rpoN1, but not rpoN2, is required for twitching motility, natural competence, growth on nitrate, and virulence of Ralstonia solanacearum

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Introduction

Ralstonia solanacearum is a plant pathogenic bacterium that causes a lethal wilt disease in more than 200 plant species including many plants of economical interest such as tomato, potato, eggplant, banana, groundnut, or trees like Eucalyptus. The bacterium lives in two separate niches: in the soil as a saprophyte and inside the plant as a parasite (Genin, 2010). To coordinate the change from a saprophytic to a parasitic mode of living, an elaborate sensory network is used by the bacterium to regulate the expression of virulence and pathogenicity genes (Schell, 2000; Genin and Denny, 2012). The regulation enables growth and spread of the bacterium inside the host plant by overcoming the plant defense response. A hallmark of pathogenicity gene regulation in R. solanacearum is the interaction among different two component regulation systems (TCRSs).
present in this bacterium (Schell, 2000). The TCRSs characterized in this bacterium are Phc, Prh, Peh, Sol, and Vsr systems (Yoshimochi et al., 2009; Genin and Denny, 2012). The TCRSs perceive signals from the environmental and regulate expression of multiple pathogenicity factors. The interplay of the regulatory components of this intricate regulatory network is still poorly understood and the nature of several inductive environmental signals is unknown (Schell, 2000; Genin and Denny, 2012; Zuluaga et al., 2013). For example, the PehSR system has been demonstrated for its role in twitching motility, flagellar motility, production of polygalacturonases, and virulence (Allen et al., 1997; Kang et al., 2002). But the signaling molecule that activates PehS (sensor kinase) is unknown and the mechanism of PehR (response regulator) regulation of the downstream genes is yet to be demonstrated.

*R. solanacearum* has a bipartite genome that comprises a chromosome of 3.7 Mb and a second replicon called a megaplasmid of 2.1 Mb (Salanoubat et al., 2002). Important pathogenicity genes are distributed both on the chromosome and the megaplasmid. For example, genes encoding the type III secretion system are megaplasmid-borne whereas the phcA-phcR-S and vsrA-D, vsrB-C virulence regulatory genes are located on the chromosome. It has also been shown that paralogous gene families can be distributed on both replicons as in the case of the GALA (ripG) effector family members (Remigi et al., 2011). There is also evidence of apparent gene duplications on the two replicons as observed for some genes encoding Type III effector proteins (ripAX family), structural components such as the Flp pilus (Wairuri et al., 2012), hemagglutinins, several metabolic enzymes (including glutamine synthetase, and sarcosine oxidase), and regulatory genes. One example of the latter class is rpoN, which encodes the sigma factor $\sigma^{54}$ (Sigma-54). Out of the two rpoN genes, the rpoN1 (RSc0408) is present in the chromosome and rpoN2 (RSp1671) is present in the megaplasmid. The role of rpoN in *R. solanacearum* virulence is not known.

Though rpoN gene is historically known for its role in nitrogen assimilation, the gene has also been shown to be involved in regulating other important functions in bacteria (Kohler et al., 1989). rpoN has been demonstrated to regulate multiple determinants in diverse pathogenic bacteria such as type I and type IV pili, flagellar motility, type III and type VI protein secretion systems, biosynthesis of exopolysaccharides or biofilm formation (O’Toole et al., 1997; Reitzer and Schneider, 2001; Kazmierczak et al., 2005; da Silva Neto et al., 2008; Dong and Mekalanos, 2012; Hao et al., 2013). A recent comprehensive computational study of different Sigma-54 interacting activators in bacteria indicated that Sigma-54 regulates processes that involve physical interaction of an organism with its environment like host colonization or biofilm formation (Francke et al., 2011).

In this study, we report the involvement of rpoN1 in virulence, twitching motility, natural transformation and growth on nitrate in *R. solanacearum* GMI1000 strain, which are functions not fulfilled by rpoN2. Our study therefore reveals that the function of the two paralogous rpoN proteins is not redundant in *R. solanacearum*.

