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The two-component system CpxAR is Essential for Virulence in the phytopathogen bacteria *Dickeya dadantii* EC3937

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Abstract

The CpxAR two-component system is present in many Proteobacteria. It controls expression of genes required to maintain envelope integrity in response to environmental injury. Consequently, this two-component system was shown to be required for virulence of several zoo-pathogens but it has never been investigated in phyto-pathogens. In this paper, we investigate the role of the CpxAR two-component system *in vitro* and *in vivo* in *Dickeya dadantii*, an enterobacterial phytopathogen that causes soft-rot disease in a large variety of plant species. *cpxA* null mutant displays a constitutively phosphorylated CpxR phenotype as shown by direct analysis of phosphorylation of CpxR by a Phos-Tag retardation gel approach. Virulence in plants is completely abolished in *cpxA* or *cpxR* mutants of *D. dadantii*. *In planta*, CpxAR is only activated at an early stage of the infection process as shown by Phos-Tag and gene fusion analyses. To our knowledge, this is the first time that the timing of CpxAR phosphorelay activation has been investigated during the infection process by direct monitoring of response regulator phosphorylation.

Keywords

CpxAR; Virulence; stress; plant pathogen; *D. dadantii*

Introduction

For bacteria, for which rapid division is a key component of fitness, the adaptation to environmental variations, but also to various hosts for pathogens, is essential. These adaptations required selective expression of a set of genes depending on the environmental injury. Two-component systems are the main systems sensing these variations directly in the

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border between the cell and the environment. They are also responsible for the plasticity of gene expression in response to these environmental variations.

Typical two component systems are characterized by a transmembrane sensor histidine kinase/phosphatase and by a cytoplasmic cognate response regulator. Under stimuli, often unknown, the sensor histidine kinase autophosphorylates and transfers its phosphate group to its cognate response regulator, which in turn regulates the expression of different target genes (Hoch, 2000). Dephosphorylation is thought to occur via the reverse way in absence of stimulus. Several two component systems, such as CpxAR (Suntharalingam *et al.* 2003; Ronnebaumer *et al.* 2009; Labandeira-Rey *et al.* 2010), are implicated in virulence and in stress response of many pathogens.

The CpxAR system regulates expression of genes required for the envelope stress response (Raivio and Sylhavy, 1997) and for motility, is required for resistance against antimicrobial peptides (Audrain *et al.* 2013) and is consequently required for virulence (Raivio 2005, Ravio 2014). The Cpx system includes the transmembrane sensor histidine kinase CpxA and its cytoplasmic response regulator CpxR. In *E. coli*, this system was shown to be involved in folding and quality control of periplasmic proteins, particularly via the regulation of the expression of *spy* or *degP* (Price and Raivio 2009). The *spy* gene encodes a periplasmic ATP-independent chaperone that prevents protein aggregation and aids in the folding of proteins. DegP, a periplasmic protease, degrades all the misfolded proteins in the periplasm. The CpxAR system is also involved in the regulation of the porin OmpF (Batchelor *et al.*, 2005). Surprisingly, despite its important role in envelope integrity maintenance and in virulence, this two-component system was often studied *in vitro* in *E. coli* or in zoopathogenic bacterial species, a few times *in vivo* in zoopathogenic bacterial species (Humphreys *et al.*, 2004, Spinola *et al.* 2010) but never in phytopathogenic bacterial species.

Our model, *Dickeya dadantii* EC3937 (formerly *Erwinia chrysanthemi*) is a necrotrophic phytopathogenic Enterobacteria and causative agent of soft-rot diseases affecting a wide range of plant species, especially crops. *Dickeya spp*. are directly responsible for from 5 to 25% of the losses of potato crops in Europe and Israel (Toth *et al.*, 2011). The pathogen is list as a A2 quarantine organism by the European and Mediterranean Plant Protection Organization (OEPP/EPPO, 1982, 1988, 1990).

The infection process occurs as follows. *D. dadantii* colonize the surface of the plant via the motility. Bacteria penetrate at a wounded site of the plant. Once in the apoplast, bacteria fight against plant defenses mainly acidic stress, oxidative stress, and antimicrobial peptides (Plessis *et al.*, 2011; Reverchon and Nasser, 2013). Bacteria synthesize a set of plant cell wall degrading enzymes (PCWDEs), particularly pectinases and cellulases (Barras *et al.*, 1994; Collmer and Keen, 1986), allowing growth of bacteria on degraded plant polymers and spread of the disease throughout the whole plant leading to maceration, the visible symptom of the disease.

In this study, we characterized the CpxAR system in *D. dadantii*. Data indicated that this two component system is essential for virulence because *cpxA* or *cpxR* mutant strains are

completely non virulent, and that activation of the CpxAR two-component system is required during the early steps of the virulence process.

Results

Putative structural features and phylogeny of CpxAR

The putative *cpxR* and *cpxA* genes of *D. dadantii* are annotated on its genome and are classically organized in an operon of two genes *cpxRA* (Glasner et al, 2011). We analyzed the family and domain of CpxA and CpxR proteins by using Pfam databases (Finn *et al.*, 2014). The CpxA (Dda3937_03052) is the putative sensor protein of 457 amino acids with 2 transmembrane segments, from amino acid 6 to amino acid 27 and from amino acid 165 to amino acid 184 separated by a periplasmic domain of 138 amino acids. The first transmembrane segment is preceded by 5 cytoplasmic amino acids. The second transmembrane segment is followed by a cytoplasmic amino acids sequence (amino acids 185 to 457) carrying three domains. A so called HAMP domain overlaps the second transmembrane segment and the beginning of the cytoplasmic amino acid sequence (amino acids 164 to 234). A histidine kinase is located between amino acids 238 and 300 and its ATPase domain is located between amino acids 345 and 454. The CpxR (Dda3937_03053) encodes the putative 232 amino acid cognate cytoplasmic regulator protein containing the response regulator domain (amino acid 4 to 112) containing the phosphorylatable D51 residue and the transregulator domain required for fixation to DNA regulatory sequences (amino acid 151 to 228). A phylogenic tree of the CpxR protein obtained by the maximum-parsimony method was constructed with all the bacterial species in which the Cpx system has been studied (in red) and with a selection of the most important pectinolytic phytopathogenic bacterial species (in green) (Fig. 1A). A response regulator from *Gloeobacter violaceus* was used as outgroup (in black) and indicate the possible location of the root. No separated group could be observed between the CpxR sequences from zoopathogens and the CpxR sequences from phytopathogen. A similar tree was observed for the CpxA homologues (Fig. 1B). No synteny was observed among species. This *in silico* analysis strongly suggests that this operon encodes the CpxAR two-component system of *D. dadantii*.

