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GENOMICS, TRANSCRIPTOMICS, PROTEOMICS

Pleiotropic control of antibiotic biosynthesis, flagellar operon expression, biofilm formation, and carbon source utilization by RpoN in *Pseudomonas protegens* H78

Yujie Liu[1] · Huimin Shi[1] · Zheng Wang[1] · Xianqing Huang[1] · Xuehong Zhang[1]

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Abstract

The rhizobacterium *Pseudomonas protegens* H78 biosynthesizes a number of antibiotic compounds, including pyoluteorin, 2,4-diacetylphloroglucinol, and pyrrolnitrin. Here, we investigated the global regulatory function of the nitrogen metabolism-related sigma factor RpoN in *P. protegens* H78 through RNA-seq and phenotypic analysis. During the mid- to late-log growth phase, transcriptomic profiling revealed that 562 genes were significantly upregulated, and 502 genes were downregulated by at least twofold at the RNA level in the *rpoN* deletion mutant in comparison with the wild-type strain H78. With respect to antibiotics, Plt biosynthesis and the expression of its operon were positively regulated, while Prn biosynthesis and the expression of its operon were negatively regulated by RpoN. RpoN is responsible for the global activation of operons involved in flagellar biogenesis and assembly, biofilm formation, and bacterial mobility. In contrast, RpoN was shown to negatively control a number of secretion system operons including one type VI secretion system operon (H1-T6SS), two pilus biogenesis operons (Flp/Tad-T4b pili and Csu-T1 pili), and one polysaccharide biosynthetic operon (*psl*). In addition, two operons that are involved in mannitol and inositol utilization are under the positive regulation of RpoN. Consistent with this result, the ability of H78 to utilize mannitol or inositol as a sole carbon source is positively influenced by RpoN. Taken together, the RpoN-mediated global regulation is mainly involved in flagellar biogenesis and assembly, bacterial mobility, biofilm formation, antibiotic biosynthesis, secretion systems, and carbon utilization in *P. protegens* H78.

Keywords Plant growth-promoting rhizobacteria · *Pseudomonas* · RpoN · Antibiotic biosynthesis · Flagellar biogenesis · Secretion systems

Introduction

The rhizobacterium *Pseudomonas protegens* H78, which was screened from the rape rhizosphere soil a Shanghai suburb, can produce several antifungal compounds, including pyoluteorin (Plt), 2,4-diacetylphloroglucinol (DAPG), and pyrrolnitrin (Prn) (Huang et al. 2017; Wang et al. 2017). The biosynthesis of secondary metabolites, including these antibiotics, is coordinated by multiple pathway-specific and global regulatory systems or factors in *Pseudomonas* spp. (Haas and Keel 2003). Two typical pathway-specific regulators, PltR and PhlF, are responsible for the activation of Plt synthesis and the repression of DAPG synthesis, respectively. PltR activates transcription of the Plt operon *pltLABCDEFG* (Li et al. 2012). In contrast, PhlF plays a transcriptional repressor role of the DAPG operon *phlACBD* (Abbas et al. 2002). In *Pseudomonas* spp., the classic Gac/Rsm regulatory cascade, which is in turn composed of the GacS/GacA two-component signal transduction system (TCS), the RsmY family of sRNAs and the RsmA/RsmE family of translational repressors, has been confirmed to globally regulate secondary mechanisms, including antibiotic biosynthesis (Haas and Keel 2003). The GacS/A TCS plays a global regulatory role in secondary

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✉ Xianqing Huang
xqhuang66@sjtu.edu.cn

[1] State Key Laboratory of Microbial Metabolism, Joint International Laboratory on Metabolic & Developmental Sciences, School of Life Sciences & Biotechnology, Shanghai Jiao Tong University, Shanghai, China

metabolism, primary metabolism, etc. in *Pseudomonas aeruginosa* M18 (Li et al. 2008; Wei et al. 2013; Zhou et al. 2010). However, in *P. protegens* H78, GacS/GacA activates Plt synthesis through both the Gac/Rsm-RsmE positive regulatory cascade and a RsmA/E-driven feedback activation loop in H78 (Wang et al. 2017). In addition, signaling molecules or systems are another important category of global regulators in *Pseudomonas* antibiotic biosynthesis. In *P. protegens* H78, secondary metabolism, including antibiotic production, is globally activated by the (p)ppGpp-mediated stringent response (unpublished data). However, the complex and unique regulatory network and mechanism of antibiotic biosynthesis are not fully clarified in *P. protegens*.

In addition to the abovementioned global regulatory systems, the house-keeping sigma factor RpoD (σ^{70}) (Schnider et al. 1995) and two alternative sigma factors, including the nitrogen metabolism-related sigma factor RpoN (σ^{54}) (Pechy-Tarr et al. 2005) and the stationary-phase sigma factor RpoS (σ^{38}) (Sarniguet et al. 1995), exert pleiotropic influences on antibiotic biosynthesis in *P. protegens* CHA0 and Pf-5. Amplification of RpoD results in a marked enhancement of Plt and DAPG in CHA0 (Schnider et al. 1995). The *rpoS* mutation causes an excessive production of Plt and DAPG and the inhibition of Prn in the Pf-5 strain (Sarniguet et al. 1995). RpoN positively regulates Plt biosynthesis and negatively regulates DAPG biosynthesis in CHA0. The influence of RpoN on Prn biosynthesis was not investigated in CHA0 (Pechy-Tarr et al. 2005). However, the regulatory mechanism of these two sigma factors in antibiotic biosynthesis remains unknown.

The RpoN protein of *Escherichia coli* is mainly composed of three functional domains, including the upstream activator protein-binding domain, the RNAP (RNA polymerase)-binding domain, and the promoter-binding HTH domain. The RpoN sigma factor recognizes a conserved promoter sequence, -24(GG)/-12(GC), and initiates target gene expression in cooperation with at least one transcriptional activator (enhancer) (Potvin et al. 2008; Taylor et al. 1996). RpoN was first observed to be related to nitrogen metabolism (Hunt and Magasanik 1985). Thereafter, an increasing number of studies suggested that RpoN is widely distributed in diverse bacteria and has pleiotropic effects on multiple bacterial traits, including flagellar biogenesis and motility (Anderson et al. 1995; Fazli et al. 2017; Jones et al. 2007; Kawagishi et al. 1997; Potvin et al. 2008; Saldias et al. 2008; Totten et al. 1990).

This study aims to investigate the global regulatory model of RpoN in *P. protegens* H78, which was grown to the mid- to late-log growth phase in KMB medium, through a comparative analysis of transcriptomes and phenotypes. The results suggest that RpoN plays a global regulatory role in antibiotic biosynthesis, flagellar biogenesis and assembly, biofilm formation, mobility, bacterial secretion systems, and carbohydrate metabolism.

