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# Structural basis for the activation mechanism of the PlcR virulence regulator by the quorum-sensing signal peptide PapR

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The quorum-sensing regulator PlcR is the master regulator of most known virulence factors in *Bacillus cereus*. It is a helix-turn-helix (HTH)-type transcription factor activated upon binding of its cognate signaling peptide PapR on a tetratricopeptide repeat-type regulatory domain. The structural and functional properties of PlcR have defined a new family of sensor regulators, called the RNPP family (for Rap, NprR, PrgX, and PlcR), in Gram-positive bacteria. To fully understand the activation mechanism of PlcR, we took a closer look at the conformation changes induced upon binding of PapR and of its target DNA, known as PlcR-box. For that purpose we have determined the structures of the apoform of PlcR (Apo PlcR) and of the ternary complex of PlcR with PapR and the PlcR-box from the *plcA* promoter. Comparison of the apoform of PlcR with the previously published structure of the PlcR–PapR binary complex shows how a small conformational change induced in the C-terminal region of the tetratricopeptide repeat (TPR) domain upon peptide binding propagates via the linker helix to the N-terminal HTH DNA-binding domain. Further comparison with the PlcR–PapR–DNA ternary complex shows how the activation of the PlcR dimer allows the linker helix to undergo a drastic conformational change and subsequent proper positioning of the HTH domains in the major groove of the two half sites of the pseudopalindromic PlcR-box. Together with random mutagenesis experiments and interaction measurements using peptides from distinct pherogroups, this structural analysis allows us to propose a molecular mechanism for this functional switch.

crystal structure | protein–DNA interaction | quorum sensor | regulation mechanism

Communication is the essence of important biological processes in the living world. In bacteria, quorum sensing enables a population to trigger physiological processes depending on cell density (1). In Gram-positive bacteria, such as Bacilli and Cocci, new quorum-sensing regulators have been found that, unlike in most described quorum-sensing systems, are controlled by their direct interaction with a reimported signaling peptide: Rap proteins involved in the development of competence and sporulation in Bacilli (2); the necrotrophic response regulator NprR, which enables necrotrophism in *Bacillus thuringiensis* (3); the sex pheromone receptor PrgX involved in the control of conjugation in *Enterococcus faecalis* (4); and the transcription regulator PlcR responsible for the production of virulence factors in *B. thuringiensis* and *Bacillus cereus* (5). These proteins share structural similarities: tetratricopeptide repeats (TPRs) forming a peptide binding domain (6) and a helix-turn-helix (HTH) DNA-binding domain (7) in the case of transcriptional regulators. They have been grouped in a new family of quorum sensors called RNPP, for Rap, NprR, PrgX, and PlcR (8).

PlcR was first identified by Lereclus and colleagues (9) as a positive transcriptional regulator of the Phosphatidylinositol-Specific Phospholipase C gene *plcA* at the onset of the stationary phase in *B. thuringiensis*. The *plcR* gene is found in bacteria of the

*B. cereus* group, which consists of several spore-forming adenine- and thymine (AT)-rich bacteria (5, 10). *plcR* codes for a 34 kDa protein that positively regulates its own expression. It was shown to be a pleiotropic regulator controlling the expression of 45 genes including virulence factors, such as enterotoxins, cytotoxins, and hemolysins (5). Virulence of a *plcR*-deficient mutant is abolished in various animal models of infection (11, 12). PlcR activates the transcription of its target genes by binding to a consensus sequence defined as wTATGnAwwwTnCATAw (5, 13). The activity of PlcR is under the control of the signaling peptide PapR (14). *papR* belongs to the PlcR regulon and is located 70 bp downstream from *plcR*. It encodes a 48 aa peptide that is secreted and then reimported through the oligopeptide permease (OppABCDF) system (15). Once inside the cell, its processed truncated form binds PlcR and promotes its recognition of the PlcR box. The minimal active form of PapR was originally shown to be the LPFEF C-terminal pentapeptide PapR5. A more extensive study focusing on the *B. thuringiensis* 407 strain revealed that the active biological form of PapR in both intra- and extracellular environments was the C-terminal heptapeptide ADLPFEF, called PapR7 (16). The activation mechanism of PlcR by PapR has been shown to be strain specific (14). PapR sequences from different strains of the *B. cereus* group show divergences in their three N-terminal residues, whereas the PFEF core is more conserved. Analysis of a phylogenetic tree built by comparison of the amino acid sequences of PlcR and PapR from 29 different strains resulted in the definition of four groups (I to IV) of PlcR–PapR pairs corresponding to four different phenotypes in the *B. cereus* group (10). However, the structural basis for this specificity remains unknown. The crystal structure of the 34 kDa PlcR protein from group I in complex with the pentapeptide PapR5 (LPFEF) was published in 2007 (8). This binary complex is a dimer similar to the structure of the other RNPP transcriptional regulator PrgX (17). Each subunit of PlcR consists of an N-terminal HTH DNA-binding domain composed of four  $\alpha$ -helices and a C-terminal regulatory domain composed of 10  $\alpha$ -helices forming five degenerated TPR motifs, each composed of a pair of antiparallel  $\alpha$ -helices. The first helix of the TPR domain comprises an N-terminal extension forming a long helix of 30 residues spanning over 40 Å, which connects the HTH- and TPR-domains. It has been named the linker helix (8). Finally, an additional helix, designated as capping helix (8), is found at the C terminus of the protein (Fig. S1).