The Cpx two component system controls expression of the *degP*, *ompF* and *spy* genes in *D. dadantii*

To confirm that the genes found *in silico* encode the CpxRA two component system of *D. dadantii*, expression of *degP*, *spy* and *ompF* were analyzed. These three genes are known to be regulated by the CpxRA two-component system in other related bacterial species (see introduction). The *cpxA* and the *cpxR* genes were inactivated by reverse genetics and we constructed and introduced an ectopic copy of the *degP::uidA*, *ompF::uidA*, and *spy::uidA* transcriptional fusions into these *cpxA* and *cpxR* null mutant strains (see materials and methods). The activity of the 3 transcriptional fusions was measured (Fig. 2 A–C). As compared to the wild-type strain, the *cpxA* mutant strain displays a 3-fold increase in the expression of the 3 genes while the *cpxR* mutant strain displays a 1.5-fold increase in *degP* expression, and a 0.8 and 4-fold decrease in expression of the *spy* and the *ompF* expressions, respectively. To confirm that the change in the regulation of the *degP*, *ompF* and *spy* genes

was the result of the inactivation of the sensor histidine kinase *cpxA* or the response regulator *cpxR*, the plasmids pNFW512 (harboring the *cpxA* wild-type gene, *cpxA*$^+$) and pNFW460 (harboring the *cpxR* wild-type gene, *cpxR*$^+$), were constructed and complementation was performed. Plasmid pCpxA (harboring the wild-type *cpxA* gene) was introduced in the *cpxA* mutant strains harboring the *degP*, *spy* and *ompF* fusions and plasmid pCpxR (harboring the wild-type *cpxR* gene) was introduced in the *cpxR* mutant strains harboring the same fusions (respectively pCpxA/*cpxA* and pCpxR/*cpxR* strains). Complementation occurred, because expression was restored at a level similar to the one observed in the wild-type strain for all the fusions. Taken together, these results strongly suggest that the inactivated genes are the *cpxA* and *cpxR* genes of *D. dadantii*. The CpxA and CpxR are known to activate expression of the genes of its regulon. The hypothesis generally given to explain the increased expression of the genes of the *cpx* regulon in *cpxA* null mutant strain is the hyperphosphorylation of the CpxR regulator protein (Klein *et al.*, 2007, Wolfe *et al.*, 2008 Labandeira-Rey *et al.*, 2010, Wolfe, 2010).

The CpxR regulator protein is constitutively phosphorylated in a *cpxA* null mutant

To examine directly the hyperphosphorylation hypothesis of CpxR in *cpxA* null mutants, we carry out Phos-Tag™ analysis. Phos-Tag™ analysis is based on a dinuclear metal complex linked to acrylamide molecules, which binds phosphate groups. In a Phos-Tag™ acrylamide gel, the Phos-tag™ linked to acrylamide molecules interacts with the phosphorylated form of the protein, which migrates more slower than the non-phosphorylated protein, allowing the separation of both forms of the protein. This method has to be adapted to each regulator (Barbieri and Stock, 2008). Purified CpxR was phosphorylated *in vitro* by the phosphor-donor phosphoramidate (PA). 5 ug of CpxR was incubated different times with or without PA, then the samples were loaded onto a Phos-Tag™ acrylamide gel and stained with coomassie blue (Fig. 2 D). Without PA, a single band corresponding to the non-phosphorylated CpxR form (called CpxR) was observed (Fig. 2 D). In contrast, a retardation of migration was observed when PA was added during 15 min and 30 min (Fig. 2 D). This shifted band corresponds to the phosphorylated CpxR form (called CpxR-P). The *in vivo* phosphorylation level was performed using the same approach (Fig. 2 E). In the wild-type strain, almost all the CpxR (89%) was not phosphorylated (Fig. 2 E). As expected, no band could be observed in *cpxR* null mutant strain (Fig. 2 E). In the *cpxA* null mutant strain, almost half (41%) of the CpxR was phosphorylated (Fig. 2 E, and Fig. 2 F). Thus, Phos-Tag data agrees with fusion data. There are yet no studies reporting direct observation of CpxR phosphorylation levels in various genetic backgrounds.

The CpxAR two-component system is required for virulence in *D. dadantii*

The impact of the inactivation of the Cpx system on the virulence of *D. dadantii* was evaluated in chicory leaves, carrots and potatoes. Each plant host was inoculated with the *D. dadantii* wild-type, *cpxA* and *cpxR* mutant strains (EC3937, NFB7515 and NFB7532, respectively) (Fig. 3). After 72h of incubation, no maceration could be observed with mutant strains in any plant host (Fig. 3). To confirm that the inactivation of the sensor histidine kinase *cpxA* or the response regulator *cpxR* provokes a loss of virulence in the three plant hosts, the cpxA/pCpxA or cpxR/pCpxR complemented strains were inoculated in to various plants. In each kind of plant, the virulence was restored to a level similar to that seen when

using the wild-type strain (Fig. 3). To understand the total loss of virulence, the impact of the inactivation of the Cpx system on two of the major virulence factors (the motility and the PCWDE production) was observed.

The motility is affected by the inactivation of the CpxAR two-component system

Motility is required for full virulence, especially for the spread of the disease throughout the plant. The impact of the inactivation of the CxpAR two-component system on motility was measured on soft agar plates (Fig 4 A). The *cpxA* and *cpxR* null mutant strains showed 2-fold lower swim diameters than the wild-type strain (Fig. 4 A). The motility was restored in the pCpxA/*cpxA* and pCpxR/*cpxR* complemented strains since similar swim diameters as the wild-type strain were observed (Fig. 4 A).

The production of the PCWDEs is reduced by the inactivation of the CpxAR two-component system

The PCWDEs are essential factors of virulence and are directly responsible for the damage (maceration) caused by *D. dadantii*. Global pectinase and cellulase activities were estimated on plates (Fig. 4 B–C).

In the pectinase plate test, haloes derived from polygalacturonate degradation by the *cpxA* and *cpxR* null mutant strains were severely reduced as compared to the wild-type strain (Fig. 4 B). The complementation with the pCpxA/*cpxA* and pCpxR/*cpxR* strains allowed restoration of halos similar to those seen in the wild-type strain.

In the cellulase plate test, haloes derived from carboxylmethylcellulase degradation by the *cpxA* and *cpxR* null mutant strains were severely reduced as compared to the wild-type strain (Fig. 4 C). The complementation with the pCpxA/*cpxA* and pCpxR/*cpxR* strains allowed restoration of halos similar to those seen in the wild-type strain.

Thus, two major virulence factors of *D. dadantii*, i. e. motility and PCWDEs production, are severely affected by loss of the CpxAR two component system.

The CpxAR system is required in the resistance of stresses delivered by plant cells against *D. dadantii.*

As a pathogen, *D. dadantii* has to defend against the plants defense. During its infectious cycle, *D. dadantii* has to protect against acidic stress, oxidative stress and antimicrobial peptide (Plessis *et al.*, 2011). To investigate the role of the CpxAR system in the response to these stresses, the resistance of the *cpxA* and *cpxR* null mutant strains was compared to the wild type strain for each stress.

No difference in resistance to acidic stress, encountered by bacteria during maceration, was observed (data not shown) assayed by comparing the growth rates of the mutant strains in M63 medium at pH 4.5 and 7. For each pH, no significant growth rate difference was observed between the two different mutant strains with growth rates values of 0.63 and 0.67 at pH 4.5 and 7 respectively.

Resistance to the oxidative stress was also assayed. Stationary-phase culture of the wild-type, the *cpxA* and *cpxR* null mutant strains were exposed to 6 mM H_2O_2 and the number of viable cells was counted after plating serial dilution on LB plates. The survival of the *cpxA* and *cpxR* null mutant strains was significantly lower than the wild-type strain (Fig. 5A). The resistance to H_2O_2 was similar to the wild-type strain for the two complemented strains pCpxA/*cpxA* and pCpxR/*cpxR* (Fig. 5A).

Resistance to the antimicrobial peptide was evaluated with polymixin B. The *cpxA* and *cpxR* null mutant strains were significantly more sensitive than the wild-type strain (Fig. 5 B). At 10 μg/ml, only half of the initial population was still alive for the *cpxA* and *cpxR* null mutants while all the population of the wild-type strain was still alive (Fig. 6B). The wild-type resistance level resumed for the pCpxA/*cpxA* and pCpxR/*cpxR* complemented strains.