Materials and methods

Primers, plasmids, strains, and growth conditions

Strains, vectors, and primers are described in Tables S1 and S2. *E. coli* was traditionally cultivated with LB at 37 °C. The wild-type strain of *P. protegens* H78 has been deposited in the China General Microbiological Culture Collection Center (CGMCC 15755). *P. protegens* H78 and derivative strains were cultured at 28 °C in KMB (20 g bacto-tryptone, 15 ml glycerol, 0.514 g $K_2HPO_4{\cdot}3H_2O$, and 0.732 g $MgSO_4$ per liter, pH 7.5) (King et al. 1954). Where needed, antibiotics were supplemented to media at the final concentration (μg ml^{-1}): kanamycin (Km), 50, ampicillin (Amp), 100, tetracycline (Tc), 30, for *P. protegens* H78 and its derivative, and Km, 50, Amp, 100, Tc, 15, for *E. coli*.

Methods in molecular biology and bioinformatics

All molecular biological operations without detailed description were carried out according to standard procedures (Sambrook and Russell 2001). The reagent kits involved in genomic or plasmid extraction and DNA fragment purification were supplied by TaKaRa. Similarly, In-Fusion HD cloning kits and Solution I DNA ligase were obtained TaKaRa. Other enzymes, including KOD plus Neo DNA polymerase (Toyobo), Easy Taq Mix DNA polymerase (TransGen), and restriction endonucleases (NEB), were used according to the suppliers' recommendations. Primers were synthesized and DNAs were sequenced by HuaDa Gene technology Co. Ltd. and Shanghai Sangon Biotech Co., Ltd. The RpoD-recognized promoters were predicted by the BPROM software (Solovyev and Salamov 2011). Sequence alignment was carried out by BLAST at NCBI.

Knock-out and complementation of the *rpoN* gene

The *P. protegens* H78 *rpoN* gene was deleted using homologous recombination technology. Two DNA fragments (527 bp and 503 bp) on both sides of the *rpoN* ORF were obtained by PCR amplification with two pairs of primers (Table S2), in which the downstream primer of fragment 1 and the upstream primer of fragment 2 are complementary in their 5′ terminal sequences. Then, an in-fusion PCR fragment, in which the *rpoN* ORF has been deleted in-frame, was produced using a mixed template of these two products and cloned into the *EcoR*I/*Pst*I site of the suicide vector pK18mobsacB carrying a Km-resistance gene and a sucrose-inducible lethal gene *sacB*. The recombinant plasmid, designated pk18-rpoN, was transferred from *E. coli* S17 into *P. protegens* H78 by conjugation. The single-crossover integration recombinants were first selected on LB agar including Amp (to counter-select S17) and Km. After the second homologous recombination

(double crossover), the Km-sensitive, Amp-resistant, and sucrose-resistant *rpoN* in-frame deletion mutants of H78 were obtained by a sensitive-resistant pair screening on two parallel agar plates which respectively contain Km and sucrose. The H78rpoN mutant was further confirmed by PCR and sequencing.

To complement the *rpoN*-deleted mutant, a fragment (1758 bp), which covers the entire coding region and promoter of *rpoN*, was amplified and cloned into the *Kpn*I/*Eco*RI site of the *Pseudomonas-E. coli* shuttle vector pME6032.

RNA-seq-based transcriptomic assay

Overnight culture solutions of *P. protegens* H78 and *rpoN* deletion strain were inoculated, at a final OD_{600} of 0.02, into the 500-ml conical flasks containing 100 ml KMB. Each strain for RNA-seq was set up with three parallel replicates. RNA sequencing was performed in triplicate for each strain. The cultures were sampled after 18 h of incubation (28 °C, 200 rpm) to the mid- to late-log growth phase. The cells were collected by centrifugation (4 °C, 5000 rpm, 3 min) with a 50-ml tube. After being quickly frozen with liquid nitrogen and transported using dry ice, the cell samples were submitted for subsequent RNA-seq analysis by the Shenzhen HuaDa Genomics Institute. Total RNA was extracted using the phenol-chloroform extraction method. Ribosomal RNA (rRNA) was depleted by DNA probe hybridization and DNase I digestion. After mRNA enrichment and fragmentation, the first and second cDNA strands were synthesized to generate the sequencing library. Cluster generation and de novo sequencing were carried out using the BGISEQ-500.

After adaptor and low-quality sequences were trimmed from the raw reads, the clean reads were mapped to the *P. protegens* H78 complete genome and all genes using HISAT and Bowtie 2, respectively. The mRNA abundance of genes was normalized using the RSEM software and represented as FPKM (fragments per kilo-base of mRNA per million reads) (Mortazavi et al. 2008). The mean FPKM values were calculated for each gene between the H78rpoN mutant and the H78 WT strain from their respective repeats. Differentially expressed genes between the H78rpoN group and the H78 group were screened by NOISeq to determine if they met the standards (fold change ≥ 2, deviation probability ≥ 0.8). Further enrichment analyses (COG, GO, and KEGG) were performed for these differentially expressed genes.

Construction of *lacZ* reporter vectors and quantification of β-galactosidase expression

To validate the transcriptomic data or assess the influence of *rpoN* on relevant target operons, ten genes, including *pltL*, *flgF*, *flgB*, *mtlE*, *prnA*, *pslA*, *csuA/B*, *cpaB*, *retS*, and *mnhA*, were selected to construct the in-frame fusion reporters of these genes with *lacZ* gene on the pME6015 plasmid (Table S1). In these *lacZ* reporters, the leader regions, containing the promoter-operator–leader region of the genes, were individually fused with the *lacZ* gene lacking the corresponding regions in pME6015.

To ascertain whether the *pltR* promoter including a putative RpoN-recognized motif (Fig. 2d) is identified by the RpoN sigma factor, three fragments, which individually cover − 73 to + 30 bp, − 40 to + 30 bp, and − 73 to − 18 bp upstream of the *pltR* transcriptional start site (TSS), were separately inserted into front of the promoterless *lacZ* gene on the pME6522 plasmid (Table S1).

For β-galactosidase analysis, culture solutions of *P. protegens* H78 and derivative strains carrying these *lacZ* reporter plasmids were inoculated into the 500-ml conical flasks with 100 ml of KMB at a final OD_{600} of 0.02 and then cultivated at 28 °C and 200 rpm. The cells were harvested at one or more time points. β-galactosidase activity was quantified according to the Miller's method(Sambrook and Russell 2001) and as previously reported by our group (Huang et al. 2004).

Quantification of Plt production

P. protegens H78 and its derivative strains were inoculated and grown according to procedures and conditions similar to those adopted in the above β-galactosidase assay. The cultures were sampled at different time points. Plt was extracted with ethyl acetate and quantified by HPLC, as described previously (Huang et al. 2004).

Assay for biofilm formation

Biofilm formation quantity was measured by using the crystal violet staining method (Tomaras et al. 2003). *P. protegens* H78, H78rpoN, and its complement strain were grown for 48 h in a 24-well plate at 28 °C in the static state. After incubation, the planktonic bacterial cells were removed, and the formed biofilm was stained with 0.1% (*w*/*v*) crystal violet. After three washes with 0.1 M PBS and drying at 37 °C for 20 min, the biofilm adhered to crystal violet was solubilized with 90% ethanol. The amount of formed biofilm was determined through the absorbance assay of crystal violet at 600 nm.