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Data deposition: The atomic coordinates have been deposited in the Protein Data Bank, [www.pdb.org](http://www.pdb.org) [PDB ID code 4F5C (PlcR apoform) and 3U3W (PlcR/PapR7/DNA complex)].

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In this article we describe the crystal structures of PlcR in its apoform (Apo PlcR) and in complex with the physiological peptide PapR7 and DNA. Comparison of these two new structures with the previously published binary complex PlcR–PapR5 reveals a subtle effect of peptide binding and a drastic conformational change of the PlcR–PapR complex upon DNA binding. Together with the characterization of PlcR mutants active in the absence of PapR, this structural analysis allowed us to propose a molecular activation mechanism for this virulence regulator.

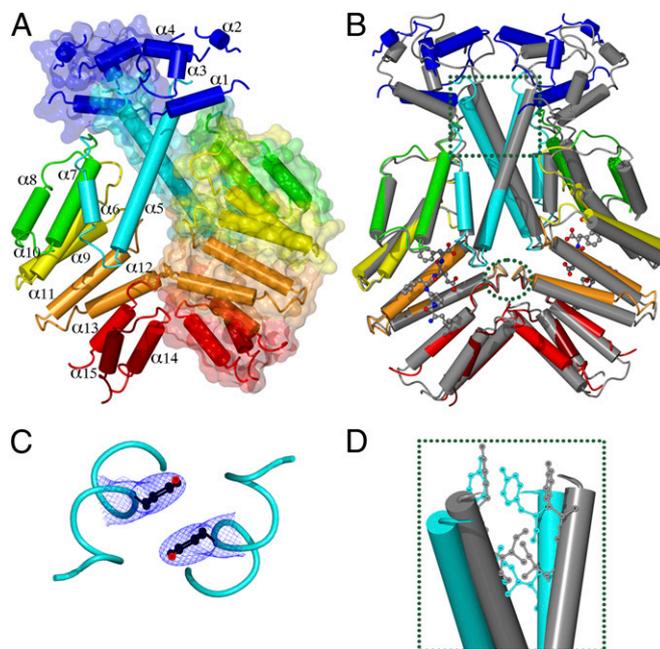
## Results

**Crystal Structure of Apo PlcR.** We solved the X-ray crystal structure of the apoform of PlcR from *B. thuringiensis* strain 407 (pherogroup I) at 3.65 Å resolution, in space group P3<sub>1</sub>, by molecular replacement, using monomer A of the PlcR–PapR5 binary complex (PDB ID 2QFC) (8) as the searching model. The diffraction and refinement statistics are given in Table S1. The crystallographic asymmetric unit contains four molecules, which are nearly identical with an average rmsd of 0.93 Å over an average of 262 aligned C $\alpha$  atoms. There is no significant electron density for loops 16–19 and 26–31 in the HTH domain, neither for loop 55–58 connecting the HTH domain to the linker helix. Lastly, the loop 250–253 connecting helices  $\alpha$ 13– $\alpha$ 14 of the last TPR is also disordered (Fig. S24).