### Materials and Methods

#### Bacterial Strains and Growth Conditions

The relevant characteristics of the plasmids and bacterial strains used in this work are listed in Table 1. *Escherichia coli* strains were grown at 37°C in Luria-Bertani medium (Ausubel et al., 1989). *R. solanacearum* strains were grown in complete BG medium or in MP minimal medium supplemented with glucose (5 g L$^{-1}$ at final concentration). The composition of BG medium (Plener et al., 2010) is as follows (g L$^{-1}$): Bacto peptone, 10; Casamino Acids, 1; yeast extract, 1. BG medium was supplemented with glucose (5 g L$^{-1}$) and triphensyltetrazolium chloride (0.05 g L$^{-1}$) for agar (15 g L$^{-1}$) plates. The composition of MP medium (Plener et al., 2010) is as follows (g L$^{-1}$): FeSO$_4$·3H$_2$O, 1.25 × 10$^{-4}$; (NH$_4$)$_2$SO$_4$, 0.5; MgSO$_4$·7H$_2$O, 0.05; KH$_2$PO$_4$, 3.4. The pH was adjusted to 7.0 with KOH. When needed, antibiotics were added to the media at the following final concentrations (mg L$^{-1}$): kanamycin, 50; spectinomycin, 40 for *R. solanacearum*; gentamicin, 10; tetracycline, 10; ampicillin, 100 for *E. coli*.

To find out if bacterial strains were capable of utilizing nitrate as nitrogen source, bacterial strains were streaked on MP minimal medium plates without (NH$_4$)$_2$SO$_4$ but supplemented with KNO$_3$ (500 µM). Difference in growth of bacterial strains having the ability to use nitrate and not having the ability to use nitrate was distinctly observed visually after 48 h of incubation.

The co-cultivation of bacteria with Arabidopsis plant cells was performed as described by Marenda et al. (1998). After growth in BG medium, bacteria were pelleted, washed and resuspended in sterile water. Hundred microliters of these suspensions, adjusted to an OD$_{600}$ of 0.3, were inoculated into 10 ml of Arabidopsis cell cultures grown to a density of 40 g L$^{-1}$ fresh cells. The co-cultures were maintained for 12 h at 28°C on a rotary shaker. The mixture was filtered on 20 µm pore size nylon membrane to separate bacteria and plant cells. The bacteria were then collected by centrifugation for β-galactosidase assays.

#### DNA Manipulations and Genetic Constructs

Standard recombinant DNA techniques were performed as described previously (Ausubel et al., 1989). Restriction enzymes, DNA ligase, and other DNA enzymes were used according to the manufacturers’ recommendations. Standard PCR reactions were set up with the following reagents: 4 µL of 5X GoTaq buffer (Promega, Madison, WI), 0.6 µL DMSO (5%), 0.4 µL dNTP (10 mM), 1 µL each primer (10 µM), 0.1 µL Taq (5U/µL), template DNA 20 ng, then the volume adjusted to 20 µL with deionized water.

lacZ reporter fusions with rpoN1 and rpoN2 were created by cloning DNA fragments of these two genes into the pCZ367 integrative vector (Cunnac et al., 2004). These constructs (Table 1) were introduced by transformation in strain GMI11000 as already described (Cunnac et al., 2004).

#### Creation of Disruption Mutants for the rpoN1, rpoN2, pehR, and RSc0407 Genes

Disruption mutants were created with the use of the Ω interposon carrying resistance gene against either Spectinomycin...
TABLE 1 | List of plasmids, R. solanacearum, and E. coli strains used in this study.

| Plasmid/Strain | Genotype                  | Antibiotic | References          |
|----------------|---------------------------|------------|---------------------|
| PLASMIDS       |                           |            |                     |
| pGEMT          | Cloning vector            | Ap         | Promega             |
| pCZ367         | Insertional vector with lacZ reporter | Ap, Gm     | Cunnac et al., 2004 |
| pGRS595        | pGEM-T with ΩKm insertion in rpoN1 gene | Km         | This work           |
| pGRS596        | pGEM-T with ΩSpc insertion in RsC0407 gene | Spc        | This work           |
| pGRS597        | pGEM-T with ΩSpc insertion in pehR gene | Spc        | This work           |
| pGRS598        | pGEM-T with ΩKm insertion in pehR gene | Kan        | This work           |
| pGRS599        | pGEM-T with ΩSpc insertion in rpoN2 gene | Spc        | This work           |
| pGRS601        | pCZ367::rpoN1             | Gm         | This work           |
| pGRS602        | pCZ367::rpoN2             | Gm         | This work           |
| E. COLI        |                           |            |                     |
| DH5α           | F− recA lacZ ΔM15         |            | Life Technologies   |
| R. SOLANACEARUM|                           |            |                     |
| GMI1000        | Wild-type strain          |            | Salanoubat et al., 2002 |
| GMI1605        | phcA::Ω                   | Spc        | Genin et al., 2005  |
| GMI1750        | pA::pTOK2                 | Tc         | Kang et al., 2002   |
| GRS552         | rpoN1::Tn5                | Km         | This work           |
| GRS553         | rpoN1::Ω                  | Km         | This work           |
| GRS554         | rpoN2::Ω                  | Spc        | This work           |
| GRS555         | rpoN2::Ω rpoN1::Tn5       | Spc, Km    | This work           |
| GRS556         | RsC0407::Ω                | Spc        | This work           |
| GRS557         | RsC0407::Ω rpoN1::ΩKm    | Spc, Km    | This work           |
| GRS561         | rpoN2::lacZ               | Gm         | This work           |
| GRS562         | rpoN2::lacZ rpoN1::Tn5    | Gm, Km     | This work           |
| GRS566         | rpoN1::lacZ               | Gm         | This work           |
| GRS567         | pehR::Ω                   | Spc        | This work           |
| GRS568         | pehR::Ω rpoN1::Tn5        | Spc, Km    | This work           |
| GRS569         | phcA::Ω rpoN1::Tn5        | Spc, Km    | This work           |
| GRS570         | phcA::Ω pehR::ΩKm         | Spc, Km    | This work           |

