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GENOMICS, TRANSCRIPTOMICS, PROTEOMICS

The (p)ppGpp-mediated stringent response regulatory system globally inhibits primary metabolism and activates secondary metabolism in *Pseudomonas protegens* H78

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Abstract

Pseudomonas protegens H78 produces multiple secondary metabolites, including antibiotics and iron carriers. The guanosine pentaphosphate or tetraphosphate ((p)ppGpp)-mediated stringent response is utilized by bacteria to survive during nutritional starvation and other stresses. RelA/SpoT homologues are responsible for the biosynthesis and degradation of the alarmone (p)ppGpp. Here, we investigated the global effect of *relA/spoT* dual deletion on the transcriptomic profiles, physiology, and metabolism of *P. protegens* H78 grown to mid- to late log phase. Transcriptomic profiling revealed that *relA/spoT* deletion globally upregulated the expression of genes involved in DNA replication, transcription, and translation; amino acid metabolism; carbohydrate and energy metabolism; ion transport and metabolism; and secretion systems. Bacterial growth was partially increased, while the cell survival rate was significantly reduced by *relA/spoT* deletion in H78. The utilization of some nutritional elements (C, P, S, and N) was downregulated due to *relA/spoT* deletion. In contrast, *relA/spoT* mutation globally inhibited the expression of secondary metabolic gene clusters (*plt, phl*, *prn*, *ofa, fit*, *pch*, *pvd*, and *has*). Correspondingly, antibiotic and iron carrier biosynthesis, iron utilization, and antibiotic resistance were significantly downregulated by the *relA/spoT* mutation. This work highlights that the (p)ppGpp-mediated stringent response regulatory system plays an important role in inhibiting primary metabolism and activating secondary metabolism in *P. protegens*.

Keywords *Pseudomonas protegens* · RelA/SpoT · (p)ppGpp-mediated stringent response system · Transcriptome · Primary metabolism · Secondary metabolism

Introduction

Pseudomonas protegens H78, which was isolated from the rape rhizosphere, can produce numerous antibiotics and siderophores, including pyoluteorin (Plt), 2,4-diacetylphloroglucinol (DAPG), pyrrolnitrin (Prn), orfamide, *Pseudomonas fluorescens* insecticidal toxin (Fit), hydrogen cyanide (HCN), pyoverdine, and pyochelin (Huang et al. 2017; Liu et al. 2018; Wang et al. 2017). Genomic analysis suggested that *P. protegens* H78 (GenBank No. CP013184) is highly homologous with *P. protegens* Pf-5 (Paulsen et al. 2005), CHA0 (Jousset et al. 2014), and Cab57 (Takeuchi et al. 2014). *P. protegens* species can produce a specific profile of antibiotics, including Plt, DAPG, and Prn (Huang et al. 2017; Jousset et al. 2014; Paulsen et al. 2005; Takeuchi et al. 2014). The Plt biosynthetic gene cluster has two types of evolutionary origins: the *P. protegens* type (Huang et al. 2017; Jousset et al. 2014; Paulsen et al. 2005; Takeuchi et al. 2014) and *Pseudomonas aeruginosa* type (M18 and LESB) (Winstanley et al. 2009; Wu et al. 2011). These two origins of *plt* clusters are identical in gene organization but moderately different in nucleotide and amino acid sequences. However, they are distinct from each other in the *pltR-pltL* noncoding sequences, aside from the *lys* box (Paulsen et al. 2005; Wang et al. 2017; Wu et al. 2011). Orfamide is a *Pseudomonas-*

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produced cyclic lipopeptide with biosurfactant, antimicrobial, and insecticidal activities (Gross et al. 2007; Loper et al. 2016). Fit is a large protein toxin biosynthesized by the *fitABCDEFGH* gene cluster in *P. protegens* CHA0 (Pechy-Tarr et al. 2008). In nature, these secondary metabolites contribute to the ecological and nutritional competition abilities of *Pseudomonas* spp.

Secondary metabolism, including antibiotic biosynthesis, is subject to the regulation of multiple global and pathway-specific regulatory systems or factors. Classically, the Gac/Rsm signal transduction regulatory system, which is sequentially composed of the two-component system (TCS) GacS/GacA, the RsmY family sRNAs, and the RsmA (Rsm, regulator of secondary metabolism) family RNA-binding proteins, plays a crucial role in the global activation of secondary metabolism in *Pseudomonas* spp. (Haas and Keel 2003). Gac/Rsm-driven global regulation is generally mediated by pathway-specific regulators, such as the LysR-type transcriptional activator PltR of the Plt biosynthetic operon (Huang et al. 2008). However, the environmental signal that stimulates the phosphorylation of the sensor kinase GacS remains unidentified. Such signalling factors or systems constitute another important array of regulators involved in the biocontrol or virulence of *Pseudomonas* spp. (Jimenez et al. 2012). A single mutation of any one of the three quorum sensing (QS) systems, including LasI/LasR, RhlI/RhlR, and Pqs/PqsR, causes an enormous increase in Plt biosynthesis in the rhizobacterium *P. aeruginosa* M18 (Chen et al. 2008; Lu et al. 2009; Yan et al. 2007). Las and Rhl belong to the LuxI/LuxR family QS system (Chen et al. 2008; Yan et al. 2007). Another Lux-type regulatory system, PhzI/PhzR, activates *phz* operon expression and phenazine biosynthesis in *P. chlororaphis* (Chancey et al. 1999). Moreover, many nucleotide second messengers, including c-di-GMP, c-di-AMP, cGMP, cAMP, and guanosine pentaphosphate or tetraphosphate ((p)ppGpp), are involved in the global regulation of bacterial cell biology (Jimenez et al. 2012).

(p)ppGpp is the modulator of the stringent response of bacteria to cope with nutritional starvation and other stresses. The RelA/SpoT homologue (RSH) enzymes are responsible for (p)ppGpp biosynthesis and hydrolysis. Two RSH enzymes, RelA and SpoT, exist in *Escherichia coli* and other β- or γ-proteobacteria (Hauryliuk et al. 2015; Steinchen and Bange 2016). The RelA protein has only synthetase activity and responds to amino acid and fatty acid starvation. SpoT is a bifunctional enzyme with both synthetase and hydrolase activities that is crucial for (p)ppGpp homeostasis. SpoT poorly responds to various stresses, such as fatty acids, carbon sources, phosphate or iron starvation, diauxic shifts, oxidative stress, and hyperosmotic shock (Hauryliuk et al. 2015; Sinha et al. 2019; Steinchen and Bange 2016).

An increasing number of studies have shown that (p)ppGpp globally regulates various aspects of bacterial physiology and metabolism: DNA replication, cell division, DNA transcription, growth, survival, adaption, competence, secondary metabolism, cell motility, biofilm formation, and pathogenicity. Bacterial RNA polymerase (RNAP) has been confirmed to be the first direct target of (p)ppGpp. rRNA transcription and ribosome biogenesis in starving bacteria are thus shut down to conserve energy and survive in adverse conditions (Atkinson et al. 2011; Dalebroux and Swanson 2012; Hauryliuk et al. 2015; Potrykus and Cashel 2008; Steinchen and Bange 2016).

