50 nM RNAP together with the indicated concentrations of ExpR in the presence or absence of 100 μM OHHL. B, effect of ExpR upon expR promoter activity. The reactions were performed in the presence of 50 nM RNAP together with the indicated concentrations of ExpR in the presence or absence of 100 μM OHHL. C, quantitative analysis of the expR P2 activity; the data were normalized to the value obtained for the bla promoter (an internal control).

bind tightly. This footprint area is, however, in the appropriate position for initiating transcription at the expR P2 promoter (Fig. 4B). The extensive footprints in the upstream region of expR promoter might be explained by wrapping of the DNA around RNAP as reported for several E. coli promoters on which RNAP footprints spanned 90 bp (−70 to +20) or even more (39). In the absence of OHHL, the addition of a combination of RNAP and ExpR resulted in an enhancement of the protection in the regions spanning −90 to −27 and −24 to −2. Notably, the pattern of the protection of the region located near the expR core promoter (−24 to −2) is closer to that obtained in the presence of ExpR alone (e.g. increase in intensity of bands at positions +6, −2, −12, and −24), whereas the pattern displayed by the upstream region is closer to that obtained in the presence of RNAP alone (e.g. decrease in intensity of bands at positions −64, −52/−57, −44, and −34). These results suggest that, in the absence of OHHL, RNAP and ExpR stabilize the binding of each other at the expR operator and that the core promoter is mostly occupied by ExpR. In the presence of the pheromone, the pattern of the digestion observed in the upstream region was similar to that obtained above, whereas an intermediate pattern, with a new decreased band at position +6 and two new increased bands at positions +19 and +29, is observed in the downstream region containing the expR core promoter. These results suggest a reorganization of the ExpR and RNAP interaction with this region in the presence of OHHL, which might favor the action of RNAP at the expense of that of ExpR, as revealed by the in vivo transcriptional fusion assay. The discrepancies observed between the band-shift assay and the DNase I footprinting protection could be explained by the probable relative instability of the ternary OHHL-ExpR-DNA complex, which would be destabilized during the band-shift assay. An atypical behavior in band-shift experiments has also recently been reported for LasR and EsaR (28, 38). Globally, the results of band-shift and DNase I experiments clearly show that ExpR and RNAP form a complex at the expR promoter and that the presence of the quorum-sensing pheromone OHHL modifies the interaction properties of this complex with DNA.

ExpR Inhibits Transcription Initiation at the expR Promoter—The effect of ExpR upon RNA polymerase activity was investigated first by using potassium permanganate footprinting on supercoiled plasmid-containing RRexpR (pSR2681). KMnO₄ preferentially targets the pyrimidine residues in the twisted regions of DNA and, thus, allows the extent of promoter opening to be measured. Upon the addition of RNAP, we observed that two bases at positions −6 and −7 in the predicted −10 element of the main promoter P2 are sensitive to KMnO₄ (Fig. 5A, lanes 2). Incorporation of 100 μM OHHL in the reaction mixture restores the KMnO₄ reactivity (Fig. 5A, compare lanes 2–4). The addition of ExpR substantially decreased the KMnO₄ reactivity of these two bases (Fig. 5A, compare lanes 3, 6, and 7). Incorporation of 100 μM OHHL in the reaction mixture restores the KMnO₄ reactivity (Fig. 5A, compare lanes 6–9). From these data, we can infer that binding of ExpR in the expR regulatory region inhibits open complex formation by RNAP at the main promoter P2 and that this inhibition is relieved by the pheromone OHHL. No KMnO₄ reactivity was shown in the region of the second minor expR promoter P1. This observation is probably due to the relatively low contribution of this promoter to expR expression, as previously reported by primer extension analysis (11).
We next used in vitro transcription to directly follow the effect of ExpR on the RNAP activity. For this purpose, we monitored expR transcription using pSR2681 DNA with RNAP and ExpR added either alone or in combination. As expected, the addition of increasing ExpR concentration decreased the transcription of the expR promoter (Fig. 5B), whereas the transcription of the reference bla promoter was not noticeably affected. Incorporation of 100 μM OHHL in the reaction mixture partially restored the transcription of the expR promoter without significantly modifying the bla promoter activity (Fig. 5, B and C). These results demonstrate that ExpR specifically inhibits the expR promoter activity in vitro by directly preventing transcription initiation and that this inhibition is relieved by the pheromone OHHL.