Ap, ampicillin resistance; Spc, spectinomycin resistance; Km, kanamycin resistance; Gm, gentamycin resistance.

or Kanamycin (Prentki and Krisch, 1984). DNA fragments encompassing the target open reading frames were PCR-amplified into the pGEMT vector. The restriction sites used for cloning the interposon and their position within the target coding sequence is shown in Supplementary Material Figure 1A and the list of primers used to amplify the gene coding sequences from the GMI1000 genome is provided in Supplementary Figure 1B. The obtained plasmids (see Table 1) were linearized and used to transform R. solanacearum as described below. Double recombination events were selected using appropriate antibiotic resistance and checked by PCR. Insertion of the Ω interposon in the rpoN1, rpoN2, pehR, RsC0887 gene sequences was confirmed by PCR amplification using one oligonucleotide specific to the interposon (5’-TGTTACCAGAGGCTTG-3’) and a second one specific to the target gene.

The rpoN::EZ-Tn5™ strain was obtained from the R. solanacearum GMI1000 library as described by Plener et al. (2012) and available at the following web address: http://iant.toulouse.inra.fr/bacteria/annotation/site/prj/ralso/tools/mutants_db/cgi/EZLucene.cgi. Insertion and fine mapping of Tn5 in the rpoN1 gene was confirmed by the PCR using primers against the supposed flanking regions of EZ-Tn5™ insertion (Supplementary Material Figure 1A).

**Procedure for R. solanacearum Transformation**
The protocol used followed the method described by González et al. (2011): Ralstonia solanacearum was grown in MP medium supplemented with glycerol (20 g L−1) as sole carbon source to reach an OD600 between 0.5 and 1.0. Fifteen microliters of bacterial suspension was mixed with 3–5 µg of linearized plasmid DNA and the mixture was deposited on a 0.45 mm cellulose nitrate filter unit placed on a BG agar medium. After 48 h of incubation at 28°C, bacteria were collected and spread on selection plates supplemented with the appropriate antibiotic.

**Twitching Motility Assay**
The procedure followed the one described by Liu et al. (2001). Briefly, saturated culture of bacteria was diluted in BG medium 104-fold. Ten microliters of the diluted culture was placed on BG-agar plate. The plates were kept covered, left at 28°C for 24 h growth and observed under a light microscope (Nikon Labophot) equipped with 5X objective.
Swimming Motility Assay
Swimming motility test for R. solanacearum strains was done of soft agar plates following the procedure described by Tans-Kersten et al. (2004). Soft agar BG medium plates were prepared with 0.15 and 0.2% agar. Due to the low agar concentration, the medium reaches a semi-solid state on plate after 30 min. The surface of the medium was just touched by the tip of the toothpick dipped in a saturated culture of the bacterium. Plates were incubated at 28°C. Bacteria proficient for swimming motility moves radially in all directions. A white radial zone distinctly observed can be measured. The semi-solid state of the soft-agar medium is stable for even 3 days. Motility proficient bacteria can cover the whole plate. In this study, swimming motility was estimated by the measurement of the radial zone after 24 h of incubation.