As in *E. coli*, (p)ppGpp biosynthesis is also catalysed by RelA and SpoT in *Pseudomonas* spp. The (p)ppGpp-mediated stringent response is involved in regulating virulence or biocontrol in *Pseudomonas* spp. (Chatnaparat et al. 2015; Manuel et al. 2011, 2012). (p)ppGpp functions as a global signal for activating the virulence of *Pseudomonas syringae* (Chatnaparat et al. 2015). Moreover, in *P. protegens* CHA0, (p)ppGpp is required for sustaining biocontrol ability (Takeuchi et al. 2012). The biosynthesis of antibiotics, including DAPG and Plt, is diminished in the *relA/spoT* double mutant, in which (p)ppGpp biosynthesis is entirely abolished. The *relA/spoT* double mutation significantly decreases the expression levels of RsmXYZ sRNAs and the *gacS/gacA* TCS in CHA0; this effect correlates the (p)ppGpp-mediated stringent response with the Gac/Rsm cascade (Takeuchi et al. 2012). However, in two other *Pseudomonas* strains, the (p)ppGpp-mediated stringent response represses the antifungal activity mediated by the biosynthesis of antibiotics and extracellular enzymes (Manuel et al. 2011, 2012). The *relA/spoT* mutant of *P. chlororaphis* PA23 shows increased production of Prn, lipase, and protease, but phenazine production is unchanged (Manuel et al. 2012). Similarly, in *Pseudomonas* sp. strain DF41, the *relA/spoT* mutation significantly enhances lipopeptide production and protease activity, thereby contributing to the biocontrol ability of the DF41 strain (Manuel et al. 2011). Therefore, (p)ppGpp-mediated regulation shows a certain level of specificity among various strains. Moreover, no genome-wide transcriptomic or proteomic profile has been reported for stringent response mutants in *Pseudomonas* spp.

In this study, we compared the transcriptomic profile of the *relA/spoT* mutant with that of the wild-type strain H78 using RNA sequencing (RNA-seq) after growing the strains to mid- to late log phase (optical density (OD_{600}) = 5.0–6.0) in King's medium B (KMB) at 28 °C. During this period, *P. protegens* H78 was still at the stage of rapid growth in the KMB medium with glycerol as carbon source and then it can grow to a maximum OD_{600} of 14 or above. In addition, systematic phenotype analysis was conducted to assess the diversity and intensity of *relA/spoT*-mediated regulatory effects on the physiology and metabolism of *P. protegens* H78. The (p)ppGpp-mediated stringent response extensively inhibited primary metabolism, including DNA replication; bacterial growth; core transcriptional and translational components; amino acid transport

and metabolism; carbohydrate and energy metabolism; and transport and utilization of phosphate, sulfate, and nitrogen. However, the stringent response plays a crucial role in maintaining cell survival and globally activating secondary metabolism, including the biosynthesis of antibiotics, iron carriers, and polysaccharides.

Materials and methods

Bacterial strains, plasmids, primers, and culture conditions

Bacterial strains, plasmids, and primers are listed in Supplemental Tables S1 and S2. *E. coli* was conventionally cultivated in Luria-Bertani (LB) medium 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 its derivative strains were grown at 28 °C in King's medium B (KMB) (King et al. 1954), which is composed of 20 g tryptone, 15 ml glycerol, 0.514 g $K_2HPO_4{\cdot}3H_2O$, and 0.732 g $MgSO_4$ per litre. Kanamycin and tetracycline were used at working concentrations of 50 and 30 μg ml^{-1} for *P. protegens* H78 and 50 and 15 μg ml^{-1} for *E. coli*, respectively. In addition, 4 mg l^{-1} ortho-nitrophenyl-β-D-galactopyranoside (ONPG) in phosphate buffer (pH 7.0) was used to quantify β-galactosidase activity.

DNA manipulation

DNA cloning and recombination were performed following standard methods (Sambrook and Russell 2001). KOD (Toyobo, Osaka, Japan), LA *Taq*, *Taq* DNA polymerases (TaKaRa, Kusatsu, Japan), DNA restriction enzymes (NEB, Ipswich, MA), DNA ligase (TaKaRa, Kusatsu, Japan), DNA markers (MBI Fermentas affiliated to Thermo Scientific, Waltham, MA), and other related enzymes were used according to the instructions for the reagents. Genomic DNA from *P. protegens* H78, plasmid DNA from *Pseudomonas* or *E. coli*, and DNA fragments from the gel were purified using the corresponding kits from TaKaRa (Kusatsu, Japan). DNA synthesis and sequencing were performed by Sangon (Shanghai, China) or HuaDa Corporation (Shenzhen, China).

In-frame deletion and heterogeneous complementation of *relA* and *spoT* in *P. protegens* H78

In vitro in-frame knockout and in vivo homologous recombination were utilized to delete *relA* and *spoT* in the *P. protegens* H78 genome according to the construct map displayed in Supplemental Fig. S1. Two flanking fragments (553 and 519 bp) of the *relA* ORF were individually amplified using the KOD Plus DNA polymerase with two pairs of primers, *relA*-F1/*relA*-R1 and *relA*-F2/*relA*-R2 (Supplemental Table S2). The respective 3′ and 5′ ends of upstream and downstream fragments share a 15-bp complementary region. The upstream and downstream fragments were mixed as templates in the subsequent fusion PCR with the primers *relA*-F1 and *relA*-R2. The fusion PCR product was inserted into the *Kpn*I-*Hin*dIII sites of the suicide vector pK18mobsacB. Afterward, the resultant vector, pK18-relA, was transferred from *E. coli* S17 into *P. protegens* H78 via biparental hybridization. The single-crossover mutant was first screened on an LB plate with 50 μg ml^{-1} kanamycin (Km). Then, the double-crossover mutant, which is sensitive to Km and resistant to sucrose, was screened in parallel between an antibiotic-free plate containing 15% sucrose and a kanamycin-containing plate. The resultant *relA*-deleted mutant was further confirmed using PCR and sequencing. Similarly, the *spoT* gene was deleted in the *relA* mutant background (Supplemental Fig. S1; Supplemental Table S1). The transcriptomic results further confirmed that *relA* and *spoT* have been successfully deleted in the *relA/spoT* mutant. The transcripts of these two genes were not detected in the *relA/spoT* mutant. However, the transcripts of *relA* and *spoT* have mean FPKM values of 1980.0 and 3249.6, respectively, in wild-type H78 (Supplemental Table S3).

relA (H78_04755) and *spoT* (H78_06273) encode the (p)ppGpp synthetase and the bifunctional (p)ppGpp synthetase/hydrolase, respectively. A fragment (2244 bp) that carries the entire ORF and upstream promoter/operator region of the *relA* gene was amplified and cloned into the *Kpn*I/*Sac*I-digested pME6032 vector. The resulting vector, p6032-*relA*, was used to complement the *relA*/*spoT* mutant of *P. protegens* H78.

RNA isolation, RNA-seq, and data analysis

P. protegens H78 and its *relA/spoT* mutant were cultivated to mid- to late log phase (OD_{600} = 5.0–6.0) in KMB at 28 °C for RNA sampling; each strain was repeated three times. RNA sequencing was performed in triplicate for each strain. RNA isolation and sequencing were carried out by Shanghai Bohao Biotechnology Company (Shanghai, China). Total RNA was extracted using the Qiagen (Dusseldorf, Germany) RNeasy Mini Kit (Cat. 4106), quantified by a NanoDrop ND-2000 spectrophotometer (Thermo Scientific, Waltham, MA), and assessed for quality by the Agilent (California, USA) BioAnalyzer 2100. The isolated total RNA was further purified using the Qiagen (Dusseldorf, Germany) RNeasy micro-kit (Cat. 74004) and the Qiagen (Dusseldorf, Germany) RNase-Free DNase Set (Cat. 79254). mRNA was enriched by removing rRNA

and further fragmented to synthesize the first and second cDNA strands for constructing the sequencing library. Cluster generation and de novo sequencing were performed using the Illumina (San Diego, CA) Hiseq 2500.