These data indicate that the Cpx system is involved in the response to the plant defense and suggest that the Cpx system is activated at an early stage of the infectious process.

Activation of the CpxAR two-component system is required in the early stage of the infectious process

To investigate when *D. dadantii* needs to activate the CpxAR two component system during its infectious process, we measured the expression level of the *degP*, *spy* and *ompF* gene before inoculation and after one, two, and three days of incubation in chicory leaves (Fig. 6). The expression level increased 4-fold for *degP* and *spy* (Fig. 6AB) and 3-fold for *ompF* (Fig. 6C) one day after inoculations and stayed at the same level during the second day after inoculation as compared to the levels observed before inoculation. The expression level of these 3 genes decrease in the third day of inoculation and display a level similar to the one observed before inoculation.

To strengthen these results, a Phos-tag approach was performed on bacteria extracted from chicory leaves after each of these three days of infection. Because chicory leaves are routinely inoculated with bacteria grown until their stationary phase, CpxR phosphorylation before inoculation (Fig. 6 D) was monitored in these physiological conditions unlike in Fig. 2E where CpxR phosphorylation was monitored in bacteria grown until mid-log phase. The CpxR phosphorylation level of the *cpxA* mutant strain was added as a control of CpxR (61%) and phosphorylated CpxR (39%). Phosphorylated CpxR (4%) was detected in bacteria extracted before inoculation (Fig. 6 D). CpxR phosphorylation increased after one day of infection (18% of phosphorylated CpxR), and after two days of infection (26% of phosphorylated CpxR) but decreased after the third day of infection (13% of phosphorylation) (Fig. 6D). Thus, the Cpx two-component system is activated at an early stage of the infectious process in response to the plants immune response of the plant, but activation decreases as the majority part of the leaf is macerated, logically suggesting a decrease in plant defense. This is the first time that the variation of the phosphorylation level of the response regulator of a two-component system has been observed in a bacterial pathogen species during its infection cycle.

Discussion

During the infectious cycle, *D. dadantii* goes from the surface of the leaf through the apoplast. To achieve a successful infection, bacteria needs to sense its new environment (*i.e*. the plant) and to adapt. Bacteria have one and two-component systems to sense and respond to environmental variations. One-component system are more likely involved in the adaptation of the metabolism to fit better to the new environment (Ulrich *et al.*, 2005). Two-component systems are the major system able to sense stresses from the environment directly in the border between the cell and the environment. Among the 32 two-component systems of *D. dadantii*, only GacAS and PhoPQ systems were shown to be essential for the virulence (Llama-Palacios *et al.*, 2003, Llama-Palacios *et al.*, 2005, Yang *et al.*, 2008, Lebeau *et al.*, 2008). For both, the inactivation of the system displays a total loss or severe decreased of virulence *in planta*. Based on our study, the CpxAR system may be added to the list of essential two-component systems required for virulence.

Inactivation of *cpxA* or *cpxR* genes provokes a change in the expression of *degP*, *spy* and *ompF*. OmpF is a porin while DegP and Spy proteins are involved in folding and quality control of periplasmic proteins. These results suggest that the CpxAR system plays a classic role in the fitness of the envelope. This result can be correlated with the phylogenic tree showing the conservation of the CpxAR system among the zoopathogenic and the phytopathogenic bacterial species. In *E. coli*, the OmpF porin expression is regulated, to a large extent, by the EnvZ/OmpR two-component system. In *D. dadantii*, the EnvZ/OmpR two-omponent system is not involved in the regulation of *ompF* expression (Condemine and Ghazi, 2007). Our study showed that in *D. dadantii*, the regulation of *ompF* expression depends at least in part on the CpxAR two component system.

The loss of the CpxAR system results in a total loss of virulence regardless the kind of plant used. This loss of virulence is the result of at least three factors. First of all, the CpxAR system is involved in the regulation – although indirect – of the PCWDEs and motility. Second, the CpxAR system is involved in the protection of *D. dadantii* against plant defenses (oxidative stress, peptide antimicrobial). Our data suggest that these plant defenses are mainly effective during the early phase of the infectious cycle. Our result show both by gene expression analysis and Phos-Tag analysis, that the CpxAR system is also activated during the early phase of the infectious cycle. Taken together, these data show clearly that the CpxAR system is activated during the early phase of the infectious cycle to counteract the damage provoked by plant defenses.

A global opposite effect on the expression of the *cpx* regulon was often observed in other bacterial species when either CpxA or CpxR were inactivated. The hypothesis generally given to explain the increased expression of the genes of the *cpx* regulon in *cpxA* null mutant strains is the constitutive phosphorylation of the CpxR regulator protein (Klein *et al.*, 2007, Wolfe *et al.*, 2008 Labandeira-Rey *et al.*, 2010, Wolfe, 2010). We demonstrate this hypothesis by a Phos-Tag gel analysis since the loss of *cpxA* displays an excess of the phosphorylated form of the CpxR regulator as compared to the CpxR phosphorylation level of the wild-type strain. *In silico* analysis indicates that CpxA possesses both kinase and phosphatase activities. The fine-tuning between both activities is required to obtain the

adapted response allowing appropriate expression of genes of the *cpx* regulon in the different environments encountered by *D. dadantii*. Activation of the CpxAR two-component system is essential to fight against plant defenses. Thus, the non-virulent phenotype of the *cpxA* null mutant was surprising since this mutant displays a constitutive activation of CpxR. Excess of CpxR-P was previously described to affect the virulence in *Haemophilys ducreyi*, *Salmonella typhimurium* or *Yersinia pseudotuberculosis* (Humphreys *et al.*, 2004; Spinola *et al.*, 2010, Liu *et al.*, 2012). In *Salmonella typhimurium*, Humphreys and collaborators proposed that excess of CpxR-P down-regulated genes involved in the virulence (Humphreys *et al.*, 2004).

The reason of the same phenotype in *cpxA* and *cpxR* null mutant in oxidative stress or polymixin B sensitivity is not clear. Same guess can be proposed for the oxidative stress or polymixin B sensitivity. An excess of CpxR-P harm the defense against oxidative stress or polymixin B because the CpxAR system affects directly the traffic into the membrane.

Moreover a similar phenotype is observed in *Salmonella typhimurium* for the PhoPQ two-component system. The inactivation of the response regulator PhoP as well as a constitutive activation of PhoPQ two-component system attenuates the virulence of the bacteria (Miller, 1991).

This indicates that the fine-tuning observed in the wild-type strain is also of importance *in planta* for survival of *D. dadantii* and that phosphorylation of the CpxR regulator must be turned off as soon as possible because excess of CpxR phosphorylation becomes deleterious when not required for *D. dadantii* in this kind of environment. Taken together, these results strongly suggest that without stress, the phosphatase activity is the main activity of the CpxA sensor needed to maintain the CpxR regulator at a very low level of phosphorylation.

Experimental Procedures

Bacterial strains, media and growth conditions

Bacterial strains are described in Table 1. Bacteria were grown at 30°C in lysogeny broth (LB) (Bertani, 2004), or in minimal medium M63 supplemented with a carbon source at a concentration of 2 $g.l^{-1}$ (Miller, 1992). Solid media were obtained by adding agar at 15 $g.l^{-1}$.

Motility tests were made on LB plates containing agar at 0.3 $g.l^{-1}$. 10^7 bacteria in 5µL were spotted onto the plate, incubated at 30°C and swim diameters were measured after 30 hours of incubation.

The solid media used to test the pectinase and cellulases activities have been described previously (Page *et al.*, 2001).