Assessment of carbon source utilization

The ability of four strains (H78, H78rpoN, H78rpoN/pME6032, and H78rpoN/pME6032-rpoN) to utilize mannitol or inositol as sole carbon source was compared in minimal medium plate. The minimal media were composed of the following chemicals per liter of liquid medium: $Na_2HPO_4 \cdot 12H_2O$ (17.17 g), $(NH_4)_2SO_4$ (5.95 g), KH_2PO_4 (2.99 g), NaCl (0.58 g), $MgSO_4 \cdot 7H_2O$(0.246 g), $FeCl_3 \cdot 6H_2O$ (16.7 mg), VB_1 (0.01 mg), $CaCl_2$ (4.3 mg), $MnCl_2 \cdot 4H_2O$

(1 mg), $ZnCl_2$ (1.7 mg), $CuCl_2 \cdot 2H_2O$ (0.43 mg), $CoCl_2 \cdot 6H_2O$ (0.6 mg), and $H_4MoNa_2O_6$ (0.6 mg). The media were solidified with 1% (*W*/*V*) of agarose. In addition, 5 g l^{-1} mannitol or 4 g l^{-1} inositol was supplemented as sole carbon source.

The bacterial cells were collected from overnight cultures by centrifugation and washed with phosphate buffer. The collected cells were first diluted to the same start density ($OD_{600} = 0.05$) and then serially diluted by 1/10. Two-microliter diluted cell suspensions was inoculated on the minimal medium plates added with mannitol or inositol and then cultivated at 28 °C. The bacterial growth was observed and photographed at 24 h, 48 h, and 72 h.

In this study, all experiments were repeated at least twice. In each experiment, at least three parallel samples were included for every strain. Every value is the average ± SD (standard deviation) of three repetitions.

Accession number of nucleic acid sequence and RNA-seq data

In this study, DNA sequences and flanking sequences of the *rpoN* gene were extracted from the complete genome (GenBank accession No. CP013184) of *P. protegens* H78. The RNA-seq data of *P. protegens* H78 and its *rpoN* mutant have been submitted to the Gene Expression Omnibus (GEO) database and the Sequence Read Archive database under the accession number GSE112907.

Results

Transcriptomic profile of the *rpoN* mutant of *P. protegens* H78

P. protegens H78 and its *rpoN*-deleted mutant were monitored for growth in KMB medium, and the result is plotted in Fig. S1. Growth of the *rpoN* deletion strain was observed to be slightly less than that of the parental strain H78 up to 50 h. However, the opposite pattern was observed after 50 h of culture (Fig. S1). The sampling point for RNA-seq was chosen during the mid- to late-log growth phase, with a cell density of $OD_{600} = 4.0$~5.3 in KMB. Cultures from these two strains, which each had three replicates, were collected. The validity of the RNA-seq data was confirmed by qRT-PCR (Fig. S2) and *lacZ* reporter analysis (Fig. S3) and subsequent experiments in this study. Transcriptome profiling suggested that 502 genes were downregulated, and 562 genes were upregulated at least twofold in terms of transcript abundance after *rpoN* deletion (Fig. 1a; Table S3, S4).

The functional classification of the *rpoN* regulon is shown in Fig. 1b. The largest functional class of the RpoN regulon is function-unknown or predicted genes. Based on a comparison between the downregulated and upregulated gene numbers in every functional category, we found that the positive regulon of RpoN is predominantly involved in signal transduction, cell surface structure biogenesis, general replication and transcription, defense mechanisms, and function-unknown or predicted genes. In comparison, the functional categories under negative control of RpoN largely involve amino acid and nucleotide acid metabolism, translational and post-translational activity, energy and coenzyme metabolism, carbohydrate and lipid metabolism, and secondary metabolism (Fig. 1b).

RpoN positively regulates *pltLABCDEFG* operon expression and Plt biosynthesis

The transcriptomic data showed that the *rpoN* deletion gave rise to a 4.5- to 40.9-fold decrease in the transcript abundance of all genes in the Plt biosynthetic operon *pltLABCDEFG*. The *pltR* gene, which encodes an activator of *pltL-G* transcription, was moderately downregulated in the *rpoN*-deleted strain in comparison with the parental strain H78 (Fig. 2a). However, the *rpoN* mutation had no obvious influence on the transcript levels of the other biosynthetic gene, *pltM*, and the Plt ABC transport operon *pltIJKNOP*. The *pltL'-'lacZ* in-frame fusion expression analysis further confirmed that the expression of the *pltL-G* biosynthetic operon is positively regulated by RpoN (Fig. 2b). As the control, the empty plasmid pME6015 has not shown any expression activity of LacZ in both H78 and its *rpoN* mutant. As a result of the downregulated expression of the *plt* operon, a remarkable drop in Plt biosynthesis was found in the *rpoN*-deleted strain compared with its parental strain H78 (Fig. 2c).

The predicted RpoN-recognized motif within the *pltR* promoter was not recognized by RpoN

An alignment of the *pltL-pltR* intergenic sequence indicated that a predicted RpoN-recognized consensus sequence (5'-TG**G**CACG-N4-TTG**C**W-3′) is immediately upstream of the *pltR* promoter (Fig. 2d). To further ascertain whether this target motif RpoN is a functional promoter that can be specifically recognized and initiated by RpoN, we constructed three *lacZ* fusion reporter vectors, which carried a region containing two potential promoters, the RpoD-recognized promoter and the putative RpoN-recognized promoter (Fig. 2d). The LacZ expression from these three reporter vectors was respectively measured in *P. protegens* H78 and its *rpoN* mutant. Compared to the background expression from the empty vector pME6522, no obvious expression was observed for the *lacZ* fusion vector carrying the putative RpoN-recognized sequence in both H78 and the *rpoN* mutant (Fig. 2e). These results suggested that this predicted RpoN-recognized sequence is not a functional promoter. Remarkably, the expression activity from a minimal RpoD-recognized promoter exhibited a threefold increase due to an almost total lack of the