Plant Pathogenicity Assays
Virulence assays were done by soil drenching method. Inoculations were performed by watering 4-week-old tomato plants (Lycopersicum esculentum cv. Marmande) with 50 ml of a suspension containing 10^7 CFU ml^{-1}. Disease development was scored daily by using a disease index scale ranging from 0 for no symptoms to 4 for completely wilted plants. Twelve plants were inoculated for each strain in at least three independent experiments. In order to analyse the data using the Kaplan–Meier representation and the non-parametric log rank test to assess the difference of the survival curves, the data was transformed as follows: all disease index lower than 2 were considered as “0” and all disease index equal of greater than 2 were considered as “1.” Statistical analysis of the results was conducted as previously described (Remigi et al., 2011). The log rank test was used to perform between-group comparisons, testing the equivalence of the Kaplan–Meier survival curves for a pair of groups. When \( p < 0.05 \) the survival curves were considered as significantly different.

\( \beta \)-Galactosidase Assays
\( \beta \)-Galactosidase assays were carried out as described by Miller (1972) with the following the modifications of Genin et al. (1992): 0.15–0.25 mL of bacterial suspension was added to Z buffer to a final volume of 0.75 mL. Cells were permeabilized with 100 \( \mu \)L of chloroform and 50 \( \mu \)L of sodium dodecyl sulfate (0.1%). The reaction was started by adding 150 \( \mu \)L of ONPG (4 mg/mL) and stopped by addition of 375 \( \mu \)L of Na2CO3 (1 M). \( \beta \)-Galactosidase activity was expressed in Miller units.

Patscan Analysis
Genome of strain GM11000 was scanned for the occurrence of the following RpoN-binding site consensus TGGCAC(A, G)NNNNTTGC(A, T), with one mismatch allowed, using the PatScan tool (D’souza et al., 1997) on the R. solanacearum GM11000 website https://iant.toulouse.inra.fr/bacteria/annotation/cgi/ralso.cgi.

Results
Two Conserved rpoN Genes in the R. solanacearum Genome
The rpoN1 and rpoN2 coding sequences in strain GM11000 have a moderate but significant relatedness (Blast2P: 44% identity; 62% similarity across the whole protein sequence). Both genes have a high G+C% (rpoN1: 63.80; rpoN2: 69.19) and are also similar with respect of their codon usage. BlastN analysis with individual coding sequences of the two genes revealed the presence of rpoN1 homologs in chromosomes and the presence of rpoN2 homologs in the megaplasmid in all the other tested R. solanacearum strains. Homologs of the two genes were also present in some other bacteria that were phylogenetically close to R. solanacearum (Table 2). However, Ralstonia eutropha, Cupriavidus taiwanensis, and Cupriavidus necator species have an rpoN1 homolog but no rpoN2 homolog. To get an insight into the evolutionary significance of rpoN1 and rpoN2, we compared the level of nucleotide identity for the two genes among representative strains of the R. solanacearum species complex and in some phylogenetically close bacteria. This analysis revealed that the identity level among these different strains was similar for rpoN1 and rpoN2 and followed a pattern comparable to the one of rpoD, encoding the essential sigma factor Sigma-70, which was used as control (Table 2). The nucleotide variation among the orthologous sequences in different strains is in agreement with the established species phylogeny which places phylotype I strains and all disease index equal of greater than 2 were considered as significantly different.

| R. solanacearum strain or related species | R. solanacearum phytype | Nucleotide identity (%) |
|----------------------------------------|------------------------|-------------------------|
|                                        | rpoN1 | rpoN2 | rpoD |
| R. sol. GM11000                        | I     | 100   | 100 |
| R. sol. FOY4                           | I     | 99    | 99  |
| R. sol. CMR15                          | III   | 98    | 98  |
| R. sol. Psi07                          | IV    | 95    | 95  |
| R. syzygi R24                         | IV    | 95    | 95  |
| Blood disease bacterium R229          | IV    | 95    | 94  |
| R. sol. CFBP29567                      | II    | 95    | 93  |
| R. sol. Po82                          | II    | 95    | 94  |
| R. sol. IPO1609                       | II    | 95    | 93  |
| R. picketi 12J                        | –     | 87    | 84  |
| R. eutropha                           | –     | 80    | 89  |
| Cupriavidus taiwanensis                | –     | 80    | 88  |
| Cupriavidus necator                    | –     | 79    | 88  |

Identity score was determined using the BlastN program.
evolutionary selection to maintain rpoN1 and rpoN2 genes has been similar in the different R. solanacearum strains.