Sequenced reads were trimmed for adaptor sequences and masked for low-complexity or low-quality sequences by Trimmomatic (v0.30) (Bolger et al. 2014). The quality of the sequenced data was assessed with FastQC (v0.10.1) (Wingett and Andrews 2018). Clean reads were mapped to the *P. protegens* H78 complete genome using Tophat (v2.0.9) (Trapnell et al. 2012). The expression abundance of genes was quantified by Htseq (v0.6.1) using the reads per kilobase per million mapped reads (RPKM) method (Mortazavi et al. 2008). Mean RPKM values were calculated for the *P. protegens* H78 *relA/spoT* mutant and the wild-type strain from their respective repeats. The RNA-seq data normalization and differential gene expression analysis between two groups (*relA/spoT* mutant and wild-type H78) were carried out using the DESeq2 normalization method (Love et al. 2014) on the bioconductor platform. The transcripts with a fold change cutoff of ≥ 2 (P value < 0.05) were considered to be differentially expressed. The differentially expressed genes were further analysed using the COG (Tatusov et al. 2000), GO (Camon et al. 2004) and KEGG pathway (Ogata et al. 1998) databases.

Construction of in-frame *lacZ* fusion plasmids

The *pltL*, *phlA*, *prnA*, *hcnA*, and *pstS* genes are, respectively, the first gene of the Plt biosynthetic operon *pltLABCDEFG*, the DAPG operon *phlACBDE*, the Prn operon *prnABCD*, the HCN operon *hcnABC*, and the phosphate transport operon *pstSCAB*. The in-frame fusions of these genes with *lacZ* were generated by cloning the first several codons and promoter/operator regions of each gene individually into pME6015. In the pME6015 plasmid, the *E. coli lacZ* gene lacks its own promoter/operator and the first seven codons (Supplemental Table S1). Moreover, these five in-frame *lacZ* fusion plasmids were individually introduced into *P. protegens* H78 and its *relA/spoT* mutant to reflect the total regulatory effect of *relA/spoT* on the corresponding operons and validate the transcriptomic data.

Assessment of bacterial growth with optical density and colony forming units

The growth of *P. protegens* H78 and its *relA/spoT* mutant was assayed at 28 °C in KMB, LB, and M9 media. In the M9 medium, 5 g l^{-1} glycerol was added as the sole carbon source. The total biomass (optical density (OD_{600})) and viable cell number (colony forming units (CFU)) of bacterial cultures were measured at different time points.

In this study, the number of viable cells in cultures of *P. protegens* H78 and its derivative strains was assessed using CFU per millilitre. At the selected time points, 1 ml of cell suspension was sampled and diluted by serial 10-fold (10^{-1}) dilutions. Then, 50 μl of sample from each dilution gradient was spread on LB agar plates. After overnight culture, colony counting was carried out on the plate in which a sufficient number of colonies were isolated well enough to ensure reliable counting.

Quantification of antibiotic production and β-galactosidase expression

Fresh overnight cultures of H78 and its derivatives were inoculated into 100 ml KMB (for Plt production) in a 500-ml Erlenmeyer flask, with a final OD_{600} of 0.05, and cultivated at 28 °C and 200 rpm. Plt was extracted using ethyl acetate and DAPG with trifluoroacetic acid (TFA) and ethyl acetate ($v/v = 1{:}9$). HPLC quantification of Plt and DAPG production was performed according to our method (Huang et al. 2004) and that of Zhang et al. (2014), respectively.

P. protegens H78 and its derivatives carrying the in-frame *lacZ* fusion vectors were inoculated and cultured under the same conditions used for antibiotic fermentation. The cultures were sampled at different time points, and β-galactosidase activity was quantified by referring to the Miller method (Sambrook and Russell 2001) and our previous description (Huang et al. 2004).

Agar plate assay for siderophore production

The chrome azurol S (CAS) method (Schwyn and Neilands 1987) was utilized to assess siderophore production as previously described (Wei et al. 2013). Approximately 5 μl of diluted overnight cultures ($OD_{600} = 0.1$) of *P. protegens* H78, the *relA/spoT* mutant and its complemented strain were separately inoculated at the centre of the CAS plate and grown at 28 °C for 56 h. The size of the fluorescent orange zone formed on the CAS plate indicated the siderophore production level.

Assessment of iron, carbohydrate, phosphorus, cysteine, and urea utilization

The iron-utilization capacity of *P. protegens* H78 and its *relA/spoT* mutant was assessed in iron-deficient succinate minimal medium (5.41 g glycerol, 9.13 g K_2HPO_4, 2.99 g KH_2PO_4, 0.92 g $(NH_4)_2SO_4$, 0.06 g $MgSO_4$, 5.4 g sodium succinate, pH 8.0, and 1 l deionized H_2O) (Zolotarev et al. 2008), which was supplemented without or with (i) 100 μM $FeCl_3$, (ii) 3.3 μM haemin and 150 μg ml^{-1} EDDHA (ethylenediamine-di(*o*-hydroxyphenylacetic) acid), or (iii)

3.0 μM haemoglobin and 150 μg ml^{-1} EDDHA (Zhao and Poole 2000).

To evaluate the influence of *relA/spoT* on the carbohydrate utilization of *P. protegens* H78, succinate (3 g l^{-1}), mannitol (5 g l^{-1}), or phenethylamine (1 ml l^{-1}) was chosen as the sole carbon source in the M9 medium (17.17 g $Na_2HPO_4{\cdot}12H_2O$, 2.99 g KH_2PO_4, 0.58 g NaCl, 5.95 g $(NH_4)_2SO_4$, 0.246 g $MgSO_4{\cdot}7H_2O$, 0.01 mg vitamin B1, 4.3 mg $CaCl_2$, 16.7 mg $FeCl_3{\cdot}6H_2O$, 1 mg $MnCl_2{\cdot}4H_2O$, 1.7 mg $ZnCl_2$, 0.43 mg $CuCl_2{\cdot}2H_2O$, 0.6 mg $CoCl_2{\cdot}6H_2O$, 0.6 mg $H_4MoNa_2O_6$, and 1 l H_2O).

For the phosphate utilization assay, 2 mM KH_2PO_4, as the sole phosphate source, was added to the medium (5 g $MgCl_2$, 0.25 g $MgSO_4{\cdot}7\ H_2O$, 0.2 g KCl, 1 g $(NH_4)_2SO_4$, 1.0 g glycerol, and 1 l H_2O, pH 7.0). To assess the ability of *P. protegens* H78 to utilize cysteine as the sole sulphur source, 0.5 mM cysteine was added into the M9 medium supplemented with 5 g l^{-1} glucose. Similarly, the ability of *P. protegens* H78 to utilize urea as the sole nitrogen source was assayed in M9 medium supplemented with 20 mM urea and 5 g l^{-1} glucose and containing no NH_4Cl.

In the above media, overnight cultures of H78 and its *relA/spoT* mutant were inoculated at a final concentration of OD_{600} = 0.03 and cultured at 28 °C and 200 rpm. Bacterial growth was measured over time.

Assay for antibiotic resistance in *P. protegens* H78

Three antibiotics, including gentamicin, kanamycin, and tetracycline, were selected to determine the influence of *relA/spoT* on the antibiotic resistance of *P. protegens* H78. The wild-type strain H78 and its *relA/spoT* mutant were grown in LB medium containing increasing antibiotic concentrations. After 12 h of shaking culture at 28 °C, the OD_{600} and CFU counts were monitored.

Statistical analysis

In this study, every experiment was separately carried out more than three times. In each experiment, at least triplicate parallel samples were included. Each value was described as the average ± SD of three repeats. Two groups of data were compared by Student's *t* test in Excel (Microsoft, Washington, USA). When the *P* value was < 0.05, the results were considered statistically significant.