Antibiotics in media were used at following concentrations: kanamycin, 25 $µg.ml^{-1}$; chloramphenicol, 25 $µg.ml^{-1}$ and gentamycin, 2 $µg.ml^{-1}$.

Cloning of the *cpxA* and *cpxR* genes

The cpxA and *cpxR* DNA fragments were amplified by PCR (cpxAFor and cpxARev, cpxRFor and cpxRRev primers respectively), and cloned in blunt end into pJET1.2 following the manufacturer's recommendations (Fermentas) to give pNFW512 and pNFW460 respectively. The cloned DNA fragments were sequenced.

Construction of the *cpxA* and *cpxR* mutations

To inactivate *cpxA*, a gentamycin DNA cassette was amplified by PCR (K7gentabsrFor and K7gentabsrRev primers respectively) digested by BsrGI and inserted into pNFW521 digested by the same enzyme (pNFW513). To inactivate *cpxR*, a gentamycin DNA cassette was amplified by PCR (K7gentahpaFor and K7gentahpaRev primers respectively),digested by HpaI and inserted into pNFW460 digested by AfeI (pNFW466). After electroporation (2.5kV) of these two last plasmids, the mutations were integrated into the *D. dadantii* chromosome by marker exchange recombination in the presence of gentamycin after successive cultures in low phosphate medium (Roeder *et al.*, 1985).

Construction of the *spy* and *degP* gene fusions

The *spy'* and *degP'* DNA fragments were amplified by PCR (spyForKpnI and spyPRevXbaI, degPForKpnI and degPRevXbaI primers respectively). The *spy'* and *degP'* DNA fragments were digested by KpnI and XbaI and cloned into pUC18Not-uidA digested by the same enzymes.

The NotI fragments of these plasmids encompassing *spy::uidA* and *degP::uidA* respectively, were cloned into pUTmini-Tn*5* Kan.

Transduction, conjugation and transformation

Transformation of *E. coli* cells was carried out by the rubidium chloride technique (Miller, 1992). Construction of strains was performed by transferring genes from one strain of *D. dadantii* to another by generalized transduction with phage ΦEC2 as described previously (Resibois *et al*., 1984). Plasmids were introduced in *D. dadantii* by conjugation or electroporation.

Transposon mutagenesis

To allow integration of a single ectopic copy of the mini-Tn*5 spy::uidA-Kan* and mini-Tn*5 degP::uidA-Kan* genes fusions, transposon mutagenesis was performed as described previously (Bouchart *et al.*, 2010). Briefly, after conjugation between an *E. coli* strain harboring the pUTmini-Tn5 Kan plasmid carrying the appropriate fusion and a *D. dadantii* strain, Kan^r mutants were selected on M63 plates containing sucrose as a unique carbon source and kanamycin.

Determination of enzyme activities

β-glucuronidase assays were performed on crude extracts obtained from bacteria disrupted by sonication 2 × 20 s (Sonifier cell disruptor B-30, Branson, 70% duty cycle, 7 microtip limit, Hold time, continuous, appropriate probe) after growth *in vitro* (LB medium) or *in*

planta and extracted from chicory leaves as described elsewhere (Bontemps-Gallo *et al.*, 2013). β-glucuronidase activity was determined by spectrometric monitoring of the hydrolysis of PNPU (4-nitrophenyl- β-D-glucuronide) at 405 nm.

The protein concentration was determined by the Bradford assay with bovine serum albumin as a standard (Bradford, 1976).

Susceptibility to acidic stress, oxidative stress and antimicrobial peptides

The suceptibility to acidic stress was assayed by measuring and comparing the growth rates of bacteria in M63 medium at pH 4.5 and at pH7.

The susceptibility to oxidative stress (H_2O_2) and to antimicrobial peptides (polymixin B) was previously described (Bontemps-Gallo *et al.*, 2014). H_2O_2 at 3 mM or polymyxin B at indicated concentrations was added to cells grown until stationary-phase in M63 glycerol medium. After different times of incubation, aliquot of bacteria were taken up, and the survival rates were determined by numeration of CFU after plating serial dilutions on LB plates.

Pathogenicity test

Potato tubers, chicory leaves or carrots were inoculated as previously described (Page *et al.*, 2001). Briefly, bacteria from an overnight culture in LB medium were recovered by centrifugation and diluted in physiological water. After wounding, plants were inoculated with 10^7 bacteria and incubated in a dew chamber at 28°C until 48h.

Preparation of polyclonal antibodies against CpxR

A DNA fragment encoding the c*pxR* gene of *D. dadantii* was amplified by PCR using the primers cpxRhisFor and cpxRhisRev. The PCR product was cloned into a His6 tag expression vector, pET100/D-Topo® (Invitrogen Life Technologies). The resulting His-tagged CpxR was expressed in *E. coli* BL21(DE3) and the protein was purified by affinity chromatography according to the manufacturer's procedure [Ni-nitrilotriacetic acid (NTA) agarose; Qiagen]. The purified CpxR was used to immunize rabbits (Eurogentec).

Analysis of CpxR phosphorylation in vitro

Phosphorylation reactions of purified His-tagged CpxR were performed with 5 μg of protein in 50 mM Tris-HCl, pH7.5, 100 mM NaCl, 10 mM MgCl2 and 2 mM β-mercaptoethanol. PA was added to a final concentration of 15 mM to initiate the reaction. After 0, 15, or 30 minutes of incubation at room temperature, the reactions were stopped by addition of SDS-PAGE loading buffer (final concentration: 50 mM Tris-HCl pH6.8, 2% (w/v) SDS, 10% (w/v) glycerol, 20 mM DTT, 0.02% bromophenol blue). The mixtures were resolved using phosphoprotein affinity gel electrophoresis as described in Barbieri and Stock, 2008, with minor modifications. Briefly, Phos-tag™ acrylamide gels were composed of a 10% resolving solution [10% (w/v) 37.5:1 acrylamide/N,N'methylenebisacrylamide, 375 mM Tris (pH8.8) and 0.1% (w/v) SDS, 125μM Phos-tag™ acrylamide and 250 μM MnCl2] and a 4% stacking solution [4% (w/v) 37.5:1 acrylamide/N,N'methylenebisacrylamide, 125 mM Tris (pH6.8) and 0.1% (w/v) SDS]. The gels were run at 4 °C under constant voltage (150

V) with standard running buffer (0.1% (w/v) SDS, 25 mM Tris and 192 mM glycine) and stained with Coomassie Blue.

Phos-Tag analysis of the CpxR phosphorylation *in vivo*

Gel retardation of the phosphorylated form of CpxR was performed as described previously (Madec et al, 2014). Briefly, 1.5×10^8 cells of *D. dadantii* cells were harvested by centrifugation and the pellet was lysed with 12.7 µl of 1M formic acid, solubilized by 5 µl of 4X SDS-PAGE loading buffer and neutralized by 2.8 µl 5 N NaOH. Sample was loaded and run onto gel containing 35 µM Phos-tag™ acrylamide and 70 µM $MnCl_2$. Then, 10 min wash with transfer buffer (25 mM Tris and 192 mM glycine) supplied with 1 mM EDTA, followed by 10 min wash with transfer buffer without EDTA were performed before transferring gel to nitrocellulose membranes using a Trans-Blot® TurboTM Blotting system (Bio-Rad) with a pre-programmed protocol (2.5 A, up to 25 V, 7 min). Western blotting against CpxR was performed using standard protocols with the rabbit anti-CpxR polyclonal antibodies at a dilution of 1:1000 and anti-rabbit secondary antibody coupled to horseradish peroxidase at a dilution of 1:10 000. Blots were imaged by chemiluminescent detection (ECL kit, GE healthcare Pharmacia Biotech).