The four molecules of the asymmetric unit form two independent dimers. Analysis of protein–protein interactions with NOXclass (18) showed that Apo PlcR is an obligate biological dimer, characterized by a total buried surface area of about 3,150 Å<sup>2</sup> (interface area of 1,570 Å<sup>2</sup>) (19). Contacts between subunits extend from the N-terminal HTH DNA-binding domain to the C-terminal TPR domain through the linker helix (Fig. 1A). These are mainly nonbonded hydrophobic interactions. Only four hydrogen bonds are observed between main chain atoms from the interacting loops  $\alpha$ 12– $\alpha$ 13 and  $\alpha$ 14– $\alpha$ 15 (Fig. 1A).

**Comparison of Apo PlcR with the Binary Complex PlcR–PapR5.** Comparison of Apo PlcR with the binary complex (PDB ID 2QFC) (8) allowed us to infer the activation mechanism of PlcR. Superposition of each PlcR monomer from both structures displayed an rmsd varying from 1.46 Å to 2.04 Å over about 240 aligned C $\alpha$  atoms, depending on the considered subunits. The main difference resides in the slightly different orientations of the HTH domains and of the long linker helix  $\alpha$ 5 (Fig. S2B). Removing the corresponding residues from the comparative superposition of the two structures decreases the rmsd to about 0.87 Å over 207 aligned C $\alpha$  atoms. In a similar manner, when comparing the dimers of Apo PlcR and of the PlcR–PapR5 complex (Fig. 1B), an rmsd value of about 1.3 Å over 410 aligned C $\alpha$  atoms of their C-terminal domains is found. When superposing only one subunit of the dimers, a minor rotation of the second subunit is observed. It is centered on the dimerization axis formed by loop  $\alpha$ 12– $\alpha$ 13 between the TPR-4 and TPR-5 motifs (Fig. 1B). As a consequence, the dimer of the binary complex PlcR–PapR5 has a reduced total buried surface area of 2,400 Å<sup>2</sup> (interface area of 1,190 Å<sup>2</sup>) compared with the 3,140 Å<sup>2</sup> (interface area of 1,570 Å<sup>2</sup>) observed in the Apo PlcR dimer.

We thus tried to understand how peptide binding induces this conformational change. A more careful comparison of the peptide-binding groove in the two PlcR structures revealed that binding of PapR induces a minor shift of the capping helix  $\alpha$ 15 (Fig. 1B). As a consequence, the loop  $\alpha$ 14– $\alpha$ 15, which is involved in dimer contacts with the neighboring helix  $\alpha$ 12 (Fig. 1A), is also slightly shifted, inducing the rotation of the neighboring subunit around the  $\alpha$ 12– $\alpha$ 13 contact region (Fig. 1B). At the opposite extremity of the protein, this small rotation results in the loss of interactions between residues from the N-terminal end of the linker helix from both subunits. In particular, a strong stacking interaction observed in Apo PlcR between the Tyr64 side-chains from each subunit is lost in the binary complex, with the distance between the two aromatic rings increasing from about 4.5 Å to 9.5 Å, respectively (Fig. 1C and D). In the Apo form, the HTH



**Fig. 1.** Apo PlcR dimer. (A) Both subunits are represented in cartoon and one as surface. The N-terminal HTH domain is colored in dark blue. The TPR motifs are colored in rainbow from TPR-1 in cyan to TPR-5 in red. The N-terminal extension of the first helix  $\alpha$ 5 of TPR1 (linker helix) is shown in cyan. The C-terminal capping helix is in dark red. (B) Superposition of the Apo PlcR dimer (colored as in A) with the binary complex PlcR–PapR5 (in gray). The bound peptide is shown as a ball-and-stick model colored by atom type. The dimer contact region is highlighted with a green circle. The superposition was done using the TPR domain of one subunit. (C) Close view of the stacking interaction between the side chains of residues Tyr64 (in ball-and-stick) with their electron density map (2Fo–Fc) contoured at 1.0 sigma. (D) Detail of Fig. 1B (green square) centered on the linker helices. The distance between the two Tyr64 symmetrical residues increases from about 4.5 Å to 9.5 Å upon peptide binding. The two Ile68 symmetrical residues are about 5 Å apart in both structures.

domains of both subunits interact via their  $\alpha$ 4 helices (Fig. 1A), resulting in an incompatible orientation of helix  $\alpha$ 3 for DNA binding. The loss of contacts between the linker helices upon peptide binding most probably unlocks the N-terminal HTH-domains, promoting their flexibility, hence allowing DNA binding.