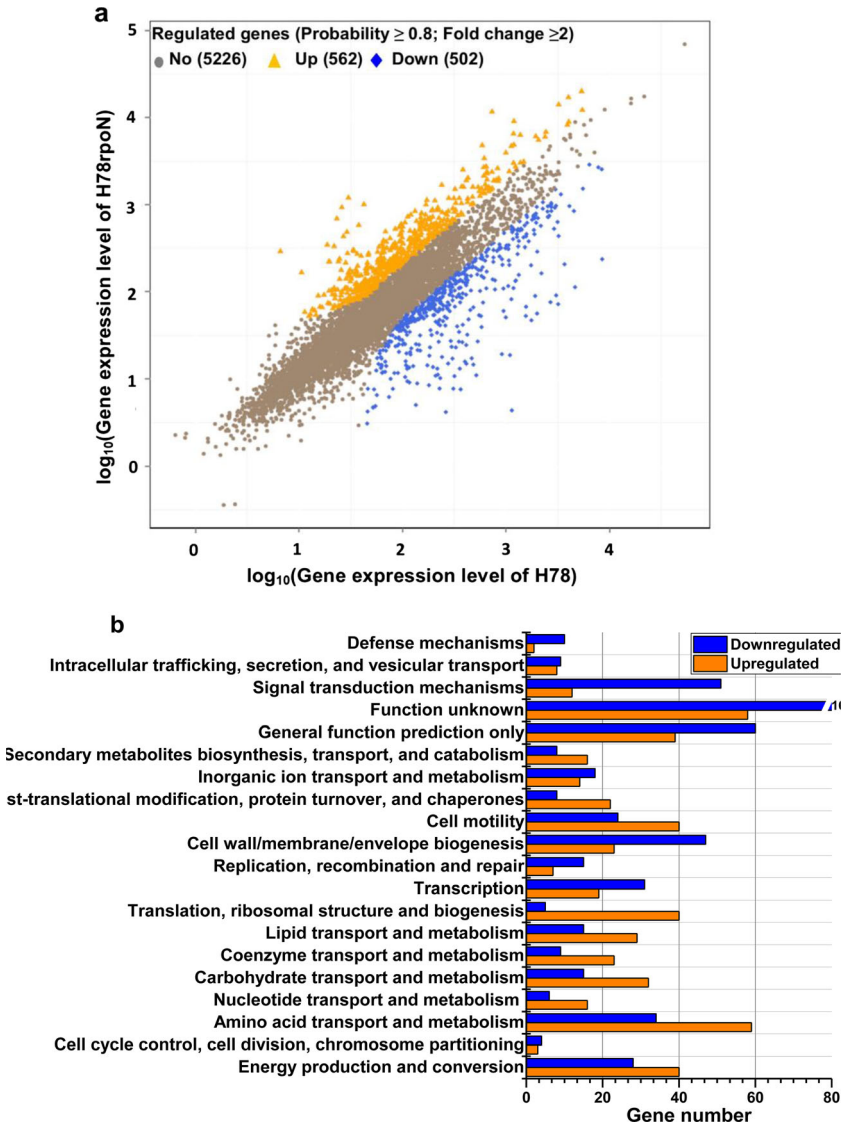

Fig. 1 Transcriptomic profiles of *P. protegens* H78 and its *rpoN*-deleted mutant H78rpoN. **a** The number of genes that was significantly upregulated or downregulated at least twofold (probability ≥ 0.8) in the H78rpoN mutant relative to the wild-type H78. **b** Functional classification of the RpoN regulon (fold change ≥ 2, probability ≥ 0.8) according to COGs (Clusters of Orthologous Groups). Downregulated and upregulated genes were counted in every functional category

putative RpoN-recognized motif and the upstream 22 bp sequence (Fig. 2e).

Negative regulation of the expression of the pyrrolnitrin operon by RpoN

Pyrrolnitrin biosynthesis originates from a precursor of tryptophan and is catalyzed by a biochemical pathway encoded by the *prnABCD* gene cluster (Hill et al. 1994). In this study, the transcriptomic data showed that the *rpoN* mutation induced a 4.2- to 15.2-fold increase in the transcript levels of all genes in the pyrrolnitrin biosynthesis operon *prnABCD* in *P. protegens* H78 (Fig. 3a). The *prnA'-'lacZ* fusion expression analysis further confirmed the significant upregulation of the expression of the *prn* operon by RpoN (Fig. 3b). The results suggested that RpoN negatively controls Prn operon expression and thus Prn biosynthesis.

RpoN globally activates gene expression involved in flagellar biogenesis and chemotaxis

Based on transcriptomic profiling, we found that the gene clusters responsible for flagellar biogenesis, assembly, and regulation are activated by RpoN in *P. protegens* H78 (Table S5). In the flagellar assembly model analyzed using KEGG, expression of almost all flagellar assembly genes significantly reduced the *rpoN* deletion strain in comparison with its parental strain H78 (Fig. S4). In the aggregation region (H78_01720 to H78_01785) of the flagellar gene cluster, a total of 45 genes were significantly downregulated from 2.4- to 62.7-fold at the transcript level in the *rpoN*-deleted mutant compared with the wild-type strain H78. These genes mainly belong to four flagellar gene clusters and one chemotaxis gene cluster, including H78_01720 to 1726 (*flgFGHIJKL*), H78_01727 to 1733 (*pse* genes), H78_01743 to 1761 (*fleSR-fliEFGHIJKLMNOPQR*), H78_01762 to 1773 (*flh* genes), and H78_01782 to 1785 (*che* genes). Similarly,