**Mutagenesis of Residues Thought to Be Involved in Structural Rearrangements Induced by Acyl-HSL Binding**—The crystal structure of the TraR of A. tumefaciens in a complex with its natural pheromone N-3-oxooctanoyl-L-homoserine lactone and with DNA has been solved (20, 21), and a number of studies have been performed on mutated forms of LuxR-type activators (15, 17–19, 40–45). This work has led to the identification of the residues involved in the binding of the signal molecule in the N-terminal domain of the regulators and to the prediction of an overall structural conservation of the ligand binding sites. Moreover, additional residues important for the dimerization or the activity of the regulators have been identified, essentially in the N-terminal domain, whereas the residues involved in DNA binding and in contacting RNAP are essentially localized in the C-terminal domain. On the other hand, recent results obtained on ExpR have showed that the purified N-terminal fragment of the protein (ExpR-N) exists as a monomer in a ligand-free state and that the interaction of the domain with the appropriate ligand OHHL results in a dimerization of ExpR-N (46). Thus, as reported for activators, ligand binding to the N-terminal fragment of the repressors induces conformational changes, which result in their multimerization. These results lead to the idea that, contrary to activators, the C-terminal region of the repressor is in a dimeric form in a ligand-free state and that interaction of the N-terminal domain with the ligand might induce conformational changes that interfere with the binding to the C-terminal domain to the target DNA. Overall, we have hypothesized that the ligand binding and the resulting structural modifications occurred by similar mechanisms in both activators and repressors of the LuxR family. Despite these studies, no information has yet been published concerning the residues involved in the transduction of the structural changes induced by the ligand binding along the N-terminal domain. To identify these putative residues, we have investigated amino acids conserved in all these regulators not directly involved in the ligand binding nor in the regulatory activity itself (DNA binding and contact with RNAP) and with a position in the YHSL binding pocket (17, 20, 21, 40), are highly conserved among the LuxR members. However, other conserved residues are located outside this pocket. Inspection on a graphic station of these amino acids in the structure of TraR with the TURBO-FRODO program (47) showed that two conserved residues of the N-terminal domain, Leu-25 and Tyr-37, are buried and are in extensive van der Waals contact via their side chains (Fig. 6B). Leu-25 is part of helix α2, and Tyr-37 is part of strand β1, which is hydrogen-bonded to the strand β5 that contains the highly conserved residue Thr-129 of the acyl-HSL binding site (Fig. 6B). This organization caught our attention because it has been shown in the closely related Fix-type proteins that a Tyr-Thr coupling in the receiver domain is a key event in the cascade of structural realignments triggered by phosphorylation (48). Thr-129 was previously shown in site-directed mutagenesis experiments to be important for TraR activity (17) and for RhlR (18). The relevance of the two other residues has not yet been investigated. Thus, site-directed mutagenesis experiments were undertaken to evaluate the impact of the three corresponding residues Ser-125, Tyr-31, and Leu-19 on ExpR activity. One substitution was conservative (S125T and Y31W), and the other was non-conservative (S125A and Y31A). We also constructed the ExpR-L19A mutant.

**Accumulation and Oligomeric State of Each Site-specific Mutant ExpR Protein in Vivo**—Each mutant ExpR was assayed for its abundance in vivo using quantitative Western immunoblots. The coding sequence of all the mutants was inserted into a low copy number plasmid pWSK129, suitable for the in vivo studies. The resulting plasmids, pSC3207 (L19A), pSC3209 (Y31A), pSC3211 (Y31W), pSC3216 (S125A), and pSC3239 (S125T) were then expressed in E. coli NM522 in parallel to pSC2912 (wild-type ExpR), and equivalent amounts of crude cell extract were used for Western blot analysis. These experiments revealed that four mutants (Y31A, Y31W, S125A, and S125T) accumulated similarly at wild-type levels, whereas mutant L19A accumulated at low but detectable levels (Fig. 7A). We next examined whether the point mutations modify the oligomerization status of ExpR by using chemical cross-linking experiments. Because these experiments require a relatively high concentration of protein, we used the T7-based protein overproduction system BL21(DE3) pLysS/pET20b(+) rather than the NM522/pWSK129 system. Clear cell lysates prepared from strains overproducing each ExpR mutant or wild-type ExpR were first submitted to quantitative Western blotting to normalize the concentration of the different forms of ExpR (data not shown). Then, clear cell lysates containing equivalent amounts of the mutated proteins and wild-type ExpR were retained for chemical cross-linking experiments. The multimeric status of ExpR was analyzed by Western blotting. In the absence of EDC (Fig. 8 lanes 1, 4, 7, 10, 13, and 16), wild-type ExpR as well as its mutant forms migrated on SDS-PAGE with an apparent molecular mass of 30 kDa (Fig. 8, band I), which is in agreement with the molecular mass predicted from the sequence (28.7 kDa). However, in the presence of 5 mM EDC (Fig. 8 lanes 2, 5, 8, 11, 14, and 17), several bands of higher molecular size appeared. In all cases two major bands with apparent molecular masses of about 60 and 68 kDa (Fig. 8, band
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II), corresponding to the dimeric forms of the proteins, were observed. The presence of the two bands is due to intramolecular cross-links within one or both protomers, which alters their mobility. In addition to the dimeric forms, one less intense band of about 130 kDa (Fig. 8, band III) and small amounts of a high molecular weight material were also detected. The 130-kDa band is consistent with the tetramer of ExpR, whereas no band corresponding to trimers was observed. A similar pattern was obtained with purified ExpR (data not shown). Because ExpR was found to be dimeric, from size-exclusion chromatography (12) we suggest that ExpR exists primarily as a homodimer.