**The rpoN1 Mutant is Deficient for Natural Transformation**

Mutant strains for the rpoN1 and rpoN2 genes were generated. The rpoN2::Ω mutant was created by insertion of the Ω interposon as described previously (Plener et al., 2012). The rpoN1 insertion mutant was obtained from an already available EZ-Tn5™ mutant library in GM1000 (Boucher and Genin, unpublished data). In this work we used the following mutants: an rpoN1::EZ-Tn5 mutant (KanR) and a rpoN2::ΩSp mutant (SpcR).

To create a double mutant, two experiments were set up (Figure 1). First, genomic DNA from rpoN2::Ω strain was used to transform rpoN1::EZ-Tn5 strain. Second, genomic DNA from rpoN1::EZ-Tn5 strain was used to transform rpoN2::Ω strain. Appropriate antibiotic medium was used to select for transformants. Surprisingly, transformants were only obtained in the second transformation experiment but not from the first transformation experiment in spite of repeating the experiment several times. As we could obtain the rpoN1 rpoN2 double mutant (rpoN1::EZ-Tn5 rpoN2::Ω; hereafter referred to rpoN1/2) in the second transformation experiment, the inability to get transformation in rpoN1::EZ-Tn5 background indicated that the GM1000 strain with the genotype rpoN1::EZ-Tn5 was likely inefficient or strongly affected for transformation. In a separate experiment, mutation in a different gene [RSc3392::Ω] also failed to transfer into the rpoN1::EZ-Tn5 background. This eliminated the possibility that the rpoN2 mutation was lethal in the rpoN1::EZ-Tn5 background and further supported the view that the rpoN1::EZ-Tn5 strain was inefficient for transformation. Any negative impact of EZ-Tn5 itself on transformation can be ruled out because three independent EZ-Tn5 mutants of GMI1000 strains were able to undergo transformation with rpoN2::Ω as recipient strain. Further, independent transformants could be obtained in GM1000 using genomic DNA from the rpoN1::EZ-Tn5 strain. These new mutants were also resistant to transformation which eliminated the possible effect of any mutation at a distant locus in the earlier strain.

To eliminate the possibility of any tightly linked locus to rpoN1::EZ-Tn5 responsible for this transformation resistant phenotype, a new rpoN1 mutant was created by insertion of the Ω interposon [rpoN1::Ω(Km)] (Supplementary Material Figure 1) and this new mutant was also found to be transformation deficient. These genetic experiments confirmed that insertion mutation in rpoN1 abolished natural transformation ability in GM1000 whereas insertion mutation in rpoN2 had no effect. As expected the rpoN1/2 double mutant was also inefficient for transformation.

A survey of the rpoN1 locus indicated that there is an ORF (RSc0407) just downstream rpoN1 and which presumably belongs to the same transcriptional unit (see https://iant.toulouse.inra.fr/bacteria/annotation/cgi/ralso.cg). A disruption of the RSc0407 gene was created (RSc0407::Ω) and proved that this rpoN1 downstream gene had no effect on R. solanacearum transformation. Altogether, these results showed that rpoN1 is required for natural competence in R. solanacearum.

**rpoN1 is Required for Twitching Motility and Growth on Nitrate**

There is no previous report in any bacteria relating the role of rpoN in natural transformation. However, it has been demonstrated that type IV pilus mutants of R. solanacearum are deficient for transformation as well as for twitching motility (Kang et al., 2002). In addition, there is a potential rpoN promoter sequence in the upstream region of the pilA gene in GM1000 genome and pilA is required for the formation of type IV pili (Kang et al., 2002). It has also been reported that rpoN is involved in the formation of type IV pili and therefore twitching motility in Pseudomonas aeruginosaa (Ishimoto and Lory, 1989; Semmler et al., 1999) and Neisseria elongata (Rendón et al., 2013). We thus studied twitching motility in rpoN1, rpoN2 and the rpoN1/2 mutants. We observed that the rpoN1 and rpoN1/2 mutants were twitching motility-deficient whereas the rpoN2 mutant was proficient for twitching motility as the wild type strain (Figure 2). This indicated that rpoN1 mutant is likely to be type IV pili deficient because type IV pili are required for twitching motility in R. solanacearum. The RSc0407::Ω mutation had no impact on twitching motility, thus confirming that the rpoN1 mutation had no polar effect on the downstream gene.