This analysis suggests that in the absence of peptide, PlcR would not be able to bind DNA. This is in agreement with previous results from DNA footprinting experiments (14) and has been confirmed here by isothermal titration calorimetry (ITC) measurements showing that the presence of PapR is required to allow binding of PlcR to DNA (Fig. S34). Together with functional assays demonstrating that the presence of the peptide is required for PlcR transcriptional activity (14), these results demonstrate that the activation mode of the peptide relies on subtle conformational changes.

**Crystal Structure of the Ternary Complex PlcR–PapR–DNA.** Crystals were obtained by mixing PlcR with the PapR7 heptapeptide (ADLPFEF) and an 18-base-pair-long dsDNA corresponding to the PlcR-box of the *plcA* promoter (5′-ctatgcaatattcatat-3′), called *plcA*18. The crystals of the PlcR–PapR7–*plcA*18 ternary complex diffracted up to a resolution of 2.4 Å in space group P2<sub>1</sub>. Calculation of the Matthews coefficient (20) suggested two molecules per asymmetric unit. The structure was solved by molecular replacement using one subunit of the PlcR–PapR5 binary complex (8) as the initial model. The dsDNA fragment was built manually. The diffraction and refinement statistics are given in Table S1.

The final model consists of a dimer of PlcR containing 280 amino acid residues and one bound PapR7 (ADLPFEF) peptide per chain, a *plcA18* dsDNA fragment, and 255 water molecules (Fig. 2A). All residues of the protein are in well-defined electron density and the two subunits of PlcR are nearly identical with a low rmsd of 0.135 Å over 280 aligned C $\alpha$  atoms. Analysis of protein–protein interactions with NOXclass (18) confirms that the ternary complex is an obligate biological dimer with a total buried surface area between the two protein subunits of 3,870 Å<sup>2</sup> (interface area of 1,934 Å<sup>2</sup>) (19).

**Peptide-Binding Mode.** Clear electron density corresponding to the PapR7 heptapeptide is visible in both TPR peptide-binding domains of the ternary complex (Fig. S4A). The peptide is bound in a deep cleft with its C terminus buried at the bottom of the cleft (Fig. 2B). The contacts between the peptide and the protein are conserved in both subunits (Fig. S4B). The hydrophobic side chains from the peptide are involved in nonbonded interactions with the protein. Both PapR7 phenylalanines are located in hydrophobic pockets. Most hydrogen bonds are made between atoms from the peptide backbone and side chains from the protein. The only specific interactions are made between the glutamate of PapR7 and residues Lys89, Gln237, and Tyr275 of PlcR. The two additional N-terminal residues A–D of PapR7 point toward the solvent. Their presence increases the total buried surface area between the peptide and a subunit of PlcR from 629.2 Å<sup>2</sup> to 730.5 Å<sup>2</sup> in the PlcR–PapR5 (PDB ID 2QFC) and PlcR–PapR7 complexes, respectively. This observation is in agreement with ITC experiments showing that PlcR displays a slightly better affinity for PapR7 than for PapR5, with  $K_d$  values of 30 nM and 85 nM, respectively (Fig. S3 B and C). It also explains why both forms of the peptide similarly activate PlcR (8, 16).