Quantification of the phosphorylated CpxR protein amount

Phosphorylated CpxR and unphosphorylated CpxR were quantified by determination of the area intensity of each band with the software Quantity One (Bio Rad) after detection by Western blot. Quantification of phosphorylated CpxR was expressed as the ratio of the phosphorylated CpxR amount divided by the sum of the phosphorylated CpxR and the unphosphorylated CpxR amounts as described elsewhere (Barbieri and Stock, 2008).

Statistical Analysis

For statistical analyses, Graph-prism6 software was used. Data were analysed by paired t-test; a value of $p<0.05$ was considered significant.

Phylogeny tree

The phylogenic tree was built using the Phylogeny.fr website (Dereeper *et al.*, 2008). The protein sequences of the 21 strains were aligned with Muscle 3.6 with default parameters. The trees were generated using the maximum likelihood based on a bootstrapping procedure of 100 bootstraps. The protein sequence of the strain *Gloeobacter violaceus* was used as outgroup. Phylogenetic trees were displayed with Dendroscope (version 3.2.10).

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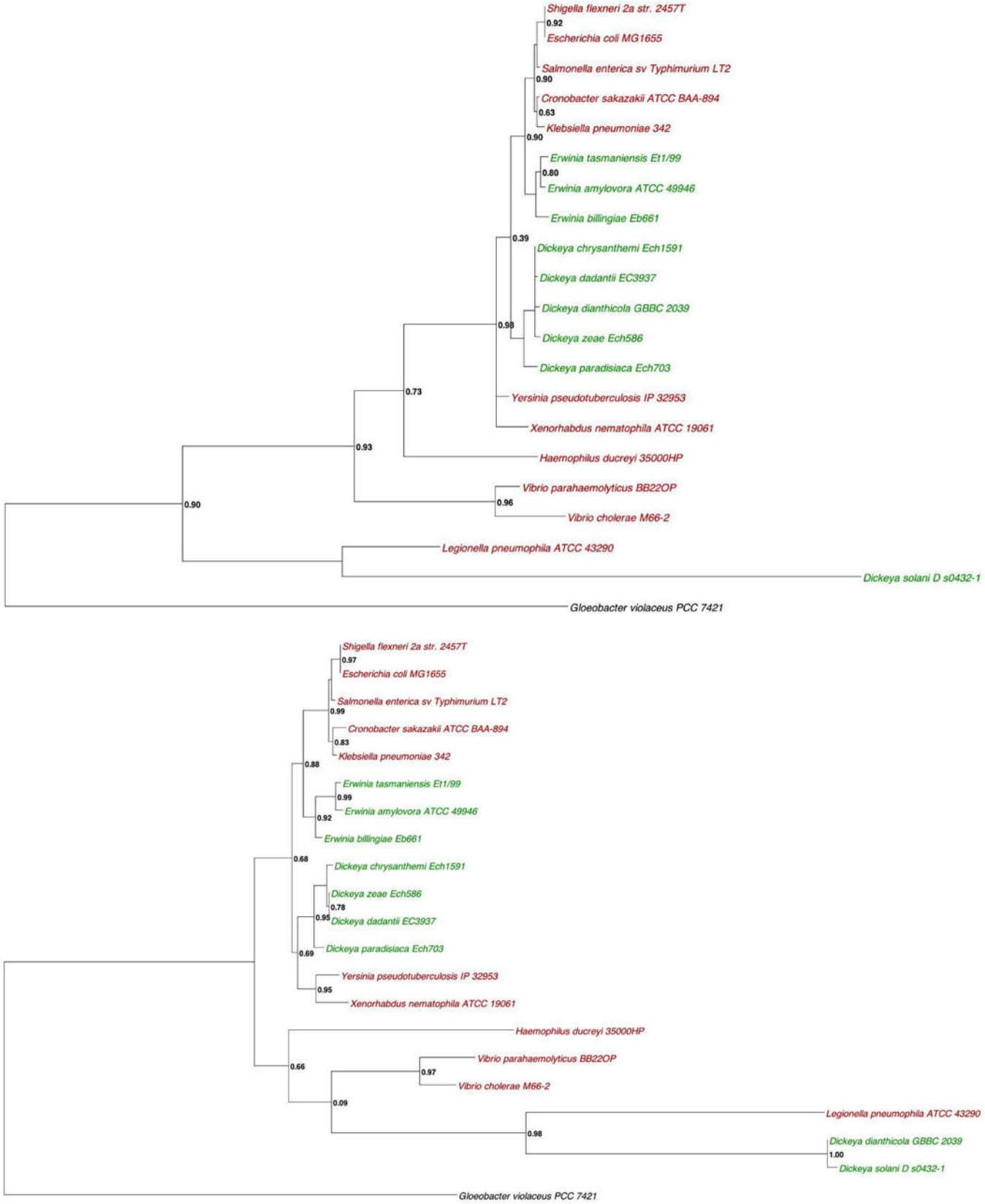

Figure 1. Rooted phylogenetic tree of CpxR (A) and CpxA (B) based on the mamixum likelihood

Numbers on knot are bootstrap confidence levels. Zoopathogen are in red. Phytopathogen are in green. In black, *Gloeobacter violaceus* used as outgroup. Both trees were constructed with the entire protein sequence from Uniprot.