ExpR (Fig. 7B, white bars). The ExpR-L19A mutant was about three times less active than the wild-type ExpR, whereas the S125T mutant was slightly more active than wild-type ExpR in the presence of L-(-)-arabinose. Other mutant proteins (Y31A, Y31W, and S125A) were to some degree less active than the wild-type protein in the same conditions. The relatively low repression levels observed with the L19A mutant is certainly due to its less pronounced in vivo accumulation and repression activity of ExpR point mutants. Values are given as a percentage of that of the strain containing the wild-type protein in the same conditions. The relatively low repression levels observed with the L19A mutant is certainly due to its less pronounced

Regulatory Activity of Each Mutant in Vivo—The mutants were further characterized by analyzing their ability to repress the expR gene expression in vivo and for their sensitivity to OHHL. Because the induction ratio was about 3–4-fold for the wild-type ExpR, we tested the mutants using saturating levels of OHHL (100 µM) to avoid missing an eventual effect of the ligand, even on mutants that display very low affinity for OHHL. The E. coli NM522 strains, carrying the pSC2908 and one of the plasmids expressing the mutant expR or the wild-type gene, were grown at 30 °C in LB medium containing ampicillin and kanamycin to an optical density at 600 nm of 0.5–0.6. The cultures were then separated into three batches, one with no addition, one supplemented with 0.005% L-(-)-arabinose, and one supplemented with both 0.005% L-(-)-arabinose and 100 µM OHHL. The resulting cultures were grown at 30 °C for an additional 4 h. Then β-galactosidase, which reflects the RRexpR::lacZ fusion activity, was assayed (Fig. 7B). In the presence of L-(-)-arabinose, all the mutants retained the ability to repress the expR gene expression in vivo, although they were not all as active as wild-type ExpR (Fig. 7B, white bars). The ExpR-L19A mutant was about three times less active than the wild-type ExpR, whereas the S125T mutant was slightly more active than wild-type ExpR in the presence of L-(-)-arabinose. Other mutant proteins (Y31A, Y31W, and S125A) were to some degree less active than the wild-type protein in the same conditions. The relatively low repression levels observed with the L19A mutant is certainly due to its less pronounced in vivo production or accumulation, as revealed by Western blot experiments (Fig. 7A). When L-(-)-arabinose and OHHL were both added to the cultures (Fig. 7B, striped bars), the repression was relieved for S125T within a
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**FIGURE 8.** Chemical cross-linking of ExpR and ExpR mutant forms. Reactions were performed with clear cell lysates containing 1 μM dimer of ExpR or mutant forms and 5 mM EDC in the presence or absence of 200 μM OHHL; control reactions were performed with 1 μM dimer of purified ExpR. After 1 h of incubation at 25 °C, the reactions were stopped by heating in an adequate volume of Laemmli sample buffer at 95 °C for 5 min. 20 μl of each sample were then subjected to electrophoresis on a 12% polyacrylamide gel. Lanes 1, 4, 7, 10, 13, and 16: no cross-linker; Lanes 2, 5, 6, 8, 11, 14, and 17: 5 mM EDC; Lanes 3, 6, 9, 12, 15, and 18: 5 mM EDC, 200 μM OHHL. ExpR and its mutant forms ExpR-S125A (S125A), ExpR-S125T (S125T), ExpR-L19A (L19A), ExpR-Y31A (Y31A), and ExpR-Y31W (Y31W) were visualized by Western blotting using anti-ExpR antiserum. Reactive bands corresponding to the positions of monomeric (I), dimeric (II), and tetrameric (III) ExpR are indicated by arrowheads. The positions of the molecular mass markers (in kDa) are shown on the left.