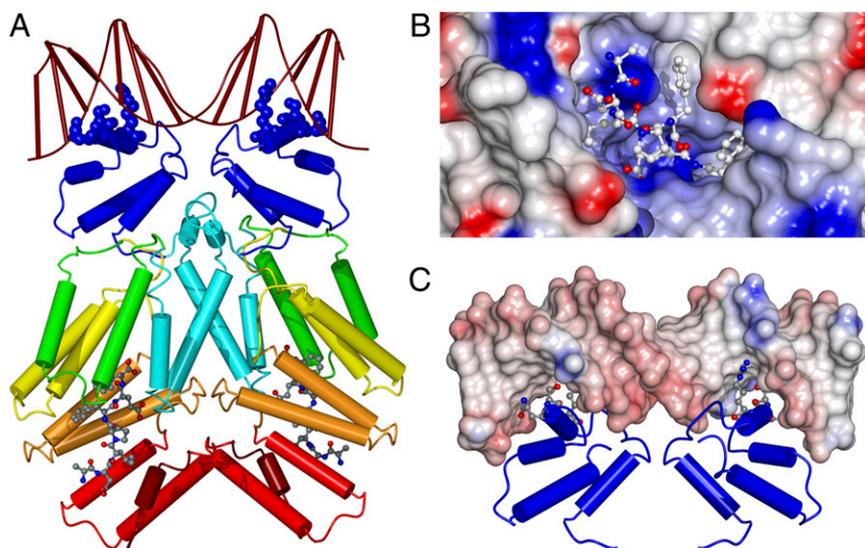
**DNA-Binding Mode.** The PlcR dimer binds to the major grooves of the designed PlcR box of its regulated gene *plcA* through its HTH domains (Fig. 2C). Analysis of the complex formed between the protein and the *plcA18* double-stranded oligonucleotide showed that the total surface area buried in this interaction covers 2,870 Å<sup>2</sup>. The bound DNA fragment displays the characteristic shape of a right-handed B-form nucleic acid structure (21), with a deviation from the regular linear helix of only 3.18°. As typically observed in HTH–DNA interactions (22), the recognition helix  $\alpha 3$  from each HTH domain is oriented almost perpendicularly to the DNA axis and specifically interacts with conserved base pairs from the major groove via the side chains of residues Gln31, Ser32, and Arg36 (Fig. S4C). Because the PlcR-box is not a perfect palindrome, the interactions network is not

exactly the same in the two half sites. The interaction between Gln31 and base A3 is observed in both half sites. Arg36 from both subunits interact with base G5, but only one interacts with base T13 as well. Ser32 interacts via its side chain with base C14 in only one of the binding sites. These specific interactions mostly involve conserved base pairs of the PlcR-box characterized by the consensus sequence wTATGnAwwwTnCATAw (5, 13). Nonspecific interactions between the HTH domains and the sugar-phosphate backbone of the DNA further stabilize the complex (Fig. S4C).

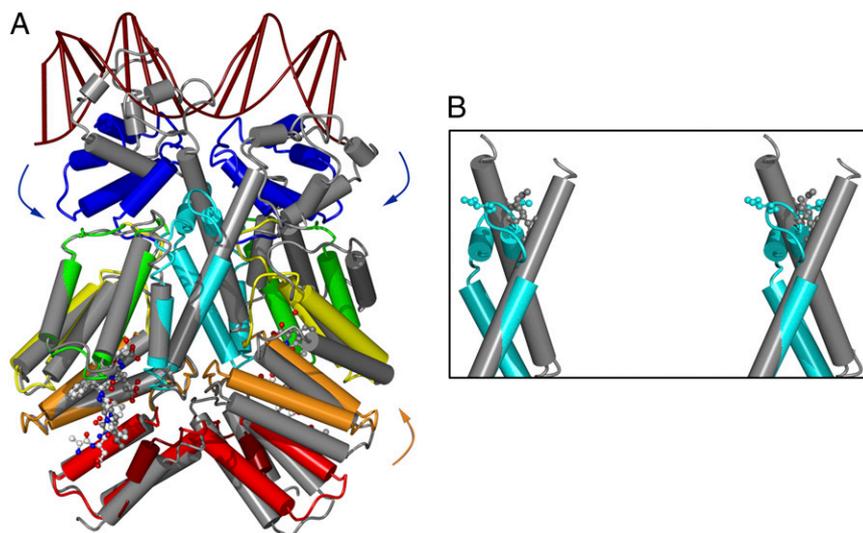
**Comparison of the PlcR Binary and Ternary Complexes.** A drastic reorientation of the HTH domains is observed when comparing the PlcR–PapR7–*plcA18* ternary complex with the structures of the PlcR–PapR5 (8) binary complex (Fig. 3A). DNA binding induces an abrupt kink in the middle of the linker helices, which bend the N terminus by about 90° (Fig. 3B). This results in the making of helix  $\alpha 5'$  (Fig. S1) and a more canonical TPR-1 motif (helices  $\alpha 5$  and  $\alpha 6$ ). Superposition of the TPR domain of one subunit from each complex outputs an rmsd of 1.3 Å over 186 aligned C $\alpha$  atoms (Fig. S2C), demonstrating that the conformation of the TPR peptide-binding domain is not greatly affected by DNA binding. It is clear, however, that the abrupt conformational change of the linker helices and the reorientation of the HTH domains promote important changes in the dimerization mode of PlcR. Superposition of the dimers of both structures displays a high rmsd of about 3.0 Å over 325–375 aligned C $\alpha$  atoms. DNA binding induces a shift of each HTH domain toward the TPR domain of the neighboring subunit, which results in the rotation of the two subunits around the dimerization axis located in the contact region involving loops  $\alpha 12$ – $\alpha 13$  (Figs. 1B and 3A). The total buried surface area of the PlcR dimer increases from 2,400 Å<sup>2</sup> on the binary complex to 3,870 Å<sup>2</sup> in the ternary complex, mainly due to novel contacts amid HTH domains and between the HTH domain from one subunit and the TPR domain from the next.