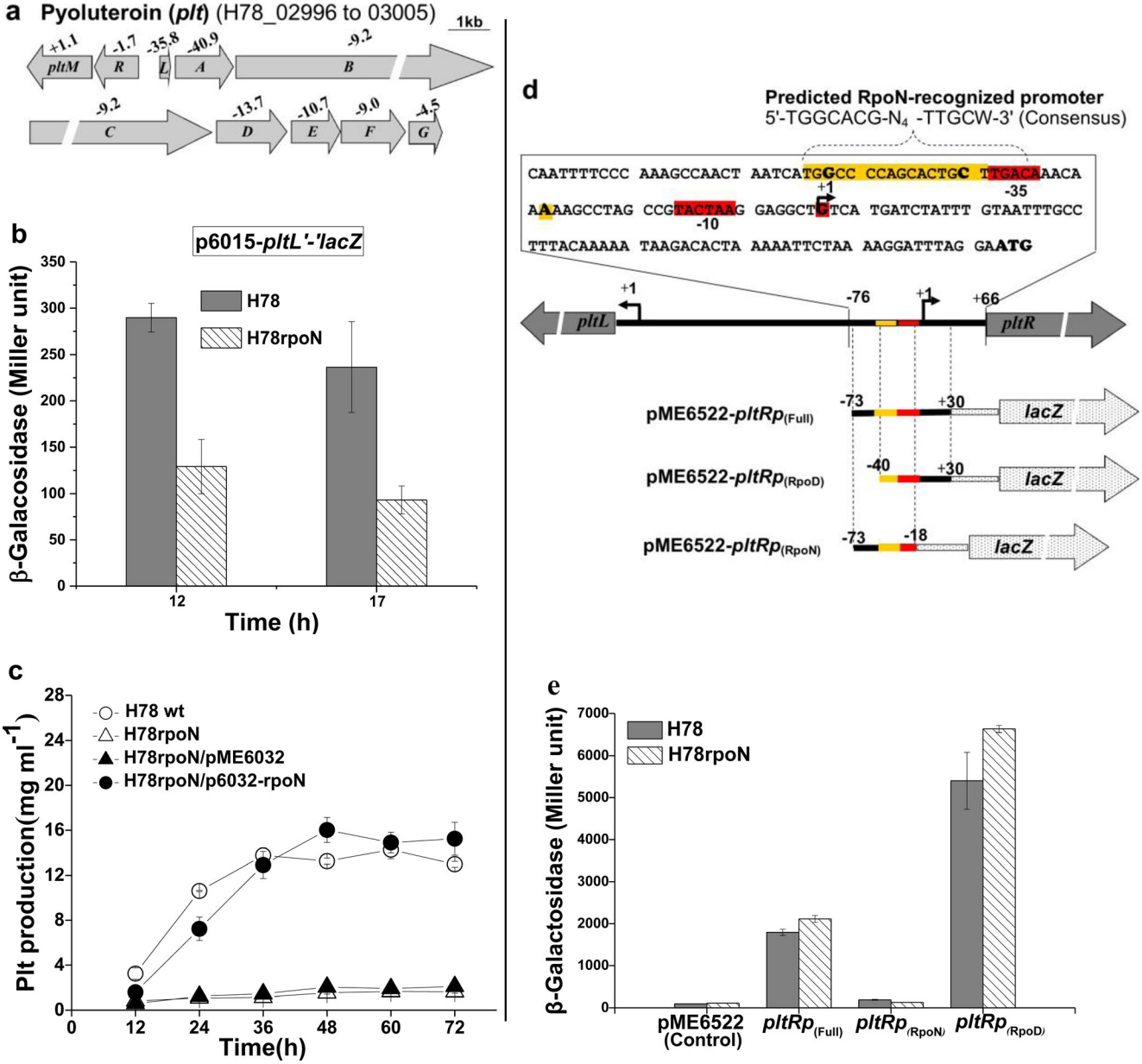

Fig. 2 Positive control of Plt biosynthesis and operon expression by RpoN (**a**–**c**) and experimental investigation of a predicted RpoN-recognized sequence that partially overlaps with the *pltR* promoter (**d**–**e**) in *P. protegens* H78. **a** The numerical values above the arrows indicate the fold changes in the transcript level of the *pltLABCDEFG* operon and its transcriptional activator gene *pltR* in the H78rpoN strain relative to the parental strain H78. **b** Expression of the *pltL'-'lacZ* in-frame fusion was assessed between H78 and its *rpoN* mutant in KMB medium. **c** Plt biosynthesis was compared between H78 and H78rpoN in KMB medium. **d** Construction map of three fusions of the putative RpoN-recognized promoter and/or the RpoD-recognized promoter with the promoter-less *lacZ* gene in pME6522. The predicted RpoN-recognized conserved sequence and the conserved -10 and -35 sequences of the RpoD-recognized *pltR* promoter are indicated in light and thick shadows, respectively. **e** β-galactosidase expression of three reporter vectors, including p6522-$pltRp_{(full)}$, p6522-$pltRp_{(RpoD)}$, and p6522-$pltRp_{(RpoN)}$, was separately quantified between *P. protegens* H78 and its *rpoN* mutant in KMB medium

another remote flagellar gene cluster, *flgBCDE*, was also substantially and positively regulated by RpoN (Table S5).

Two operons, *flgFGHIJKL* and *flgBCDE*, were predicted to contain an RpoN-recognized promoter upstream of *flgF* and *flgB*, respectively (Fig. 4a). These two operons were selected to further confirm the positive regulation of flagella biogenesis by RpoN. The *flgF'-'lacZ* and *flgB'-'lacZ* reporter vectors were constructed and transferred into *P. protegens* H78 and its *rpoN* mutant to assess their β-galactosidase expression. As exhibited in Fig. 4b, the *flgF'-'lacZ* and *flgB'-'lacZ* expression was nearly completely suppressed in the *rpoN* deletion strain relative to the wild-type H78. This finding clearly indicated that RpoN plays an activator role in the expression of the *flgFGHIJKL* and *flgBCDE* operons.

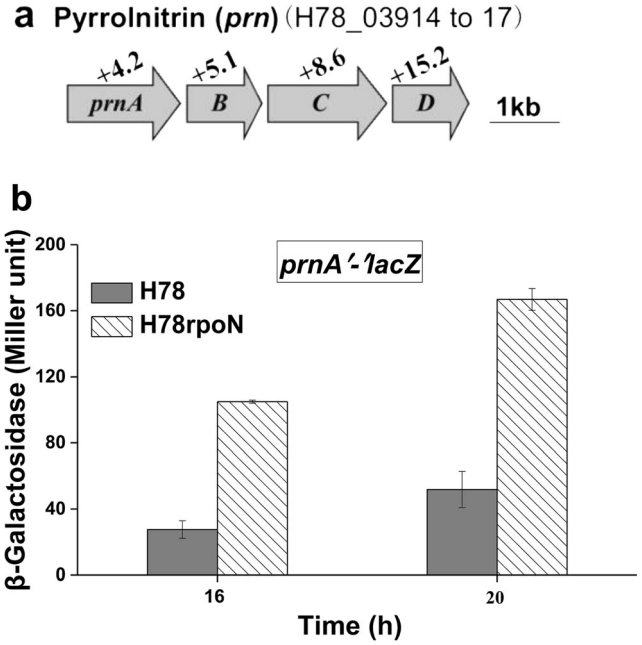

Fig. 3 Negative regulation of pyrrolnitrin biosynthetic gene expression by RpoN. **a** Fold changes in the transcript level of the *prnABCD* operon in H78rpoN relative to its parental strain H78 are shown above the gene clusters. **b** Expression of the *prnA'-'lacZ* in-frame fusion was quantified in *P. protegens* H78 and H78rpoN in KMB medium

Positive control of biofilm formation by RpoN

Biofilm formation is closely related to rhizosphere colonization activity and influenced by multiple factors in bacteria including *Pseudomonas*. Here, we compared the biofilm quantity of *P. protegens* H78 and its *rpoN* mutant. The data presented in Fig. 5 show that *rpoN* deletion seriously impaired the biofilm formation ability of *P. protegens* H78. The total biomass of the biofilm formed by the *rpoN* mutant was far below that of the parental strain H78. In turn, biofilm formation of the *rpoN* deletion strain was recovered to the wild-type level by the exogenous expression of *rpoN* on the plasmid pME6032-rpoN. These results implied that RpoN plays a key role in activating the ability of *P. protegens* H78 to form biofilms.

Negative regulation of H1-T6SS, piliation, and exopolysaccharide biosynthesis by RpoN

The genomic region from H78_06283 to 6304 encodes a typical type VI secretion system (T6SS), which is homologous to H1-T6SS of *P. aeruginosa* (Wei et al. 2013). The transcript levels of all genes in this gene cluster were markedly enhanced in the *rpoN* deletion strain in comparison with the wild-type strain H78 (Fig. S5a). Two gene clusters, *tad-cpa* and *csu*, encode the biogenesis pathways of type IV Flp/Tad pilin (Giltner et al. 2012) and type I Cup (chaperone-usher pili) pilin, respectively (Tomaras et al. 2003). All genes of these two pilin gene clusters were substantially upregulated in terms of transcript abundance in the *rpoN*-deleted strain compared with the parental strain H78 (Fig. S5b, c). In addition, the *psl* operon, which is responsible for exopolysaccharide biosynthesis, was similarly upregulated by the *rpoN* mutation in *P. protegens* H78 (Fig. S5d).