The initial discovery of the rpoN gene in bacteria was related to its role in nitrogen assimilation (Buck et al., 2000). We studied growth of different R. solanacearum strains on 500 μM nitrate. The rpoN1 as well as rpoN1/2 mutants did not grow when nitrate was supplied as the only nitrogen source while rpoN2 and RSc0407::Ω mutants exhibited growth similar to GM1000 in this medium (Supplementary Material Figure 2).

**rpoN1 Mutants are Impaired in Virulence on Tomato Plants**

To understand the role of rpoN1 and rpoN2 in R. solanacearum virulence, we independently inoculated tomato plants with

![FIGURE 1 | Genetic crosses in R. solanacearum GM1000 to obtain an rpoN1/rpoN2 double mutant. Two transformation experiments were set up to obtain the rpoN1/rpoN2 double mutants using either the rpoN1::Tn5 or the rpoN2::Ω as receptor strains. Transformation was successful only in case II.](Image 314x577 to 541x709)
rpoN1, rpoN2, rpoN1/2, RSc0407::Ω mutants and compared their virulence phenotype with the wild type GMI1000 by a soil drenching inoculation method (Figure 3). A Kaplan–Meier survival analysis shown in Figure 3A revealed that the rpoN1 and rpoN1/2 mutants were strongly reduced in pathogenicity. A log-rank test, aimed to verify the hypothesis of similarity of the survival curves, confirmed that the difference observed with the rpoN1 mutant, but not the rpoN2 mutant, was statistically significant (Figure 3B). The virulence deficiency observed in rpoN1 and as well as in rpoN1/2 double mutants could be expected because it was previously reported that twitching motility is required for full virulence of R. solanacearum (Kang et al., 2002), which is in agreement with the observed rpoN1 and rpoN1/2 twitching motility-deficient phenotype. The growth proficiency of rpoN1 mutants was compared with the GMI1000 wild-type strain in complete medium. The generation time of both rpoN1 mutant strains and GMI1000 was similar (i.e., 2.0 h). On medium without antibiotic rpoN1 mutant and GMI1000 strains formed small and big colonies, respectively, which can be easily observed after 2 days of growth on solid medium (Supplementary Material Figure 3A). This small colony phenotype presumably resulted from the twitching motility-deficiency of the rpoN1 mutant and suggested that the virulence deficiency rpoN1 is not due to growth deficiency.

The rpoN1 Mutant Phenocopies the pehR Mutant for Twitching Motility

To understand any other reason apart from the twitching motility responsible for the virulence deficiency in rpoN1 mutants, we decided to compare it with the pehR mutant, since pehR is a component of the virulence regulatory network in R. solanacearum (Allen et al., 1997) which is also deficient for twitching motility (Tans-Kersten et al., 2004). We constructed a pehR disruption mutant in strain GMI1000 and compared it to the rpoN1 mutant with respect to twitching motility. This pehR mutant was also deficient for twitching motility (Supplementary Material Figure 3B). Virulence deficiency observed for the rpoN1 and pehR mutants was also found to be of similar magnitude in soil drench inoculation assay (Supplementary Material Figure 4). rpoN1 and pehR mutants were both deficient for twitching motility but the pehR mutant strain grows on nitrate unlike rpoN1.

pehR mutants are deficient for swimming motility (Allen et al., 1997). In complete medium GMI1000 exhibits no motility so we took advantage of the phcA regulatory mutant that expresses constitutive motility (Brumbley et al., 1993). To compare the role of rpoN1 and pehR in R. solanacearum swimming motility, we therefore created phcA/pehR and phcA/rpoN1 double mutants. Swimming motility assays were performed on semi-solid agar plates and motility of the strains was compared based on the measurement of the bacterial halo diameter formed after 24 h (Supplementary Material Table 1). This assay revealed that the pehR mutation, but not rpoN1, abolished the constitutive motility observed in a phcA mutant background. This phenotypic analysis of the rpoN1 and pehR mutants supported the view that both genes are jointly involved in regulating twitching motility in R. solanacearum whereas pehR appears to regulate swimming motility independently of rpoN1 and rpoN1 regulates nitrate assimilation independently of pehR.