Our structural analysis thus shows that the subtle conformational changes induced by peptide binding allow the protein to switch from a locked-inactive conformation to a more flexible unlocked-active conformation able to undergo a large conformational change upon DNA binding.

**Characterization of Constitutively Activated PlcR.** To learn more about the activation mechanism of PlcR, we performed a random mutagenesis of the *plcR* gene and screened for PlcR mutants active in the absence of peptide. Plasmids harboring mutated versions of *plcR* were introduced in strain *B. thuringiensis* 407



**Fig. 2.** Ternary complex PlcR–PapR7–*plcA18*. (A) The PlcR dimer is shown in cartoon colored as in Fig. 1A. The bound PapR7 peptide (ADLPFEF) is represented as a ball-and-stick model colored by atom type. The bound *plcA18* DNA fragment (5'-ctatgcaatatttcattat-3') is shown as worm and sticks colored in dark red. Residues Gln31, Ser32, and Arg36 directly involved in DNA binding are illustrated as spheres, colored in blue. (B) Electrostatic potential surface representation of the TPR domain of one subunit of the ternary complex following the typical charge color-code (negative, red; positive, blue; neutral, white) with the bound PapR7 represented as a ball-and-stick model colored by atom type. (C) Electrostatic potential surface representation of the *plcA18* DNA bound to the HTH domains of the PlcR dimer represented in cartoon and colored in blue. Residues Gln31, Ser32, and Arg36, which bind the major grooves of DNA, represented as a ball-and-stick model.



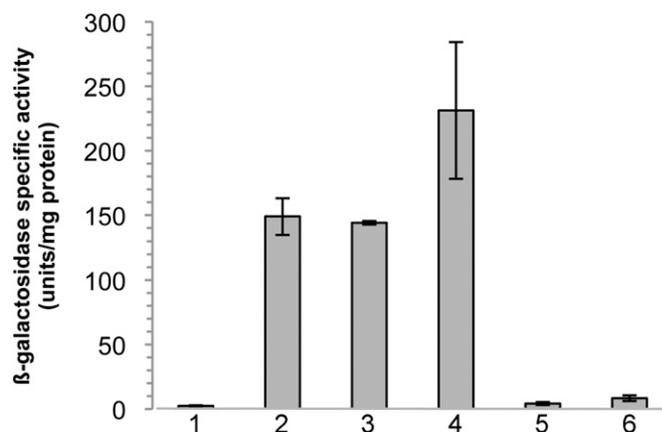
**Fig. 3.** Comparison of the binary and ternary complexes of PlcR. (A) Superposition of the ternary complex PlcR-PapR7-*plcA*18 (colored as in Fig. 2A) with the binary complex PlcR-PapR5 (in gray). Both models are represented in cartoon. The bound peptide in each structure is shown in ball-and-stick colored by atom type (PapR7 in white, PapR5 in gray). (B) Stereo-view of the superposed linker helices in the kink area. The side chains of residues Ile68 from both dimers are shown in spheres (colored as in Fig. 3A). The distance between the two Ile68 symmetrical residues increases from about 5 Å to 27 Å in the binary and ternary complexes, respectively.