Accession number of nucleic acid sequences and RNA-seq data

In this study, the DNA sequences of *relA*, *spoT*, and other related genes were extracted from the *P. protegens* H78 complete genome (GenBank accession No. CP013184). The raw reads and analysed data from RNA-seq have been deposited in the Sequence Read Archive database (Accession No. SRP091880) and the Gene Expression Omnibus database (Accession No. GSE89004) in NCBI.

Results

Effects of *relA/spoT* deletion on the transcriptomic profile of *P. protegens* H78

Whole-genome sequencing analysis of *P. protegens* H78 showed that two (p)ppGpp biosynthesis- and hydrolysis-related genes, *relA* and *spoT*, are single-copy genes, and no other homologous genes exist in the H78 genome (GenBank No. CP013184) (Huang et al. 2017). The *relA/spoT* deletion mutant of *P. protegens* H78 was constructed by homologous recombination (Supplemental Fig. S1) and validated by PCR and sequencing. The *relA/spoT* mutant exhibited an almost complete disappearance of the biosynthesis of Plt, an antibiotic we studied extensively. This outcome encouraged us to use RNA-seq to investigate the influence of the RelA/SpoT-mediated stringent response on genome-scale transcriptional expression in *P. protegens* H78. Transcriptomic profiles were compared between H78 and its *relA/spoT* mutant, and the strains were cultured until mid- to late log phase at an OD_{600} of 5.0–6.0 in KMB at 28 °C. The RNA-seq data was validated using quantitative real-time PCR (qRT-PCR) and *lacZ* reporter analyses (Supplemental Fig. S2).

As shown in Supplemental Tables S3 and S4, the transcript abundance of 1463 genes, which accounted for nearly 23% of all annotated genes in the H78 genome, was significantly modified by more than 2-fold in the *relA/spoT* mutant compared with that in the wild-type strain H78. Among the *relA/spoT* regulons, 767 genes were downregulated, and 696 genes were upregulated. The functional categories of the *relA/spoT* regulons were plotted relative to downregulation and upregulation according to PseudoCAP (Fig. 1a). The *relA/spoT*-inhibited genes were involved in DNA replication and cell division, amino acid metabolism, energy and coenzyme metabolism, translational and posttranslational modification, and cell motility. However, the (p)ppGpp-mediated stringent response predominantly activated the expression of genes related to secondary metabolism, cell envelope biogenesis, transcriptional factors, and regulators (Fig. 1a).

Most of the operons or gene clusters under the control of *relA/spoT* are indicated in Fig. 1b. The biosynthetic gene clusters of secondary metabolites, including Plt (*plt*), DAPG (*phl*), Prn (*prn*), pyochelin (*pch*), pyoverdine (*pvd*), haemophore (*has*), Fit (*fit*), and orfamide (*ofa*), were globally upregulated by RelA/SpoT. The negative regulons of *relA/spoT* included numerous primary metabolism-related gene clusters, such as DNA replication- and cell division-related gene clusters

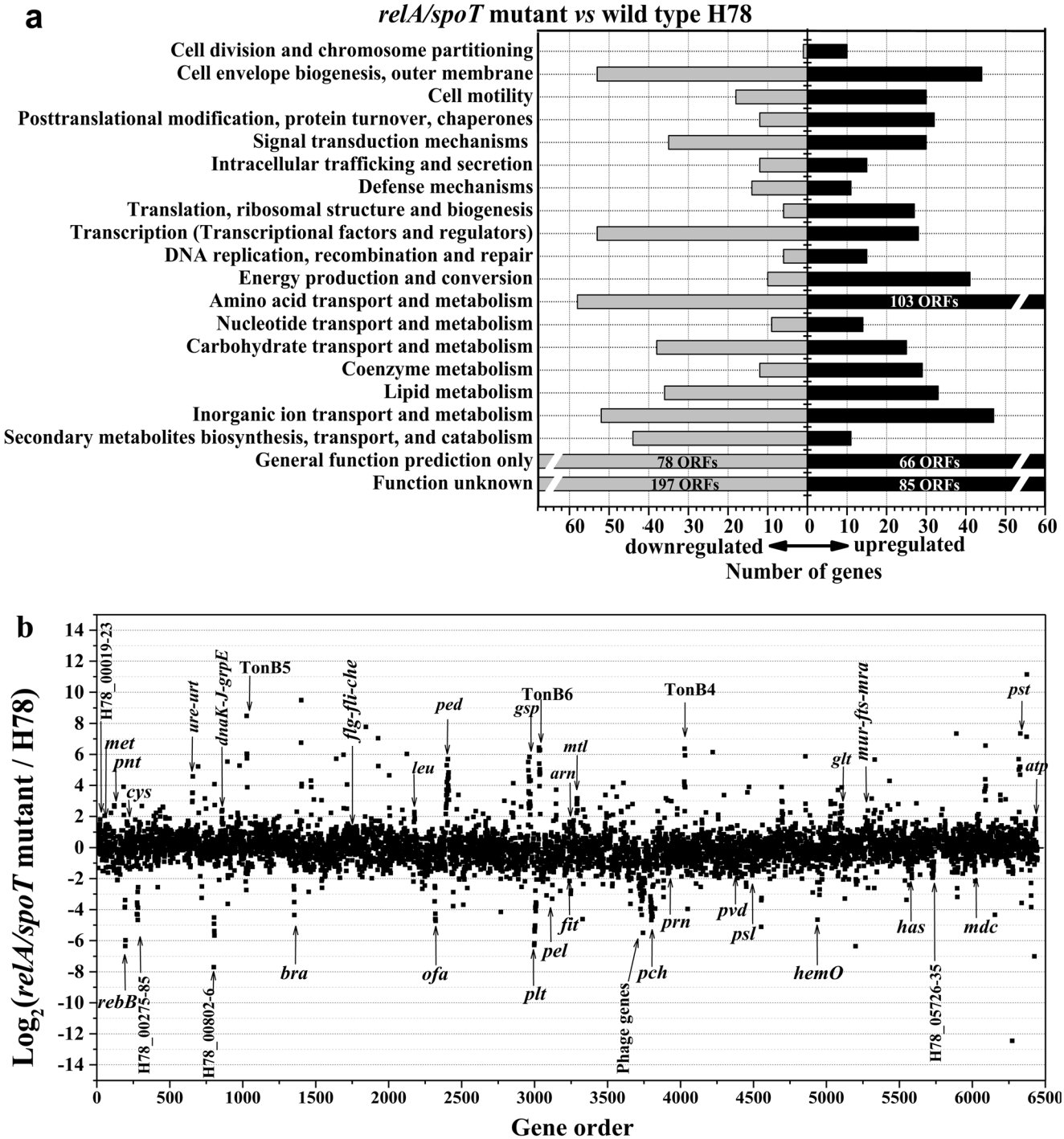

Fig. 1 Transcriptomic profiles of *P. protegens* H78 and its *relA/spoT* mutant. **a** Functional classification of the *relA/spoT* regulon (fold change ≥ 2; *P* value < 0.05). **b** Scatter plot comparing the transcriptomes of *P. protegens* H78 and its *relA/spoT* mutant. The *x*-axis indicates gene number in the H78 genome; the *y*-axis indicates the $\log_2$ fold change of the transcript abundance of each gene in the *relA/spoT* mutant relative to its parental strain H78. Significantly regulated gene clusters with known functions are marked

(*dnaK-dnaJ-grpE* and *mur-fts-mra*); energy and carbohydrate metabolic gene clusters (*pnt*, *atp*, *ped*, and *mtl*); amino acid metabolic gene clusters (*met*, *cys*, *leu*, *arn*, and *glt*); nitrogen, phosphate and sulphur transport and utilization gene clusters (*ure-urt*, *phoR/phoB-pst*, and *cys*); membrane transport and secretion gene clusters (*tonB-exbD-exbB*$_{4-6}$ and *gsp*); and a flagellum and chemotaxis gene cluster (*flg-fli-che*). Therefore, the RelA/SpoT-mediated stringent response mainly inhibits primary metabolism and activates secondary metabolism in *P. protegens* H78.