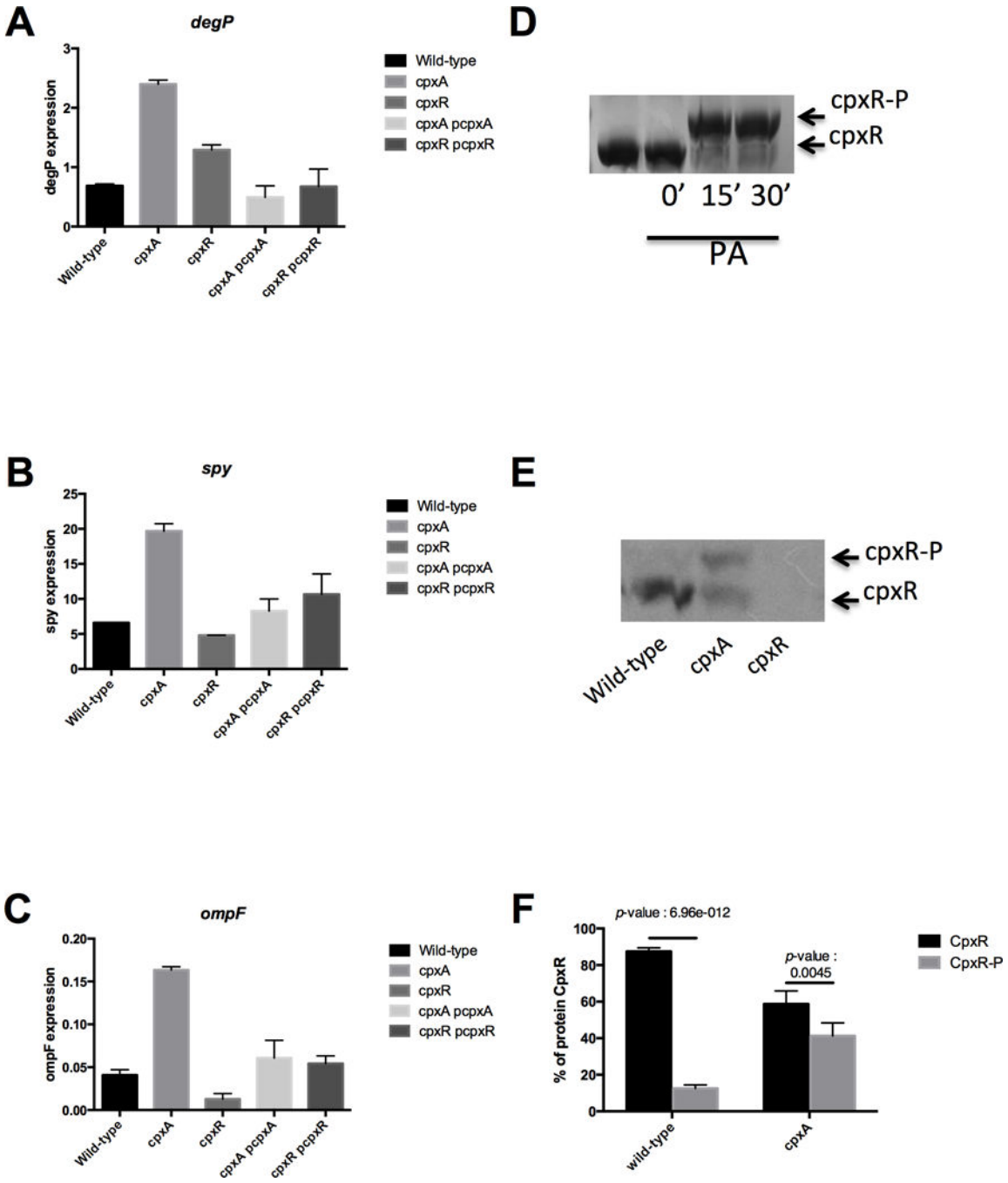

Figure 2. Effect of the *cpxA* or *cpxR* inactivation on the *degP* (A), *spy* (B) and *ompF* (C) gene expression and on the phosphorylation level of the CpxR regulator (D-F)

(A–C) For expression of the *degP::uidA*, *spy::uidA* and *ompF::uidA* gene fusions, bacteria were grown to the mid-log phase and lysed by sonication. B-glucuronidase activity was measured with PNPU as a substrate. Specific activity was expressed as the change in OD_{405} per minute and per milligram of protein. Results are the average of four independent experiments. (D) Six micrograms of purified CpxR regulator was incubated in the presence of 15 mM phosphoramidate (PA) for 0 min, 15 min and 30 min or without PA for 30 min

before loading onto the Phos-tag acrylamide gel (125 μM). After migration, the gel was stained with Coomassie Blue Brilliant to revealed the CpxR and CpxR-P. (E) Separation of CpxR and CpxR-P by Phos-Tag™ gel after extraction from bacteria *in vivo*. Cell lysate of wild-type, *cpxA* null mutant, and *cpxR* null mutant of *D. dadantii* were loaded into Phos-Tag acrylamide gel (35 μM). Both forms of CpxR were revealed by Western-blot. This results presented are from one of the three independent experiments performed. (F) Quantification of CpxR and CpxR-P extracted from the wild-type and the *cpxA* null mutant strains. Results are the average of four independent experiments.

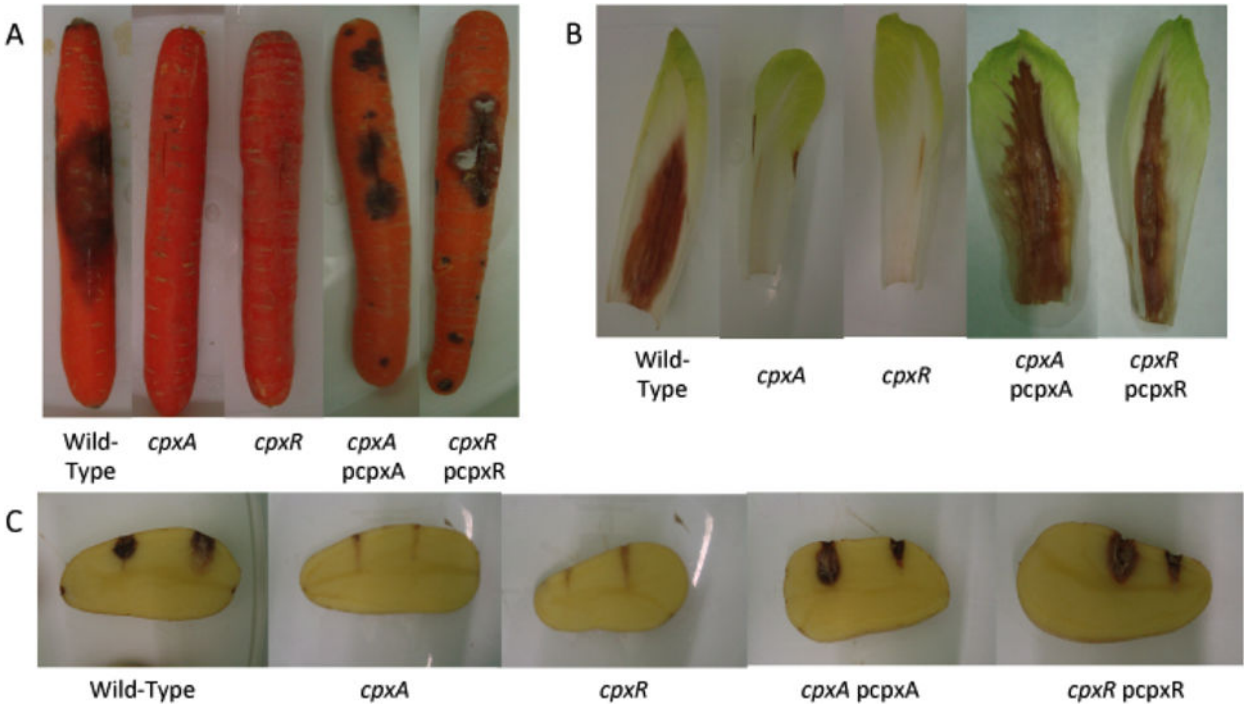

Figure 3.

Pathogenicity of the wild-type, *cpxA* null mutant, *cpxR* null mutant and complemented strains on carrots (A), chicory leaves (B) and potato tubers (C) after 3 days.