*ΔplcRpapR plcA'lacZ* lacking PlcR and PapR and carrying a transcriptional fusion between the promoter of *plcA* and the *lacZ* reporter gene. The *plcA* gene is under the direct control of PlcR, and activation of its promoter directly reflects activity of the PlcR protein. One clone in particular showed a strong induction of the reporter gene. PlcR activity in this clone was evaluated using  $\beta$ -galactosidase assays (Fig. 4A, lane 3). The activity of the mutant is similar to the activity of the wild-type PlcR-PapR complex. Mutations in the *plcR* gene were identified by sequencing and translated to I68N/L185S/M272T. Subsequent functional characterization of the proteins carrying single mutations revealed that I68N is necessary and sufficient for PlcR activity in the absence of PapR (Fig. 4A, lanes 4–6). In parallel, we introduced the Y64A mutation in PlcR. This mutant presented a slightly higher activity compared with that of wild-type PlcR (Fig. S5A) in the absence of peptide, confirming the importance of this structurally identified residue (Fig. 1B) in the activation mechanism. However, this mutation had a much weaker effect than I68N on the activity of PlcR. ITC experiments showed that PlcR (I68N) binds PapR7 with a strong affinity ( $K_d = 6$  nM) (Fig. S5B). They also confirmed that the mutant PlcR (I68N) binds DNA almost as efficiently in the presence of peptide (Fig. S5C) as in its absence (Fig. S5D), whereas the wild-type PlcR requires the presence of PapR to bind DNA (Fig. S3A). Interestingly, the ITC signal is exothermic with the I68N mutant protein and endothermic with wild-type PlcR. The large conformational change observed upon DNA binding could explain the positive enthalpy change ( $\Delta H$ ) displayed by the wild-type protein. The mutant protein in turn is most probably in the active conformation characterized by the broken linker helix, and the negative  $\Delta H$  directly reflects the favorable protein–DNA interaction.

These results demonstrate that it is possible to activate PlcR in the absence of peptide by a single amino acid substitution located in an essential area of the protein structure—that is, the kink region of the linker helix (Fig. 3). In a similar way to peptide binding, mutations I68N and to a lower extent Y64A increase the flexibility of PlcR and promote an active conformation of the protein compatible with DNA binding.

**Specificity Assays.** It has been shown that PlcR from the phylogenetic group I, the focus of our study, is activated by its cognate heptapeptide PapR-I (ADLPFEF), but can also be partially activated by PapR-II (SDMPFEF) and to a lesser extent by PapR-IV (SDLPFEH) but not by PapR-III (NEVPFEF) (16). To elucidate which part of the bound peptide is directly involved in the activation process, we compared the affinity of these three peptides for PlcR-I with that of its cognate peptide PapR-I. ITC measurements demonstrated that PapR-II displays a  $K_d$  of

77 nM of the same order as the reference  $K_d$  value of 30 nM observed for PapR-I. However, the PapR-III and PapR-IV peptide have a strongly decreased affinity with  $K_d$  values of 0.97  $\mu$ M and 1.1  $\mu$ M, respectively (Fig. S6). Because the pentapeptide (LPFEF) has been shown to activate PlcR-I almost as efficiently as the heptapeptide (ADLPFEF) (16), we can assume that the two first residues of the latter are not crucial for the activation of the protein. The third residue is then the only difference that could explain why PapR-III is inactive and PapR-II is active. Having a short apolar residue V at this position instead of the L residue found in PapR-I impairs the activation, whereas its substitution into a longer M residue in PapR-II only promotes its decrease. The poor affinity of PapR-IV can be explained by the substitution of the conserved PFEF motif into PFEH, which introduces a polar charged histidine residue in the hydrophobic pocket of the last phenylalanine residue. This clearly demonstrates that the conserved PFEF motif is essential for peptide binding. It also suggests that the L residue preceding this motif might be directly involved in the activation mechanism. This hypothesis is supported by our structural study showing that the