The *relA/spoT* mutant exhibits upregulated expression of the *dnaK-dnaJ-grpE* and *fts-mur-mra* clusters

Transcriptomic profiling showed that the expression levels of two chaperone systems, DnaK-DnaJ-GrpE and GroEL/ES, which are involved in protein quality control and DNA replication (Calloni et al. 2012; Konieczny and Zylicz 1999), were significantly upregulated in the *relA/spoT* mutant compared with those in the parental strain H78 (Fig. 2a). Another gene cluster involved in cell division and peptidoglycan biosynthesis, *fts-mur-mra*, showed a more than 2-fold increase in mRNA levels in the *relA/spoT* mutant (Fig. 2b). The negative regulatory effect of *relA/spoT* on *fts-mur-mra* expression was further confirmed by qPCR with the method and primers described in Supplemental experimental procedure and Table S2. The results indicated that three selected genes (*ftsQ*, *murF*, and *mraZ*) in this gene cluster were significantly upregulated at the transcriptional level by the *relA/spoT* mutation (Fig. 2c).

The well-known cell division gene *ftsZ* showed a 2.3-fold increase at the transcriptional level in the *relA/spoT* mutant relative to the wild-type H78 (Fig. 2b). The negative targets of *relA/spoT* include other genes that are involved in DNA replication and cell division, such as the DNA gyrase subunit B-encoding gene *gyrB*, the single-stranded DNA-binding protein-encoding gene *ssb*, the HU family gene *hupA*, and the cell division gene *ftsX*. Moreover, the RelA/SpoT-mediated stringent response participates in the regulation of DNA repair. The DNA repair gene *recO* was upregulated by 2.8-fold in the *relA/spoT* mutant. Most significantly, the H78_01844 gene encoding the helicase subunit of the DNA

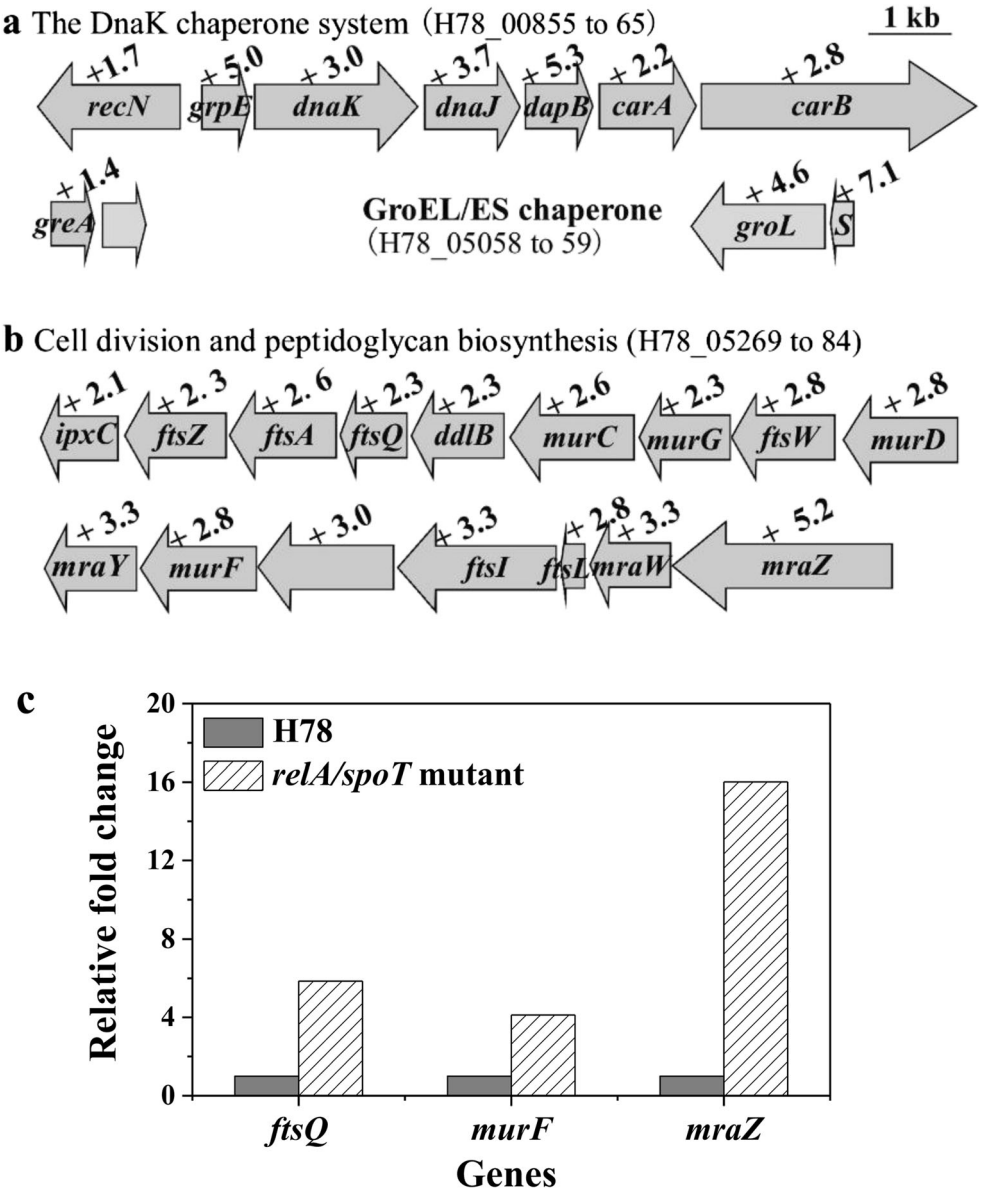

Fig. 2 **a**, **b** Negative regulatory effect of *relA/spoT* on the expression of the *grpE-dnaK-dnaJ* and *groL/groS* chaperone gene clusters (**a**) and the cell division-related *fts-mur-mra* gene cluster (**b**). The fold change of the transcript abundance in the *relA/spoT* mutant relative to wild-type H78 is shown at the top of the arrows. **c** The relative fold change in the mRNA levels (quantified by qPCR) of three genes (*ftsQ*, *murF* and *mraZ*) in the *fts-mur-mra* cluster in the *relA/spoT* mutant relative to the wild-type H78

excision repair complex was dramatically upregulated by 219.1-fold after *relA/spoT* mutation (Supplemental Table S4).

The *relA/spoT* deletion partially increases bacterial growth and significantly reduces the cell survival rate of the H78 strain

The involvement of RelA/SpoT in DNA replication and cell division brings into question about the influence of RelA/SpoT on bacterial growth and cell survival in *P. protegens* H78. The OD_{600} and viable cell number (CFU ml^{-1}) of H78 and its *relA/spoT* mutant were monitored in three media at 28 °C: KMB (the medium used for RNA-seq analysis), LB, and M9. As expected, the growth of the *relA/spoT* mutant was moderately upregulated compared with that of its parental strain H78 in the three media (Fig. 3a, c, and e). However, the number of live cells was significantly reduced in the *relA/spoT* mutant relative to the wild-type strain H78 (Fig. 3b, d, and f). These results suggested that the (p)ppGpp-mediated stringent response plays an important role in reducing bacterial growth and increasing the cell survival rate in *P. protegens* H78.