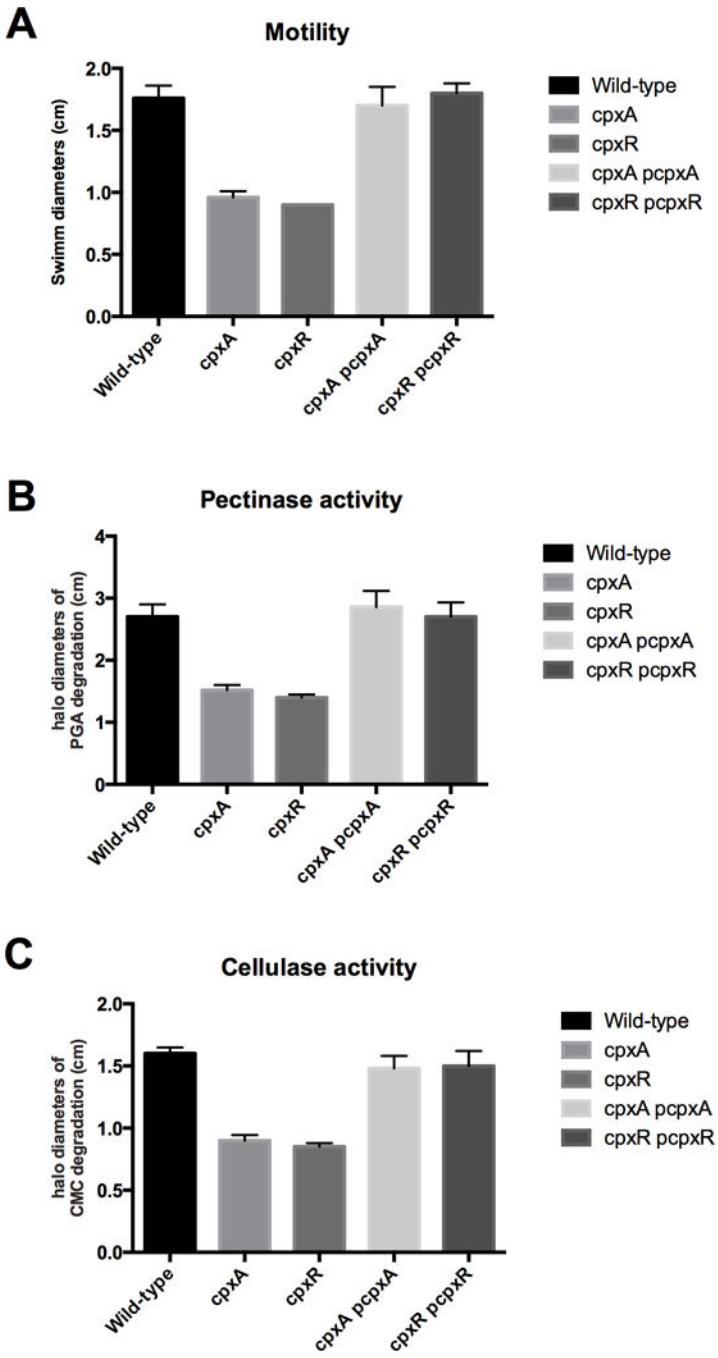

Figure 4. Motility (A), pectate-lyase (B) and cellulase activities (C) of the wild-type, cpxA null mutant, cpxR null mutant and complemented strains

(A) Motility was measured in LB semisolid plates. Swim diameters were measured after 30h of incubation at 30°C. (B-C) Exoenzyme activities were estimated on plates by measurement of the halo diameters, expressed in cm of substrate degradation.

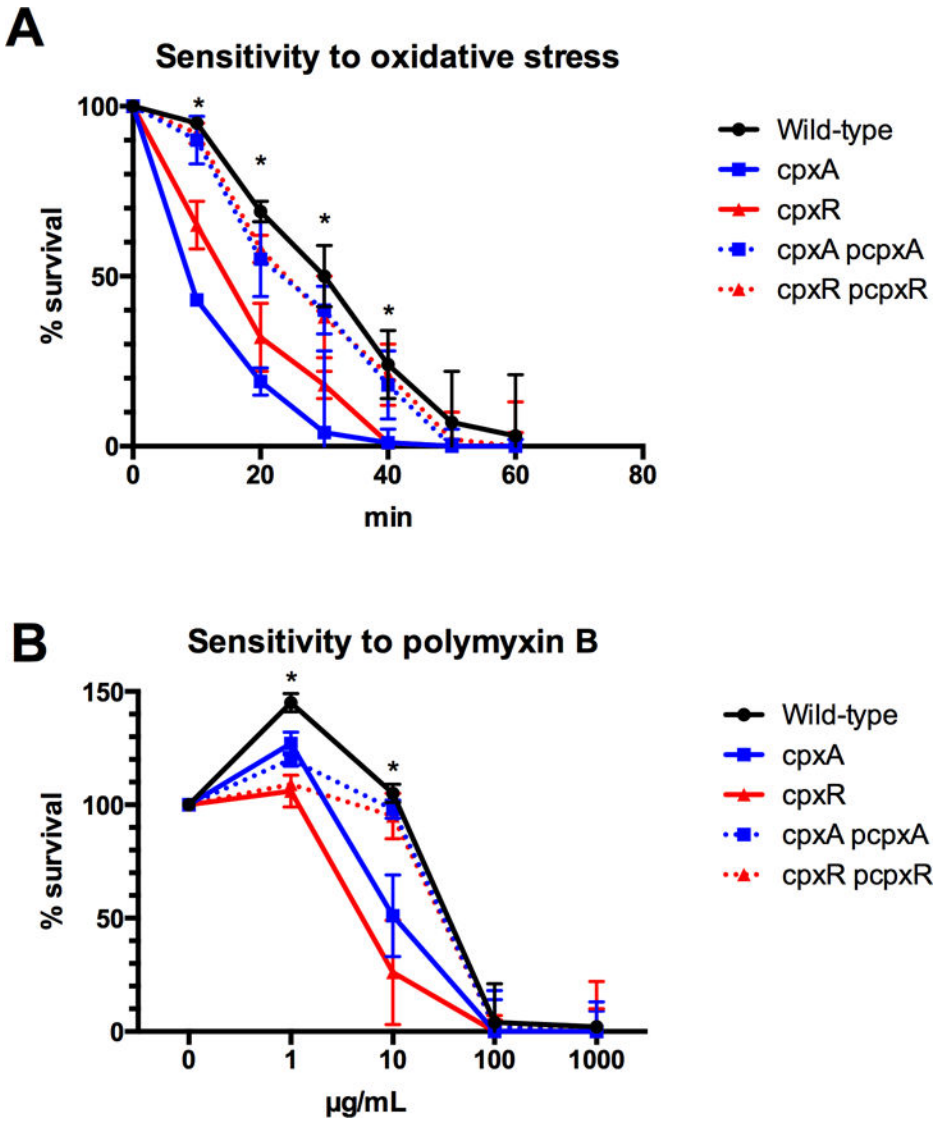

Figure 5. Sensitivity tests of the wild-type, *cpxA* null mutant, *cpxR* null mutant and complemented strains to H_2O_2 or polymyxin B

(A) For sensitivity to H_2O_2, bacteria were grown until stationary-phase in M63 glycerol medium. The culture was incubated with 3 mM of H_2O_2 and survivals were determined by numeration. Survival rate was expressed as the number of CFU counted at each time/the number of CFU before addition of H_2O_2. Results are the average of three independent experiments (B) For sensitivity to polymyxin B, bacteria were grown until stationary phase in M63 glycerol medium. The culture was incubated with the indicated concentrations and survivals were determined by numeration. Survival rate was expressed as the number of CFU counted at each time/the number of CFU before addition of polymyxin B. Results are the average of three independent experiments. Asterisks indicate that a significant difference exists only between the wild-type and the *cpxA* or *cpxR* null mutants.