rpoN1 Controls the Expression of rpoN2

In order to identify candidate rpoN regulatory targets, the whole GMI1000 genome sequence was scanned with a consensus motif found in σ54-dependent promoters (TGGCACRNNNNTGCW) (Barrios et al., 1999). A total 79 hits were found. Out of these 79 hits, 40 sites were considered as potential Sigma-54 target sequence because the consensus motif was located in the sense orientation in the 450 bp region immediately upstream of an annotated gene (Salanoubat et al., 2002). The list of the 40 identified genes is given in Table 3. In agreement with the former results, this list contains the pilA gene encoding the structural component of Type IV pili and the RSp1219–RSp1223 operon which was found to control nitrate assimilation in R. solanacearum (Dalsing and Allen, 2014). Among these 40 rpoN-regulated gene candidates, more than one-third encode hypothetical proteins of unknown function and six encode uncharacterized transcription regulators.
TABLE 3 | List of predicted σ^{54} factor-target sequences in the R. solanacearum GMI1000 genome.

| Gene      | Sequence element       | Position upstream of start codon | Product                                |
|-----------|------------------------|----------------------------------|----------------------------------------|
| RSc0133   | TGGCGCATTCATGGCA       | 73                               | Hypothetical                           |
| RSc0223   | TGGCCAGCGCGTGTGA       | 14                               | Hypothetical                           |
| RSc0030   | TGGCCAGCTCCCTGCA       | 146                              | Dicarboxylate transporter              |
| RSc0341   | TGGCCAGCATGGTGCA       | 68                               | Hypothetical                           |
| pIA      | TGGCCACGCTCCCTGCA      | 47                               | Type 4 pilin                           |
| RSc0731   | TGGCCAGCGTTTGCAT       | 22                               | Thioesterase                           |
| RSc0753   | TGGCCAGCGATTGCA        | 162                              | Hypothetical                           |
| RSc0796   | TGGCCAGCAATTGCA        | 34                               | Nucleoside permease operon            |
| RSc0940   | TGGCCAGACTGGTGCA       | 10                               | Ribosome small unit GTPaseA           |
| RSc0950   | TGGCCAGATTTTTCGCA      | 19                               | Hypothetical                           |
| RSc1121   | TGGCCAGCATCTTGCT       | 377                              | Hypothetical                           |
| RSc1878   | TGGCCACGCTTGCAT        | 100                              | Hypothetical                           |
| RSc2041   | TGGCCACGCTTGCAT        | 50                               | Transporter                           |
| RSc2102   | TGGCCACAAACTGCA        | 55                               | Transcription regulator               |
| RSc2118   | TGGCCAGCTCTGCA         | 178                              | Transporter                           |
| RSc2173   | TGGCCAGGTGCTGCA        | 280                              | ABC transporter operon                |
| RSc2194   | TGGCCACGCTCTGCA        | 54                               | Hypothetical                           |
| RSc2312   | TGGCCAGCTCTGCA         | 153                              | Transcription regulator               |
| RSc2320   | TGGCCACAAAGTTTGCAT     | 407                              | Transcription regulator               |
| RSc2441   | TGGCCACGCTTGCATT       | 430                              | Transporter                           |
| RSc2641   | TGGCCACGCTTGCAT        | 71                               | Hypothetical                           |
| RSc2930   | TGGCCACGCTTGCAT        | 79                               | Mechanosensory ion channel            |
| RSc3128   | TGGCCACGCTTGCAT        | 68                               | Dehydrogenase                         |
| RSc3203   | TGGCCACGCGCTTCCAT      | 108                              | Hypothetical                           |
| RSc3410   | TGGCCACGCGCTTCCAT      | 69                               | ABC transporter operon                |
| RSp0054   | TGGCCACGCTTGCAT        | 88                               | Transcription regulator               |
| RSp0092   | TGGCCACGCTTGCAT        | 57                               | Hypothetical                           |
| RSp0219   | TGGCCACGCTTGCAT        | 157                              | Hydroxylase                           |
| RSp0229   | TGGCCACGCTTGCAT        | 70                               | Dehydrogenase                         |
| RSp0285   | TGGCCACGCTTGCAT        | 248                              | Transcription regulator               |
| RSp0337   | TGGCCACGCTTGCAT        | 94                               | Porin                                 |
| RSp0635   | TGGCCACGCGATTGCA       | 70                               | Acyltransferase                       |
| RSp0830   | TGGCCACGCGATTGCA       | 48                               | Galactarate dehydratase               |
| RSp1093   | TGGCCACGCGATTGCA       | 55                               | Hypothetical                           |
| RSp1094   | TGGCCACGCGATTGCA       | 79                               | Hypothetical                           |
| RSp1223   | TGGCCACGCGATTGCA       | 151                              | Nitrate assimilation operon           |
| RSp1234   | TGGCCACGATTGCTGAT      | 253                              | Lipase                                |
| RSp1355   | TGGCCACGATTGCTGAT      | 253                              | Hypothetical                           |
| rpoN2     | TGGCCACGCGATTGCA       | 63                               | σ^{54}-related protein                |
| Rsp1674   | TGGCCACGCGATTGCA       | 40                               | Hypothetical                           |

Genes followed by an asterisk correspond to the first gene of probable operonic gene units.