Negative targets of RelA/SpoT include some core transcriptional and translational elements and are involved in amino acid metabolism

In the transcriptomic profiles of H78 and its *relA/spoT* mutant, *relA/spoT* negatively regulated some general bacterial transcriptional and translational factors. Two genes, *rpoB* and *rpoC*, which respectively encode the core RNAP subunits ß and ß′, were significantly upregulated by 2.2-fold in the *relA/*

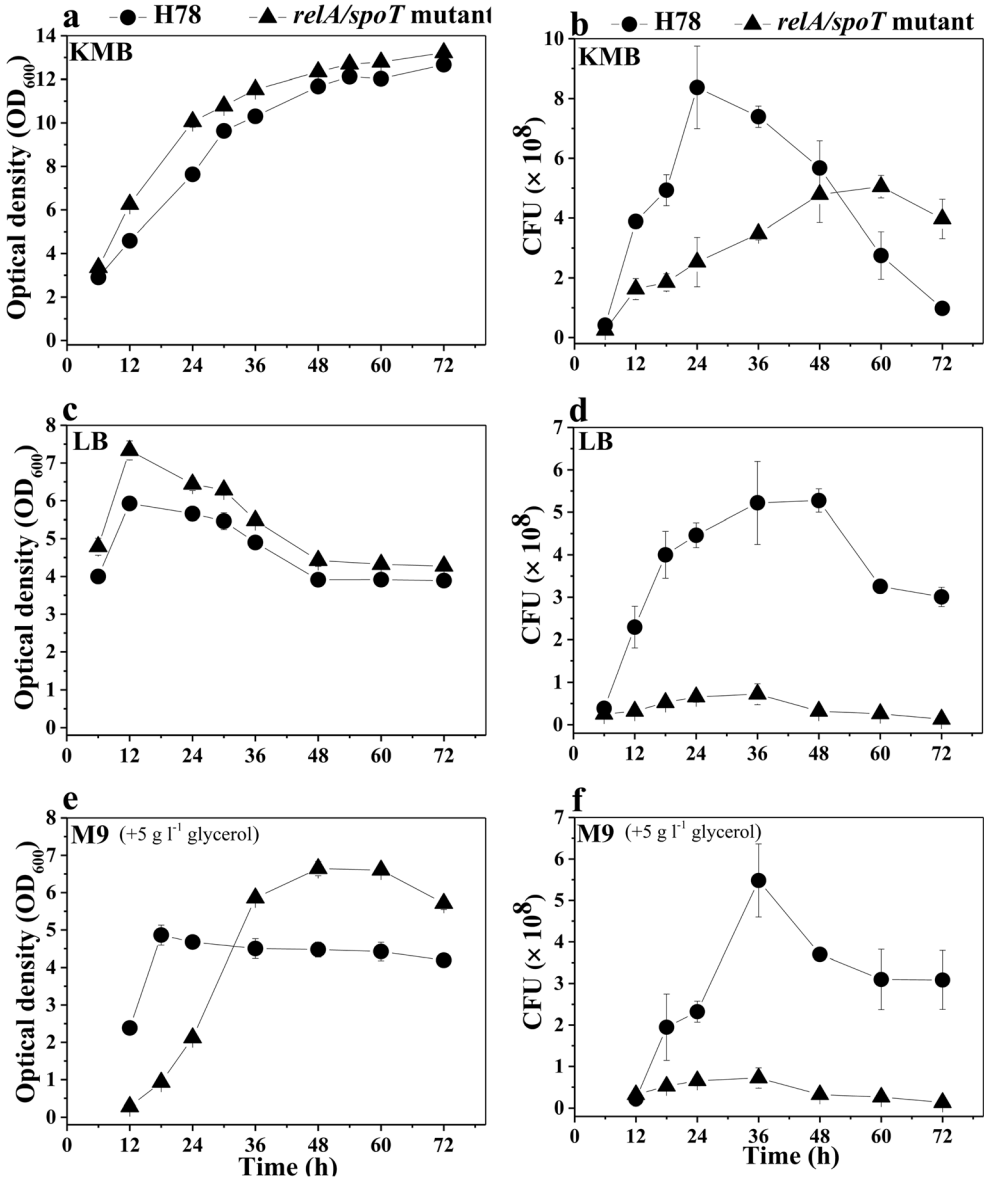

Fig. 3 Influence of *relA/spoT* on bacterial growth and cell survival in *P. protegens* H78. Bacterial growth (optical density (OD_{600})) and viable cell number (colony forming units per millitre (CFU ml^{-1})) of *P. protegens* H78 and its *relA/spoT* mutant, which were inoculated at an OD of 0.03 and were measured at 28 °C in three media: LB (**a**, **b**), KMB (**c**, **d**), and M9 (**e**, **f**). In the M9 medium, 5 g l^{-1} glycerol was added as the sole carbon source

spoT mutant compared with H78. Similarly, *relA/spoT* inhibited the expression of genes encoding general translational factors, such as the 50S ribosomal proteins L17 (H78_05744, *rplQ*), L25 (H78_05366), and L31 (H78_00458, *rpmE*); the 30S ribosomal protein S20 (H78_05530, *rpsT*); 16S rRNA methyltransferases (H78_00022, *rsmB*, and H78_00436, *rsmE*); the translational elongation factors Ts (H78_01218, *tsf*) and TU (H78_05788, *tuf*); an aminoacyl-tRNA hydrolase (H78_05367, *pth*); and a methionyl-tRNA formyltransferase (H78_00023, *fmt*) (Supplemental Table S4). These data further confirmed that the (p)ppGpp-mediated stringent response can regulate DNA replication, transcription, and translation. Among the *relA/spoT* regulons related to amino acid transport and metabolism, 103 genes were significantly upregulated by at least 2-fold in the *relA/spoT* mutant compared with H78 (Fig. 1a). These genes that are negatively regulated by RelA/SpoT were mainly involved in the transport and metabolism of amino acids, including arginine, histidine, glutamate, leucine, methionine, and tryptophan.

The RelA/SpoT-mediated stringent response globally activates antibiotic biosynthesis and gene expression

The results from RNA-seq revealed that the most significant downregulation caused by the *relA/spoT* mutation was observed for the Plt gene cluster. The transcript abundance of all 14 genes in the Plt biosynthesis (*pltLABCDEFG*) and transport operons (*pltIJKNOP*) decreased by 11.7- to 78.2-fold in the *relA/spoT* mutant compared with that in the wild-type H78 strain. The other two regulators, the LysR-type transcriptional activator PltR and the TetR-type transcriptional regulator PltZ, were respectively downregulated by 3.2- and 6.9-fold at the transcript level in the *relA/spoT* mutant (Fig. 4a). Similarly, three gene clusters that are respectively responsible for the biosynthesis of pyrrolnitrin (*prn*), orfamide (*ofa*), and *P. fluorescens* insecticidal toxin (*fit*) were significantly downregulated by more than 2-fold in the *relA/spoT* mutant (Fig. 4c–e). Unexpectedly, no significant influence on the expression of the *phlACBDE* operon involved in DAPG biosynthesis was observed (Fig. 4b). However, expression of the DAPG hydrolase-encoding gene *phlG* and the *tetR* family repressor gene *phlF* was reduced by 20.2- and 2.2-fold, respectively, at the transcript level in the *relA/spoT* mutant compared with the H78 strain (Fig. 4b).