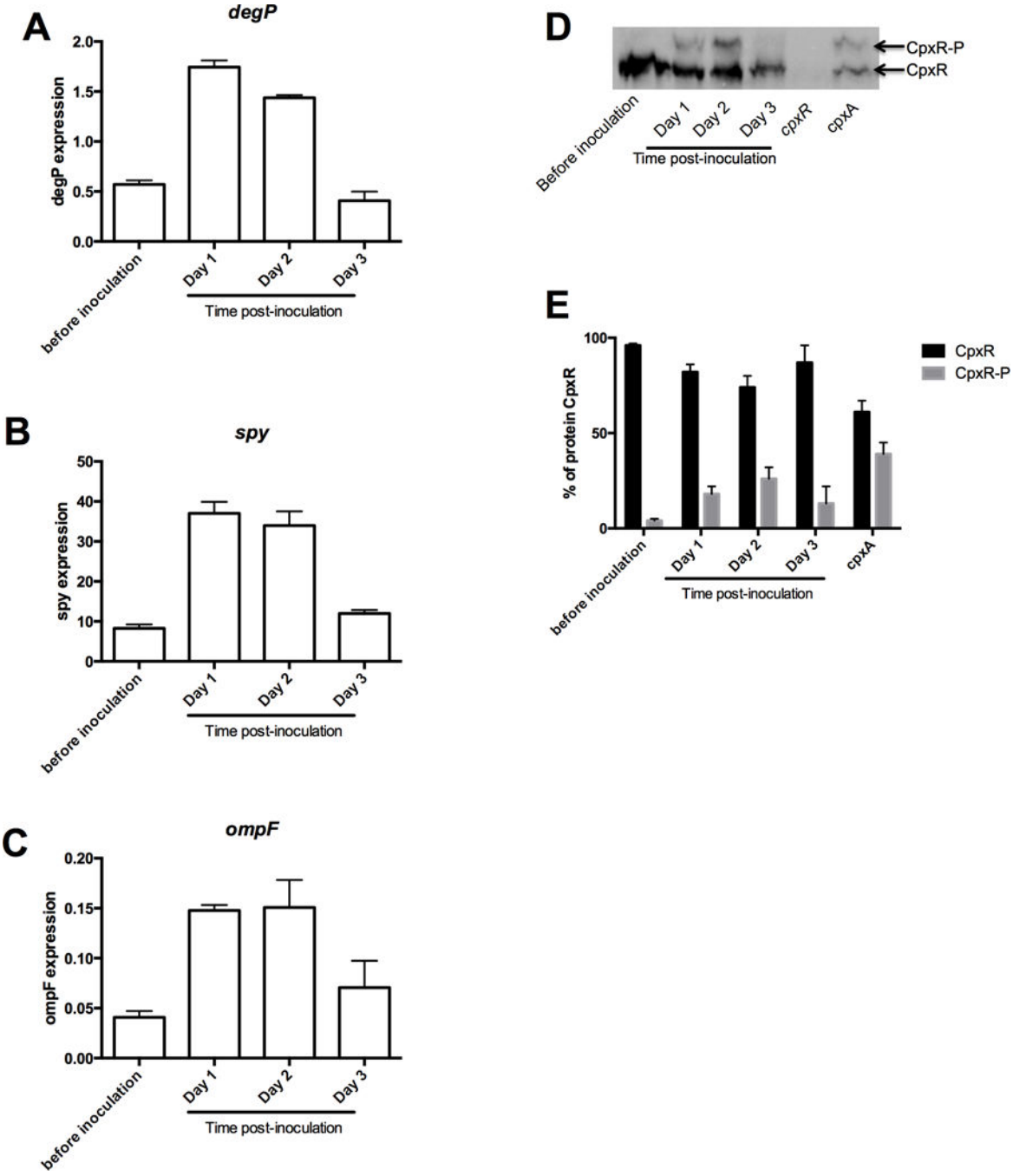

Figure 6. Expression of degP (A), spy (B) and ompF (C) gene and phosphorylation level of CpxR (D-E) during infection in chicory leaves

(A–C) B-glucuronidase activity was measured with PNPU as a substrate. Specific activity was expressed as the change in OD_{405} per minute and per milligram of protein. Results are the average of three independent experiments. (D) Separation of CpxR and CpxR-P by Phos-Tag™ gel after extraction from bacteria during the infectious cycle. Cell lysate of wild-type strain before inoculation and after 1, 2 or 3 days after the inoculation, *cpxR* null mutant, *cpxA* null mutant from *in vitro* stationary growth phase culture of *D. dadantii* were

loaded into Phos-Tag™ acrylamide gel (35 μM). Both forms of CpxR were revealed by Western-blot. (E) Quantification of CpxR and CpxR-P extracted from wild-type before inoculation and after 1, 2 or 3 days after the inoculation, *cpxR* null mutant, *cpxA* null mutant from *in vitro* culture of *D. dadantii*. Results are the average of three independent experiments.

Table 1

Strain, Plasmid and Primer

Strain	Genotype [a, b]	Source or Reference
Dickeya dadantii		
EC 3937	Wild-type	Laboratory collection
A 4229	*ompF::uidA-Kan*	Condemine and Ghazi, 2007
NFB 7012	mTn5*degP::uidA-Kan*	This study
NFB 7039	mTn5*spy::uidA-Kan*	This study
NFB 7515	*cpxA::gm*	This study
NFB 7532	*cpxR::gm*	This study
NFB 7539	*cpxA::gm ompF::uidA-Kan*	This study
NFB 7543	*cpxR::gm ompF::uidA-Kan*	This study
NFB 7548	*cpxR::gm* mini-Tn5*degP::uidA-Kan*	This study
NFB 7549	*cpxA::gm* mini-Tn5*degP::uidA-Kan*	This study
NFB 7550	*cpxR::gm* mini-Tn5*spy::uidA-Kan*	This study
NFB 7551	*cpxA::gm* mini-Tn5*spy::uidA-Kan*	This study
Escherichia coli		
BL21(DE3)	*ompT, hsdSB, gal, dcm*	Invitrogen
Top 10 F’	*F{lacIq, Tn10) mcrA,* Δ*(mrr-hsdRMS-mcrBC),* Φ80*lacZ*ΔM15, Δ*lacX74, recA1, araD139,* Δ*(ara- leu)7697, galU, galK, rpsLendA1 nupG*	Invitrogen
S17-λpir	*recA1, thi, pro, hsdR-M+, RP4:2-Tc::Mu-Kan::Tn7, λpir*	De Lorenzo *et al.*, 1994
Plasmids		
pUC18Not-uidA	‘*uidA,* Amp	Bontemps-Gallo *et al*., 2014
pJET 1.2	Amp	Fermentas
pNFW460	pJET2.1 *cpxR*	This study
pNFW466	pJET2.1 *cpxR::gm*	This study
pNFW512	pJET2.1 *cpxA*	This study
pNFW513	pJET2.1 *cpxA::gm*	This study
pUTmini-Tn*5*-Kan	mini-Tn5Spe, oriR6K, Kan, Amp	De Lorenzo *et al.*, 1990
Primer sequences[c]		
cpxAFor	CGCGAGCTGACATCCCTATT	This study
cpxARev	TCGAAAAAGCTCTCCAGCGT	This study
cpxRFor	CTATCATCCAGCCCCTGAC	This study
cpxRRev	CGGATGCTGTTTCAGGTTAC	This study
K7gentabsrFor	TCTCTGTACACCCCCATCCCCCTGTTGAC	This study
K7gentabsrRev	TCTCTGTACACGCAAGCTAGCTTGGCTGC	This study
K7gentahpaFor	GTGTGTTAACCCCCATCCCCCTGTTGAC	This study
K7gentahpaRev	GTGTGTTAACCGCAAGCTAGCTTGGCTGC	This study
spyForKpnI	AAGGTACCTGATGGCTCCTGCCGCCGGCGA	This study
spyRevXbaI	GTTCTAGAAGCCGACGCTACCCAGCGCCAG	This study

Strain	Genotype [a, b]	Source or Reference
degPForKpnI	TTGGTACCACAAACTCTCCAGCAAGCATTG	This study
degPRevXbaI	TGCTCTAGACTCAACGCCAACGCACTCAGC	This study
cpxRhisFor	CACCATGAACAAAATCCTGTTGGTTGATGACG	This study
cpxRhisRev	CGGTAACCCGAATCATGCGGTGG	This study

[a] The *degP-uidA, cpxP-uidA and spy-uidA* gene fusions are carried by a mini-Tn*5* Kan

[b] Amp, ampicillin resistance, Kan, kanamycin resistance, Gm, gentamicin resistance

[c] Restriction sites are underlined