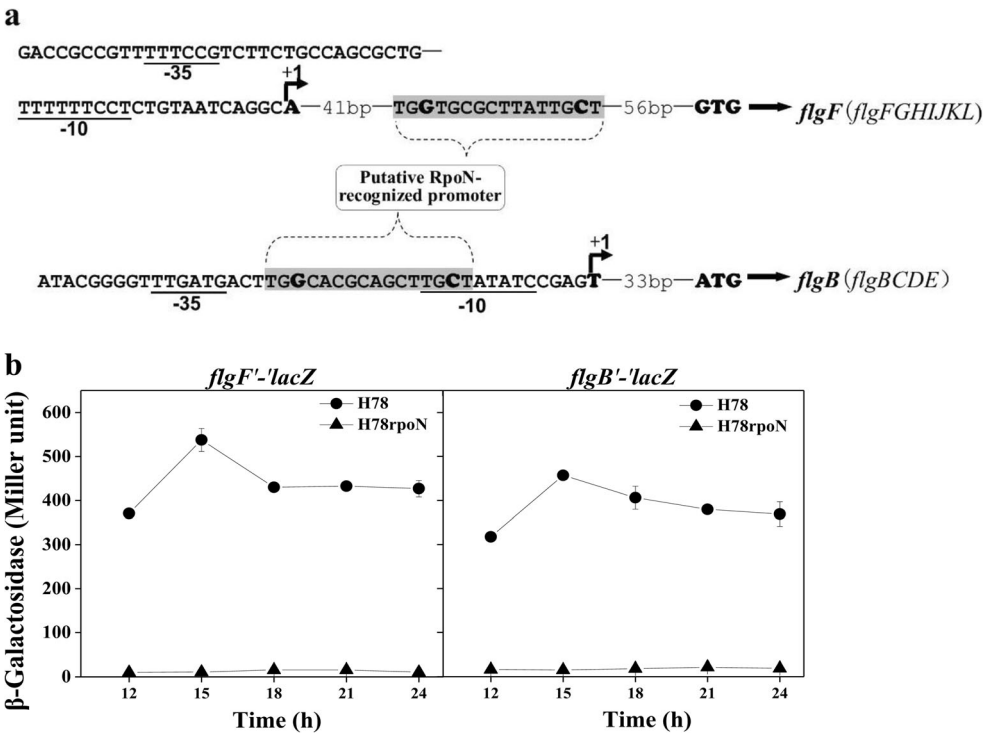

Fig. 4 Activation of the expression of operons involved in flagella biogenesis and assembly by RpoN. **a** Identification of the putative RpoN-recognized promoters in two flagellar operons including *flgFGHIJKL* and *flgBCDE*. The -10 and -35 elements of two predicted RpoD-recognized promoters were marked with underlines. The putative transcriptional start sites and the translational start codons were shown with thickened bases. **b** The β-galactosidase expression originating from two *lacZ* reporter vectors, p6015-*flgF'-'lacZ* and p6015-*flgB'-'lacZ*, was compared between *P. protegens* H78 and H78rpoN in KMB medium

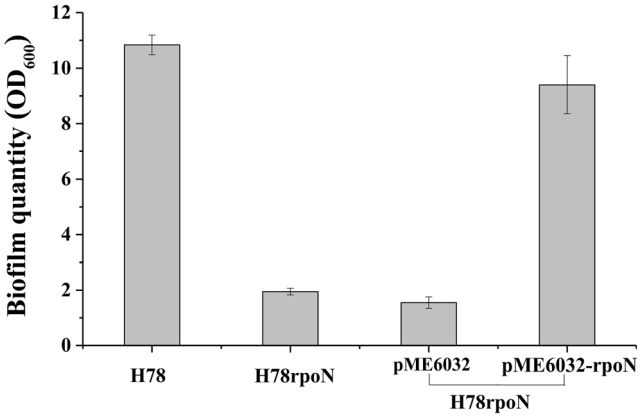

Fig. 5 Activation of the biofilm formation ability of *P. protegens* H78 by RpoN. The quantity of biofilm formed by four strains, including *P. protegens* H78, H78rpoN, and H78rpoN carrying the empty vector pME6032 or the *rpoN* expression vector pME6032-rpoN, was assessed in LB media at 48 h

Positive control of the utilization of carbon sources, including mannitol and inositol, by RpoN in *P. protegens* H78

The transcriptomic results (Fig. 6a, b) suggested that the *rpoN* deletion induced a strong downregulation of the expression of two operons, *mtl* and *iol*, which are involved in mannitol (Brunker et al. 1998) and inositol utilization (Kroger and Fuchs 2009), respectively, in *P. protegens* H78. This finding led us to further evaluate the effect of RpoN on the ability of *P. protegens* H78 to utilize mannitol and inositol as carbon sources. As the negative control, H78 and its derivative strains did not exhibit any growth in the minimal medium without the addition of any carbon source. As shown in Fig. 6c, *P. protegens* H78 was able to grow on the minimal media with mannitol or inositol as sole carbon source. However, a serious growth defect occurred in the *rpoN* mutant in the minimal medium supplemented with 5 g l^{-1} mannitol or 4 g l^{-1} inositol. Unexpectedly, the introduction of the empty vector pME6032 caused a further inhibition in the growth of the *rpoN* mutant. Nevertheless, when compared with the *rpoN* mutant carrying the empty vector pME6032, the *rpoN* mutant carrying the *rpoN* expression vector (pME6032-rpoN) was greatly improved in growth in both media. It is shown that the growth inhibition of the *rpoN* mutant can be reversed by the exogenous expression of *rpoN* gene (Fig. 6c). The above results clearly indicated that RpoN positively regulates the ability of *P. protegens* H78 to utilize mannitol or inositol as a carbon source.

Discussion

The alternative sigma factor RpoN, which was originally identified as a regulator involved in nitrogen metabolism under nitrogen-limiting conditions, has been increasingly reported as an important global regulator of various bacterial cell activities (Hao et al. 2013; Jones et al. 2007). This study revealed that RpoN plays a global regulator role in flagellar biogenesis and assembly, motility, biofilm formation, antibiotic biosynthesis, carbohydrate utilization, and secretion systems (Fig. 7).

In *P. protegens* H78, RpoN is involved in many common functions among various bacteria. That is, RpoN globally activates the expression of gene clusters involved in flagellar biogenesis, motility, and biofilm formation (Fazli et al. 2017; Hao et al. 2013; Jones et al. 2007; Kawagishi et al. 1997; O'Toole et al. 1997; Totten et al. 1990). In *P. aeruginosa*, RpoN, together with external regulators and flagellar regulators including FleQ, FleS, FleR, and FliA, constitutes a four-tiered (class I–IV) regulatory hierarchy to coordinate the transcript level of flagellar regulon. Classes I (*fleQ*), II (*flhA*, *flgA*, *flhF-fleN*, *fliEFGHIJ*, and *fliLMNOPQRflhB*), III (*flgBCDE*, *flgFGHIJKL*, and *fliK*), and IV flagellar genes (*fliCfleL* and *flgMN*) are transcriptionally activated by the external regulators, FleQ and RpoN, FleR and RpoN, and FliA (σ^{28}), respectively (Dasgupta et al. 2003). In H78, most of the flagellar genes are activated by RpoN directly or indirectly through internal regulators (Table S5 and Fig. 4). For example, two genes of the FleS/FleR TCS individually showed 12.8- and 2.4-fold decrease at the transcriptional expression in the *rpoN* deletion strain compared with the wild-type H78 (Table S5). The FleSR TCS, which itself belongs to the class II flagellar regulon, is responsible for the activation of class III genes in cooperation with RpoN (Dasgupta et al. 2003). Flagella-driven motility and relevant biofilm formation activities are naturally activated by RpoN.