Three in-frame *lacZ* fusions, *pltL'-'lacZ*, *phlA'-'lacZ*, and *prnA'-'lacZ*, were constructed to reflect the total regulatory activities of the *pltLABCDEFG*, *phlACBDE*, and *prnABCD* operons, respectively, at both the transcriptional and posttranscriptional levels. ß-Galactosidase expression from these three *lacZ* fusions in pME6015 was comparatively measured between H78 and its *relA/spoT* mutant grown in KMB at 28 °C. Expression of the *pltL'-'lacZ* and *prnA'-'lacZ* fusions was almost completely inhibited by the *relA/spoT* deletion (Fig. 4f, h). These results suggested that the (p)ppGpp-mediated stringent response globally activates the expression of the *pltLABCDEFG* and *prnABCD* operons. In addition, expression of the *phlA'-'lacZ* fusion was greatly downregulated in the *relA/spoT* mutant compared with that in H78 (Fig. 4g). This result seemed to contradict the above RNA-seq data, wherein the transcript abundance of the *phlACBDE* operon was not significantly influenced by the *relA/spoT* deletion. This outcome might be caused by the indirect regulation of the *phlACBDE* operon by *relA/spoT* at the posttranscriptional level.

Plt and DAPG production in H78 and its derivative strains were measured in KMB at 28 °C, and the results are described in Fig. 4i, j. The *relA/spoT* mutation caused complete inhibition of Plt production in the H78 strain. Moreover, the Plt production of the *relA/spoT* mutant could be recovered and even exceed the wild-type level due to the heterologous expression of the *relA* gene in pME6032. Here, the introduction of the empty plasmid pME6032 as a control caused a small increase in Plt production in the wild-type H78 strain (Fig. 4i). Given the *relA/spoT* mutation, DAPG production was greatly reduced in H78. Moreover, the inhibition of DAPG production in the *relA/spoT* mutant could be reversed by introducing the *relA* gene in pME6032 (Fig. 4j). These results suggested that the (p)ppGpp-mediated stringent response plays a key role in globally activating antibiotic biosynthesis in *P. protegens* H78.

Positive regulatory effects of the RelA/SpoT-mediated stringent response system on siderophore biosynthetic gene expression, siderophore production, and iron utilization

P. protegens H78 can produce two siderophores (pyochelin and pyoverdine) and one haemophore. The transcriptomic profiles of H78 and its *relA/spoT* mutant revealed that the most significant reduction of transcript levels occurred for the pyochelin biosynthetic gene cluster (*pchDHIEFKCBA*) in the *relA/spoT* mutant. The transcript abundance of all genes within the *pch* cluster decreased more than 8-fold in the *relA/spoT* mutant compared with that in the wild-type H78 (Fig. 5a). The biosynthetic gene clusters of pyoverdine (*pvd*) and haemophore (*has*) were moderately downregulated by the *relA/spoT* mutation at the transcript level (Fig. 5b, c). Interestingly, ten pairs of FecI/FecR (sigma factor/regulator) related to iron uptake were positively regulated by *relA/spoT* (Supplemental Table S5).

The positive regulatory effect of *relA/spoT* on the abovementioned iron carrier gene clusters and iron utilization activators implies that iron utilization may be positively controlled by the (p)ppGpp-mediated stringent response system. Siderophore production by *P. protegens* H78, the

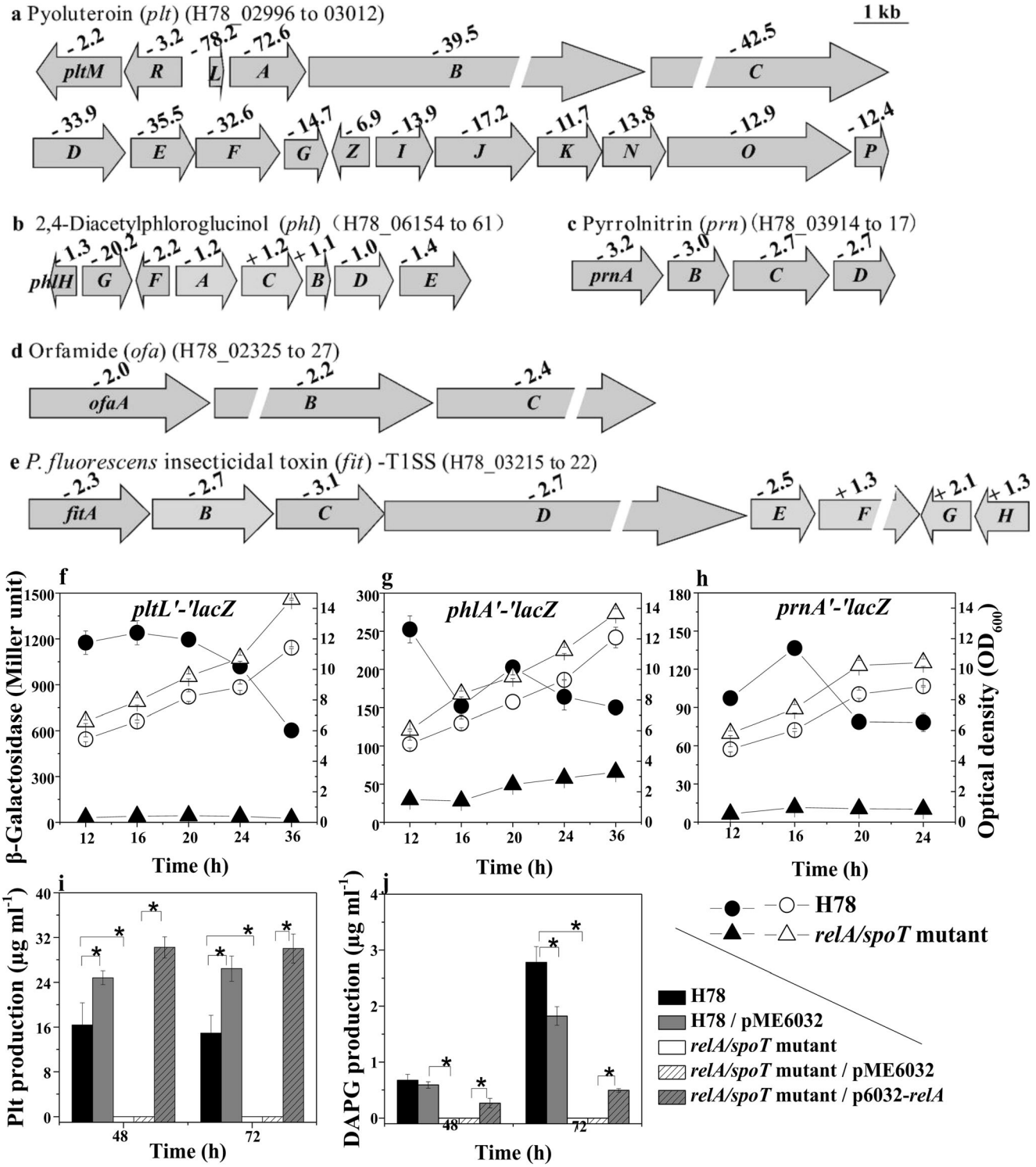

Fig. 4 Positive regulatory effect of RelA/SpoT on antibiotic gene expression and biosynthesis in *P. protegens* H78. **a**–**e** Fold change in the transcript levels of antibiotic biosynthetic gene clusters, including *plt* (**a**), *phl* (**b**), *prn* (**c**), *ofa* (**d**), and *fit* (**e**), in the *relA/spoT* mutant relative to the wild-type H78. **f**–**h** Bacterial growth (open symbols) and β-galactosidase expression (solid symbols) of *P. protegens* H78 and the *relA/spoT* mutant, which carry the *pltL'-'lacZ* (**f**), *phlA'-'lacZ* (**g**), or *prnA'-'lacZ* (**h**) in-frame fusion in pME6015, were assayed in KMB at 28 °C. **i**, **j** Plt (**i**) and DAPG production (**j**) in the H78 strain, the *relA/spoT* mutant and their derivative strains harbouring the empty plasmid pME6032 or the *relA* expression plasmid p6032-*relA* were quantified in KMB at 28 °C. $*P < 0.05$, significant difference