**FIGURE 9.** A, effect of ExpR and ExpR mutant forms on the KMnO4 reactivity of the expR P2 promoter. The expR regulatory region from pSR2681 was incubated with 50 nM RNAP and purified proteins either in the presence or absence of 100 μM OHHL; the concentration of the purified proteins used were 25 nM for ExpR and the conservative mutants (ExpR-Y31W (Y31W), ExpR-S125T (S125T)), and 50 nM for the three non-conservative mutants (ExpR-L19A (L19A), ExpR-Y31A (Y31A), and ExpR-S125A (S125A)). B, quantitative analysis of the expR P2 reactivity; the data were normalized to the value obtained for the bla promoter reactivity (an internal control).

similar range to that of the wild-type ExpR, whereas a lower level was observed for the Y31W mutant. By contrast, for all the non-conservative mutations, namely L19A, Y31A, and S125A, OHHL had no significant effect, suggesting that the pheromone could not bind to these mutated proteins or that fixation of the pheromone does not induce conformational changes that normally lead to the inactivation of the wild-type ExpR.

**Effects of the Different Mutants on the RNAP Activity in Vitro—**We next used potassium permanganate footprinting experiments on supercoiled plasmid pSR2750 to directly follow the effect of the different point mutations on the regulatory activity of ExpR. These investigations were performed with purified mutant proteins and the native ExpR. We worked at a concentration for which each different protein displays a similar effect on the expR promoter reactivity and with saturating concentration of OHHL. Such conditions permit clear differentiation of proteins responding to the ligand from the others (Fig. 9A). In the presence of the three non-conservative substitutions, ExpR-L19A (Fig. 9A, lane 7; Fig. 9B), ExpR-Y31A (Fig. 9A, lane 10; Fig. 9B), or ExpR-S125A (Fig. 9A, lane 16; Fig. 9B), the KMnO₄ reactivity was significantly reduced compared with that obtained with RNAP (Fig. 9A, lane 1; Fig. 9B). However, the decrease in KMnO₄ reactivity obtained with these three mutants was at least 2-fold less pronounced than that induced by the ExpR wild-type protein (Fig. 9A, lane 4, and Fig. 9B). This clearly revealed that the non-conservative mutants display a repression activity on the expR gene expression even if it is less pronounced than that induced by the ExpR wild-type protein (Fig. 9A, lane 4, and Fig. 9B). Moreover, the similar repression levels obtained with the three non-conservative mutants confirm that the in vivo repression defect of the L19A mutant is due to a less pronounced in vivo accumulation in this mutant. In the presence of OHHL, the reactivity pattern obtained with the mutants was not significantly modified (Fig. 9A, lanes 8, 11, and 17; Fig. 9B), in contrast to a significant relieve of the inhibition of the KMnO₄ reactivity observed with the wild-type form (Fig. 9A, lane 5, and Fig. 9B). The two conservative mutants ExpR-Y31W (Fig. 9A, lanes 13 and 14; Fig. 9B) and ExpR-S125T (Fig. 9A, lanes 19 and 20; Fig. 9B) behaved as the wild-type ExpR. These results indicate that the three non-con-
sensitive mutants retained their repression activity, albeit not modulated by the pheromone OHHL. Taken together these in vivo and in vitro experiments show that the residues Leu-19, Tyr-31, and Ser-125 play an important role in the modulation of ExpR activity by OHHL.

DISCUSSION

In the last decade it has become clear that proteins of the LuxR family can be classified into two main subfamilies. Members of the first subfamily, the vast majority of cases, fold in the active form and stimulate transcription of target genes only when complexed with their cognate ligands. This subfamily includes the two biochemically and structurally well-characterized activators LuxR of V. fischeri (3, 26) and TraR of A. tumefaciens (20, 21). The second subfamily mainly includes the quorum-sensing regulators found in Erwinia-type bacterial species, including EsaR of P. stewartii (formerly Erwinia stewartii) (28), ExpR and VirR of E. carotovora spp. carotovora (49–51), and ExpR of E. chrysanthemi (11, 12). This subfamily is distinctive in its ability to dimerize, to bind DNA, and to regulate target gene expression in the absence of a signal ligand. Members of the second subfamily are less well characterized at the biochemical and structural levels.