Another category of the RpoN regulon that is of interest for us is antibiotic biosynthesis. This study indicates that RpoN exerts a significant positive control over Plt biosynthesis in *P. protegens* H78. In contrast, the *rpoN* mutation induces a notable upregulation on the expression of the Prn biosynthetic operon *prnABCD* in H78. A potential balance between Plt and Prn may be coordinated by RpoN. The *phl* gene cluster, which is responsible for DAPG biosynthesis, was not influenced by RpoN in H78. However, in *P. protegens* CHA0, RpoN exerts both significant positive regulation of Plt biosynthesis and negative regulation of DAPG biosynthesis and thus results in an overall balance between Plt and DAPG. The regulation of *prn* operon expression and Prn biosynthesis by RpoN was not reported in CHA0 (Pechy-Tarr et al. 2005). It can be concluded that the RpoN-mediated regulation of antibiotic biosynthesis displays a certain level of strain specificity in *Pseudomonas*.

However, to date, the potential regulatory pathway and mechanism of RpoN in antibiotic biosynthesis remain unknown in *P. protegens*. Based on the fact that the putative -12/-24 motif within the *pltR* promoter was confirmed not to be a true RpoN-recognized promoter, we primarily excluded the possibility that RpoN directly regulates the Plt biosynthetic

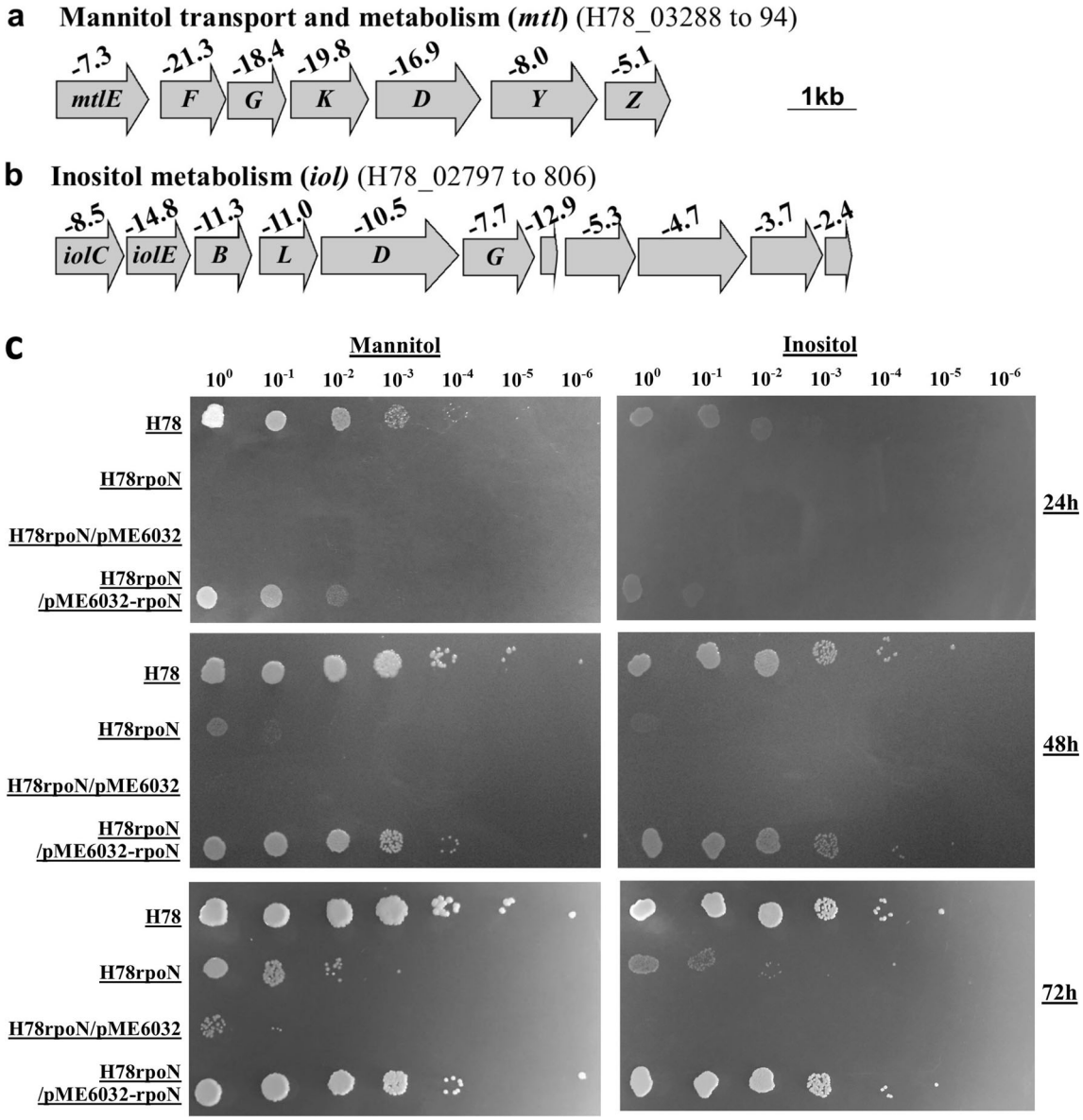

Fig. 6 Activation of the ability of *P. protegens* H78 to utilize two carbon sources, including mannitol and inositol, by RpoN. **a**, **b** Fold changes in the transcript levels of the mannitol (*mtl*) and inositol (*iol*) utilization operons in the H78rpoN mutant relative to the H78 strain are shown above the arrows. **c** The cell growth of four strains, including *P. protegens* H78, H78rpoN, H78rpoN harboring the empty vector pME6032, or the *rpoN* expression vector pME6032-rpoN, was assayed in the minimal medium plate supplemented with 5 g l^{-1} mannitol or 4 g l^{-1} inositol at 28 °C. The collected and washed cells from overnight culture were first diluted to the same start density (OD_{600} = 0.05) and then serially diluted by 1/10. Two-microliter drops was inoculated on the tested plates. Cell growth was periodically observed and photographed at 24 h, 48 h, and 72 h

operons *pltRM* and *pltLABCDEFG*. However, the transcriptomic data revealed that two *csrA*/*rsmA* family members, *rsmA* and *rsmE*, which are involved in the regulation on the biosynthesis of Plt and other antibiotics (Wang et al. 2017), were upregulated 2.2- and 4.0-fold, respectively, at the transcriptional level by *rpoN* deletion (Table S4). Similarly, the sensor RetS, which antagonizes the sensor GacS of the GscS/GacA TCS, was significantly upregulated 3.1-fold in the *rpoN*-deleted strain relative to the parent H78 strain (Table S4). The Gac/Rsm cascade, which is composed of the TCS GacS/A, the RsmX/Y/Z sRNAs, and the RNA-binding proteins RsmA/E, is involved in global regulation of secondary metabolism including antibiotic biosynthesis (Haas and Keel 2003; Wang et al. 2017). However, further work is needed to ascertain whether these global regulators, including RsmA, RsmE, and RetS, mediate the control of antibiotic synthesis by RpoN in H78.