**Fig. 4.** Characterization of the PlcR(I68N) mutant protein.  $\beta$ -galactosidase was assayed on cells Bt407 *ΔplcRpapR plcA'lacZ* carrying pHT304.18-PxyI with each gene of interest: *plcR*, *plcR*<sub>I68N/L185S/M272T</sub>, *plcR*<sub>I68N</sub>, *plcR*<sub>L185S</sub>, or *plcR*<sub>M272T</sub> sampled at the beginning of stationary phase. PapR7 was added to the culture of cells producing PlcR-wt at the time of inoculation. The bars are labeled 1–6 corresponding to 1, PlcR<sub>wt</sub>; 2, PlcR<sub>wt</sub> + PapR7; 3, PlcR<sub>I68N/L185S/M272T</sub>; 4, PlcR<sub>I68N</sub>; 5, PlcR<sub>L185S</sub>; 6, PlcR<sub>M272T</sub>. The results are the mean values of two independent experiments, and error bars represent SDs.



observed, pointing out their important role in adaptative and virulence processes (27–32). This clearly identifies these regulators as major targets for the search of new molecules with applications in medical, food, and biotechnology areas. The precise understanding of the mode of activation of the RNPP regulators will provide necessary insights for designing quorum-quenching molecules useful as antimicrobial drugs, decontaminating agents or activators of expression of genes of interest.

## Materials and Methods

**Samples Preparation.** *B. thuringiensis* 407 wild-type and mutant PlcR proteins were overexpressed as a C-terminal His6-tagged recombinant protein in *Escherichia coli* strain C41 (DE3) (Invitrogen) and purified by a two-step purification procedure (immobilized-metal affinity and size exclusion chromatography). Details of the expression and purification protocols are given in *SI Materials and Methods*. Preparation of dsDNA fragments containing the PlcR-box of the *plcA* gene and used for ITC experiments, cocrystallization assays, and mutagenesis experiments is detailed in *SI Materials and Methods*, as well as the protocol used for the preparation of the PapR peptides.

**Crystallographic Analysis.** Apo PlcR was crystallized at a concentration of 4 mg/mL in a solution containing 0.1 M sodium chloride, 0.1 M trisodium citrate pH 5.6, 40% (vol/vol) PEG 400 with a protein–reservoir solution ratio of 1:2. The PlcR–PapR7–*plcA*18 ternary complex crystallized in a condition containing 0.16 M calcium acetate, 0.08 M sodium cacodylate pH 5.6, 8% (wt/vol) PEG 8000 and 5% (vol/vol) glycerol with a protein–reservoir solution ratio of 1:2. More details on the crystallization experiments are given in *SI Materials and Methods*. X-ray diffraction data of Apo PlcR were recorded at the SOLEIL synchrotron beamline PROXIMA 1. The dataset of the ternary complex was collected at the ESRF synchrotron beamline ID29 (Grenoble, France). Details of diffraction data collection and processing as well as structure refinement procedures are given in *SI Materials and Methods*. Final statistics are listed in Table S1.

**Structural Coordinates and Figures.** The coordinate and structure factors of the apoform of PlcR and of the ternary complex PlcR–PapR7–*plcA*18 have been deposited with the Protein Data Bank (RCSB PDB) under the ID codes 4F5C and 3U3W, respectively. All structural figures were created with CCP4MG (33). Electrostatic potential surfaces were calculated by the Poisson–Boltzmann solver within CCP4MG and colored accordingly.

**ITC.** Affinities of PlcR to PapR5, PapR7, noncognate peptides, and selected dsDNA were measured by ITC at 20 °C. The dissociation constant  $K_d$  was calculated using the “One Set of Sites” curve-fitting model. Details of the measurement protocols are given in *SI Materials and Methods*.

**Mutational Analysis.** For the isolation of PlcR mutants active without PapR, random mutagenesis was achieved by amplifying the *plcR* gene by PCR with Standard Taq polymerase using *B. thuringiensis* 407 chromosomal DNA as template and primers *plcRa*3 and *plcRmuta* (Table S2). Single mutations were introduced in the coding sequence of *plcR* by directed PCR mutagenesis. More details are given in *SI Materials and Methods*.

**Beta-Galactosidase Activity Assays.** Cells were prepared as described in *SI Materials and Methods*. A total of 5  $\mu$ M PapR7 was added at the start of the culture when stated.  $\beta$ -galactosidase assays were performed as described previously (29).

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