The central finding of this study is that ExpR of E. chrysanthemi regulates its own gene expression by repression and OHHL-mediated de-repression. We have established that the ExpR regulatory action occurs by direct modulation of the RNAP activity at the transcription initiation level. These data fit closely with our earlier prediction that ExpR functions as a repressor of its cognate gene expression (11). Furthermore, we have identified candidate amino acids involved in the transduction of conformational changes induced by the ligand binding along the N-terminal domain.

The effect of ExpR on the expR promoter was established by measuring the levels of expR expression in vivo and by in vitro DNA binding studies. Specifically, ExpR represses an RExpR::lacZ fusion expression in E. coli in a concentration-dependent manner, and ExpR responds to exogenously provided OHHL for de-repression (Fig. 1B). Previous analysis has shown that purified ExpR exists as a homodimer and exhibits specificity for target genes, the strongest affinity being observed for the expR operator, which contains a well-conserved lux box-like sequence designated as the expR box (11, 12). In addition, higher order oligomeric complexes of ExpR, which were also found with CarR and EsaR, are detected only when assaying ExpR at a relatively high concentration, without OHHL. By contrast, the presence of the ligand results in the dissociation of the higher order oligomeric complexes. These last results, which have been confirmed in this current work (Fig. 2), diverge from those obtained with CarR and EsaR. In the case of CarR, a stabilization of the higher oligomeric complexes was reported in the presence of the ligand, and it was postulated that this stabilization is required for target gene activation by the CarR-ligand complex (22). No dissociation of the EsaR-DNA complexes in the presence of the ligand has been reported in band-shift assays, whereas a partial inhibition of the EsaR-DNA complex formation by the ligand was observed in fluorescence quenching and by surface plasmon resonance experiments (28).

Although no definitive explanation has been provided for this discrepancy, our band-shift studies and new data about the differences in ligand binding by the LuxR members (16–19, 26, 38) suggest that the structural modifications induced by OHHL binding on EsaR would not be sufficient to dissociate the EsaR-DNA complexes but would modify the protein regulatory activity. This hypothesis is reinforced by our results in DNase I footprinting experiments that reveal a specific binding of ExpR on the expR operator even in the presence of OHHL (Fig. 4). These results, which differ from those obtained in band-shift experiments, could be explained by the relatively high stringency of these last experiments due to the migration through the acrylamide gel in an electrical field, giving rise to the dissociation of the ExpR-DNA complexes. Thus, the behavior of the LuxR members associated with their cognate ligands might not be expected to be homogeneous in band-shift experiments and would depend on the strength of the binding of the ligand to the regulator and/or on the structural modifications induced by the ligand binding on the protein. Because the in vitro band-shift assays obtained with ExpR support the in vivo gene fusion results, we used this approach to determine the stoichiometry with which ExpR binds target DNA or OHHL. Purified native ExpR appears to bind two molecules of OHHL per dimer, like TraR, CarR, and LasR (22, 27, 38) (Fig. 28). Moreover, we have shown that ExpR binds its cognate gene in a non-cooperative fashion (Fig. 2A), as previously reported for LuxR, TraR, and partially for LasR (26, 27, 38). By contrast, CepR from Burkholderia cenocepacia (52) and LasR (only on some target operators) exhibit cooperative binding properties (38). Clearly, different mechanisms are used by quorum-sensing regulators of the LuxR family to bind to their target genes. However, the real significance of the optional cooperative binding in the modulation of target gene expression remains to be elucidated.

Next, we investigated whether the regulation of expR expression by ExpR occurs through the direct inhibition of RNAP activity. DNase I footprinting, KMnO4 reactivity, and in vitro experiments (Figs. 4 and 5) revealed that, in the absence of OHHL, ExpR prevents RNAP access to the expR promoter, thereby directly repressing transcription initiation. The presence of OHHL renders the expR promoter accessible to RNAP and then restores the initiation of transcription. These data provide for the first time an example of direct modulation of the repressive activity of a LuxR member by a pheromone. Furthermore, DNase I footprinting experiments have revealed that ExpR and RNAP mutually stabilize their binding at the expR promoter, and this, therefore, suggests that ExpR is able to establish direct contact with RNAP. Thus, as recently suggested for EsaR and ExpR from the E. carotovora subsp. carotovora (53), ExpR from E. chrysanthemi may have retained the ability to function as a transcriptional activator. Whether such a mechanism is used to activate pectinase gene expression is currently under investigation.