Our RNA-seq data show that RpoN negatively regulates the expression of gene clusters involved in type VI secretion systems (T6SSs), Flp/Tad-T4b piliation, Csu-T1 piliation, and

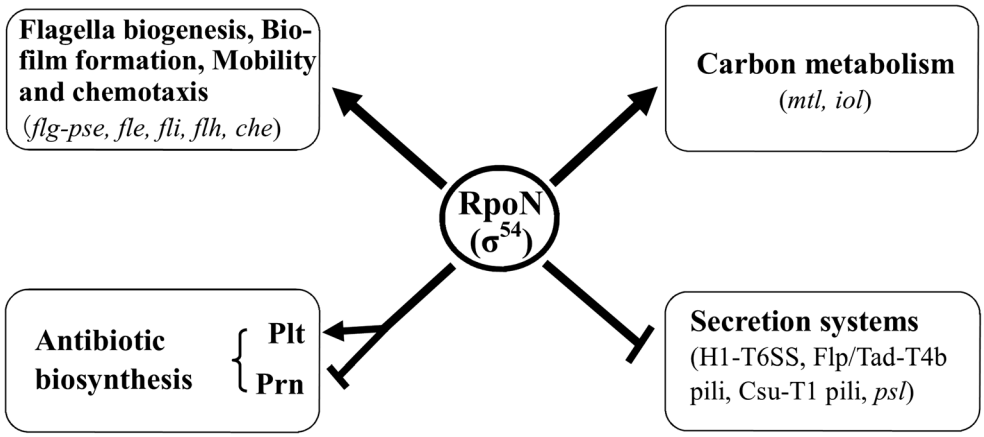

Fig. 7 A model summarizing the global regulatory effects of RpoN in *P. protegens* H78. Bold arrows, positive control; bold lines with flatted end, negative control. *flg*, *fle*, *fli*, and *flh,* the flagellar biosynthesis and assembly operons; *pse*, the pseudaminic acid biosynthesis operon; *che*, the chemotaxis operon; *plt*, the pyoluteorin biosynthetic operon; prn, the pyrronitrin biosynthetic operon; *mtl*, the mannitol transport and utilization operon; *iol*, the inositol transport and utilization operon; H1-T6SS, the operons (H78_06284 to H78_06304) encoding the type VI secretion system that is homologous to H1-T6SS of *P. aeruginosa*; Flp/Tad-T4b pili, the *tad* and *cpa* operons for type IV Flp/Tad pilin biogenesis; Csu-T1 pili, the *cupB1-B6* operon for type I pilus biogenesis; and *psl*, the polysaccharide biosynthesis and export operon

exopolysaccharide biosynthesis in *P. protegens* H78 (Table S4, Fig. S5). *P. protegens* has only one T6SS, H1-T6SS. However, *P. aeruginosa* possesses three T6SSs, H1- to H3-T6SS (Wei et al. 2013). In *P. aeruginosa* PAO1, these T6SSs were divergently regulated by RpoN. RpoN activates the expression of H3-T6SS left (one of two putative H3-T6SS) and represses the expression of H2-T6SS and H3-T6SS right. The expression of H1-T6SS is not influenced by RpoN (Sana et al. 2013). Interestingly, in *Vibrio cholera* carrying only one T6SS, RpoN positively controls the expression of two genes encoding T6SS-secreted proteins (*hcp* and *vgrG3*) but does not influence expression of the main T6SS gene cluster (Dong and Mekalanos 2012). In H78, Flp/Tad-T4b and Csu-T1 piliation were negatively regulated by RpoN. In contrast, an early paper reported that RpoN is required for pilin formation in *P. aeruginosa* (Ishimoto and Lory 1989). In addition, the *psl* operon involved in extracellular polysaccharide biosynthesis is under the negative control of RpoN in *P. protegens* H78 (Table S4, Fig. S5). However, one previous study showed that exopolysaccharide biosynthesis is not regulated by RpoN in a floc-forming *Aquincola tertiaricarbonis* strain (Yu et al. 2017). To summarize, except for RpoN-activated flagellar biogenesis and flagella-driven motility, other RpoN-mediated regulatory phenotypes vary greatly among different strains.

Generally, RpoN exerts direct regulation by recognizing the -12/-24 conserved element with the assistance of an enhancer protein that binds upstream of the -12/-24 element and is specific for each regulon (Potvin et al. 2008; Taylor et al. 1996). In rare cases, such enhancers may be not required for the RpoN-dependent promoters. For example, the transcription of *Pseudomonas* sp. *atzR* (the LysR-type transcriptional activator of the *atzDEF* operon) is directed by the RpoN-dependent promoter, which is a minimum 27 bp 12/-24 core box lacking the upstream enhancer-binding activation sequence (Porrua et al. 2009). The global and pathway-specific regulators that are directly regulated by RpoN may mediate the global control of RpoN. The abovementioned RpoN-driven regulatory cascade of flagellar biogenesis is typical of such a regulatory mode (Dasgupta et al. 2003). In addition, direct activation or antagonism among alterative sigma factors is also an important regulatory mode. In this study, our RNA-seq data indicate that the *rpoN* deletion resulted in a 2.0-fold downregulation of *rpoH* (σ^{32}) and a 2.4-fold upregulation of *algU* (*rpoE* or σ^{22}) (Table S3 and S4). It has previously been implied that the *rpoH* gene, which carries an upstream sequence that perfectly matches the RpoN-recognized conserved box, may be under the direct control of RpoN (Pallen 1999). Similarly, RpoS is directly controlled by RpoN (Smith et al. 2007). RpoN and RpoS (σ^{38}) were shown to antagonistically control motility and transcriptome in *E. coli* (Dong et al. 2011).

The transcriptomic results showed that a similar number of genes is upregulated (562 genes) or downregulated (502 genes) in the *rpoN* mutant of *P. protegens* H78. However, it should be mentioned that RpoN can activate or upregulate the expression of some operons without the RpoN-recognized promoters, such as *pltL-G* and *pltR*. Similarly, the RpoN-recognized promoters were also not identified upstream of the mannitol and inositol utilization operons (*mtlEFGKDYZ* and *iolCEBLDG*), which can be activated by RpoN. In contrast, the other antibiotic operon, *prnABCD*, which was not found to contain the RpoN-recognition sites, is under the negative control of RpoN. We can hypothesize that the RpoN regulon without the RpoN-dependent promoter might be indirectly regulated through the RpoN-controlled global or pathway-specific regulators. In addition, more than half of the RpoN regulon were shown to be significantly downregulated by RpoN in H78. The RpoN-mediated negative

regulatory mechanisms might be involved in many aspects, including the antagonism between RpoN and other sigma factors such as RpoD, the absence of enhancer-binding proteins, the conservation degree of the RpoN-recognized sequences, and the indirect regulation (Leang et al. 2009).

In summary, this study provides the first RpoN-mediated transcriptomic model of *P. protegens*. It demonstrated that the RNA polymerase sigma factor RpoN plays a global regulatory role in flagellar biogenesis and assembly, biofilm formation, antibiotic biosynthesis, carbohydrate utilization, T6SS, pilin formation, and exopolysaccharide biosynthesis in *P. protegens* H78. In the future, we are interested in exploring how RpoN is incorporated into the Gac/Rsm global regulatory network (GacS/GacA-RsmXYZ-RsmAE) of antibiotic biosynthesis by three regulators, including RsmA, RsmE, and RetS. Clarification of the molecular regulatory mechanisms of RpoN in antibiotic biosynthesis will help to improve antibiotic production by genetic and metabolic engineering.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants by any of the authors.

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