Interestingly, one of the potential σ^{54}-dependent promoter regions was found to be upstream of rpoN2. We studied the expression of rpoN1 and rpoN2 using reporter fusions with lacZ gene (Materials and Methods). β-galactosidase expression was monitored after cultivation of bacteria in complete and minimal media, as well as in presence of Arabidopsis plant cells. Results in Figure 4 show that rpoN1 and rpoN2 display a different expression pattern: rpoN1 is significantly expressed in the three conditions tested (albeit expression appears higher in minimal medium) whereas rpoN2 is not expressed in complete medium and is specifically induced in minimal medium. In the latter condition, the expression of rpoN2 is completely abolished in an rpoN1 mutant background, thus confirming that rpoN1 regulates the expression of rpoN2 in strain GMI1000. No specific induction of rpoN1 and rpoN2 expression could be observed when bacteria were co-cultivated with plant cells.
Discussion

The role of rpoN (σ^54 or also σ^N) was historically uncovered with the study of the regulation of nitrogen metabolism but it has been subsequently found to be involved in many other biological activities in many diverse Proteobacteria (Buck et al., 2000). Evidence of rpoN involvement in bacterial pathogenesis and virulence, mainly through the control of flagellar motility, is also documented (Kazmierczak et al., 2005). However, rpoN does not share a common role in plant pathogens: in Pseudomonas syringae, Pectobacterium carotovorum, and Erwinia amylovora rpoN is required for virulence and is involved in the regulation of the type III secretion system (Hendrickson et al., 2000a,b; Chatterjee et al., 2002; Ramos et al., 2013) but in Xanthomonas campestris pv. vesicatoria, disruption of rpoN has no impact on virulence (Horns and Bonas, 1996). A recent study revealed that in P. syringae rpoN also strongly activated the vast majority of genes involved in flagellar synthesis and motility, as well as many genes activated in planta including phytotoxins and the siderophore pyoverdin (Yu et al., 2014).

In R. solanacearum rpoN mutants still induce a hypersensitive response on tobacco (data not presented) which indicates that σ^54 is not required for the functionality of the type III secretion system. However, we show in this study that rpoN1 significantly contributes to R. solanacearum pathogenesis and controls multiple traits in this bacterium. This pleiotropic phenotype was observed with two mutants carrying independent mutations in the rpoN1 gene and further analyses showed that it was independent from the rpoN1 downstream gene (RSc0407) (Supplementary Material).
highly similar and functionally interchangeable (Michiels et al., 1998). In X. oryzae pv oryzae rpoN2 is located close to the flagellar regulon and is required for swimming motility, having therefore a distinct role from the paralogous rpoN1 gene (Tian et al., 2015).

Two copies of rpoN are generally found in β-proteobacteria having two or more replicons such as R. solanacearum, R. pickettii, and many Burkholderiales. However, the second copy is absent in bacteria taxonomically related to R. solanacearum such as R. etufropha and Cupiavidus species, which are non-pathogenic. In this work we show that rpoN1 and rpoN2 are not functionally redundant in R. solanacearum since rpoN1 is required for twitching motility, natural transformation and growth on nitrate whereas rpoN2 mutant is proficient for these phenotypes. We also provide genetic evidence that expression of rpoN2 is dependent upon rpoN1 and has a distinct expression profile, being specifically induced when bacteria are grown in minimal medium or in presence of plant cells. The rpoN2 mutant is not altered in virulence and no specific phenotype could be associated to this second rpoN copy, so its role remains enigmatic. However, the broad conservation and stability of this gene in the R. solanacearum species complex, which suggest a long co-evolution of the two rpoN copies in the species, prompt us to speculate that the adaptation of the bacterium to a specific niche or environmental condition might have selected a defined regulatory role for rpoN2 during the pathogen lifecycle. Future investigations aimed to determine the specific target promoters of rpoN1 and rpoN2 at the transcriptomic level will help to define the distinct roles of these two σ54-RNA polymerases during the interaction of R. solanacearum with its host plants and its environment.

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Supplementary Material

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fmicb.2015.00229/abstract

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