To identify putative residues involved in the transduction of the structural modifications induced by ligand binding along the N-terminal domain of the LuxR members, we have mutated three residues of ExpR (Leu-19, Tyr-31, and Ser-125), highly conserved in the LuxR members, with the relative positions Leu-25, Tyr-37, and Thr-129 in the TraR tri-dimensional struc-
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ture. Among these residues, only Thr-129 has been previously investigated and was shown to be involved in ligand binding in the TraR protein via the establishment of a water-mediated hydrogen bond between the residue and the 3-oxo group of the N-(3-oxo-octanoyl)-homoserine lactone lactone (17, 20, 21). The corresponding residue Ser-135 in RhlR from P. aeruginosa has also been investigated by site-directed mutagenesis and was shown to be important for the activator multimerization, ligand binding, and activity (18). Mutagenesis of ExpR Ser-125 to a conserved residue (S125T) led to a protein as active as the wild type both in vivo and in vitro. However, replacement of this residue by a non-conserved amino acid (S125A) abolished the sensitivity of the resulting mutant to OHHL. Thus, as previously reported for TraR, it could be hypothesized that a water-mediated hydrogen bond between Ser-125 and the 3-oxo group of OHHL is important for ligand binding by ExpR. The two other investigated residues Leu-19 and Tyr-31, according to the structural data, are not involved in ligand binding. Mutagenesis of these two residues by the non-conservative amino acids, L19A and Y31A, resulted in mutant forms that adopt a dimeric conformation and retain a significant repressive activity in the absence of OHHL. The presence of OHHL does not affect their activity, which is fully consistent with our prediction. Therefore, based on the positions of the equivalent residues in the TraR three-dimensional structure, we hypothesize that the ExpR Leu-19, Tyr-31, and Ser-125 are all involved in the transduction of conformational changes induced by the ligand binding along the N-terminal domain. The signal would propagate from strand β5, containing Ser-125, to helix α2, containing Leu-19, via strand β1 containing Tyr-31 (Fig. 6B). Because the crystallographic structure of the homodimer of TraR is asymmetric, conformational rearrangements starting from the center of the N-terminal domain (i.e. the lactone moiety binding site) should have distinct consequences in each monomer. Although strands β1 and β5 have a similar location in the dimer, helix α2 is located at the interface between the N-terminal and the C-terminal domains in one monomer and close to the dimerization interface in the other. From our results we suggest that in one monomer (monomer C according to the PDB structure 1L3L of Zhang et al. (21) shown in Fig. 6B) concerted changes induced by acyl-HSL binding displaces helix α2 and involves direct structural rearrangements in the C-terminal domain. Interestingly, strand β1 also makes contact with helix α6 in both monomers A and C (Fig. 6B). This helix is an essential component of the N-terminal dimerization interface. Thus, a concerted rearrangement between strands β1 and β5 may have a strong impact on both the DNA binding site and the dimerization surface. Interestingly, a similar mechanism, called the Tyr-Thr coupling (48), is proposed for the activation of the response regulators of the closely related FixJ family. LuxR-type and FixJ-type proteins contain a similar C-terminal DNA binding domain with a helix-turn-helix motif (14, 20). Their N-terminal domains have a distinct folding, α + β in LuxR-type proteins as opposed to β/α in FixJ-type proteins (Structural Classification of Proteins (SCOP) data base classification (54)). Activity is modulated via the N-terminal domain, which is phosphorylated in FixJ-type proteins (55). Numerous crystallographic structures of activated and inactivated FixJ-type proteins are available (56–60). A critical structural rearrangement triggered by a conserved threonine residue of the active site on a conserved tyrosine of an adjacent strand is systematically observed upon phosphorylation. The movement of the tyrosine is essential in the dimerization process of the N-terminal domains. Thus, according to our results, it can be speculated that both LuxR-type and FixJ-type proteins have a common mechanism of rearrangements subsequent to modification. The structure of a LuxR-type protein with and without bound pheromone needs to be determined for this hypothesis to be confirmed.

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