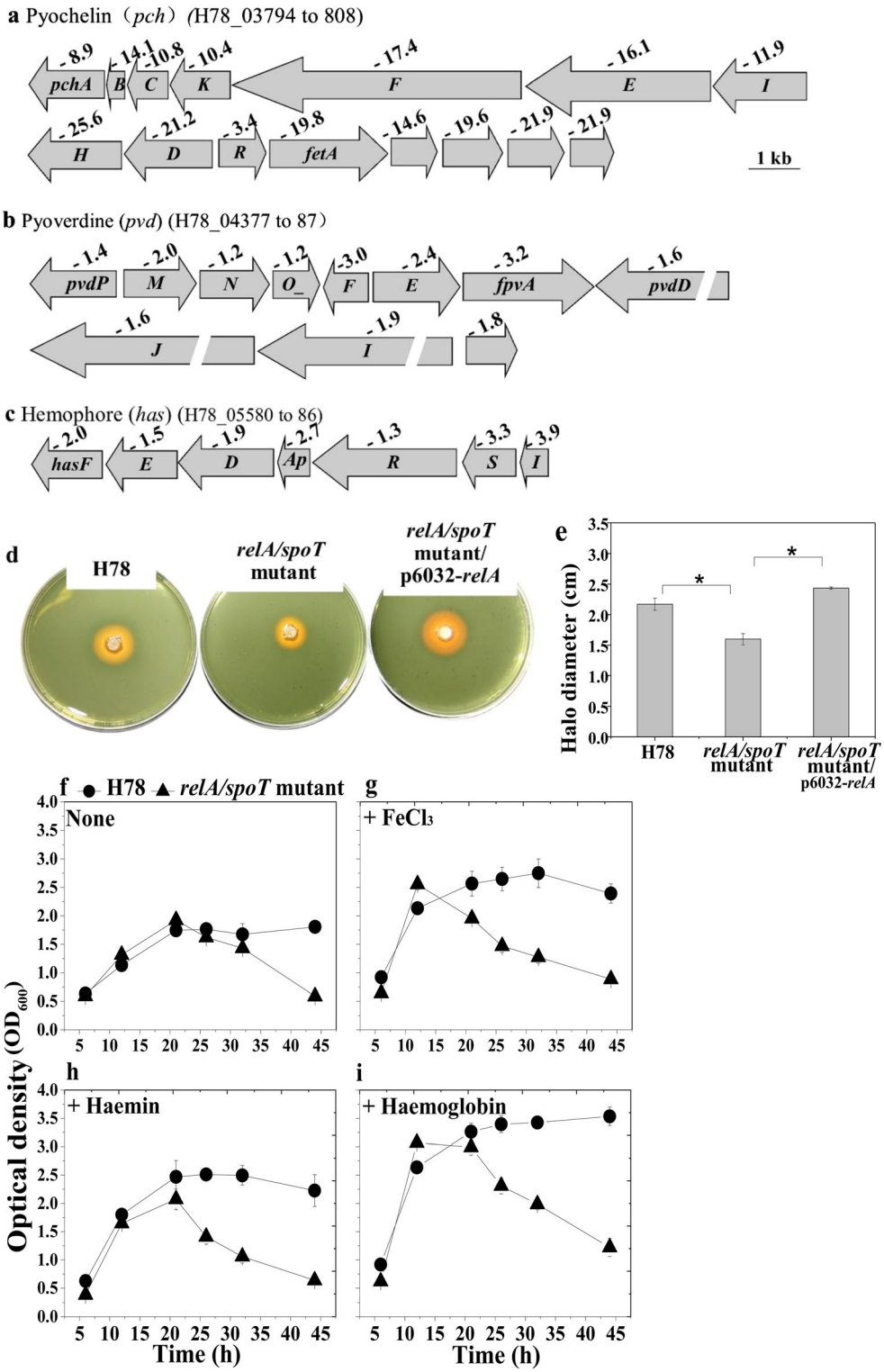

Fig. 5 Positive regulatory effect of *relA/spoT* on iron carrier biosynthesis and iron utilization in *P. protegens* H78. **a**–**c** Fold change in the transcript levels of iron carrier biosynthetic gene clusters, including *pch* (**a**), *pvd* (**b**), and *has* (**c**), in the *relA/spoT* mutant relative to the wild-type H78. **d** Siderophore (pyochelin and pyoverdine) production in H78, *relA/spoT* mutant and its derivative strain the *relA* expression plasmid was assayed in a CAS plate. **e** The fluorescent halo diameter (cm) from in H78, *relA/spoT* mutant and its derivative strain the *relA* expression plasmid. Every value represents mean ± SD. $*P < 0.05$, significant difference. The experiment was repeated independently three times with similar results. **f**–**i** Cell growth of *P. protegens* H78 and its *relA/spoT* mutant at 28 °C in iron-deficient succinate minimal medium supplemented without (**f**) or with (1) 100 μM $FeCl_3$ (**g**), (2) 3.3 μM haemin and 150 μg ml^{-1} EDDHA (**h**), and (3) 3.0 μM haemoglobin and 150 μg ml^{-1} EDDHA (**i**)

relA/spoT mutant, and complemented strains was measured on a solid plate using the chrome azurol S method (Fig. 5d, e). Deficiency of *relA/spoT* reduced the size of the fluorescent circle, indicating reduced level of siderophore production. Siderophore production in the *relA/spoT* mutant can be restored to the wild-type level by the exogenous expression of the (p)ppGpp synthetase gene *relA* in pME6032 (Fig. 5d, e). To further assess the influence of *relA/spoT* on the iron utilization ability of *P. protegens* H78, the wild-type H78 strain and its *relA/spoT* mutant were cultivated in iron-deficient succinate minimal medium with $FeCl_3$, haemin, or haemoglobin as the sole source of iron

(Fig. 5f–i). Growth of the H78 strain was significantly inhibited by the deficiency of *relA/spoT* in all three tested media that were individually supplemented with three different iron sources. Moreover, bacterial growth of the *relA/spoT* mutant rapidly declined after 21 h. However, the wild-type strain H78 maintained a steady growth rate (Fig. 5g–i). These results indicated that the (p)ppGpp-mediated stringent response positively regulates iron carrier production and iron utilization in *P. protegens* H78.

The RelA/SpoT regulon is involved in carbon and energy metabolism

Four gene clusters related to carbohydrate metabolism, *ped* (2-phenylethanol catabolism), *mtl* (mannitol transport and utilization), *suc* (succinate metabolism), and *iol* (inositol metabolism), were greatly upregulated at the transcript level because of *relA/spoT* deletion (Supplemental Fig. S3a–d). Similarly, three energy metabolic gene clusters, *atpBEFHAGDC*, *pntAB*, and *cyoABCDE*, which are individually responsible for the biosynthesis of ATP, NAD(P)H, and cytochrome O ubiquinol oxidase, were also remarkably inhibited by *relA/spoT* in H78. The *relA/spoT* mutation significantly increased the mRNA abundance of all genes in the *atp*, *pnt*, and *cyo* operons (Supplemental Fig. S3e–g). These results implied that the (p)ppGpp-mediated stringent response predominantly plays a negative regulatory role in carbohydrate and energy metabolism in *P. protegens* H78.

The RelA/SpoT-mediated stringent response regulates the ability of *P. protegens* H78 to utilize phenylethanol, mannitol, or succinate as the sole carbon source

Based on the negative regulatory effect of *relA/spoT* on the *mtl*, *suc*, and *ped* gene clusters, we predicted that *relA/spoT* may similarly inhibit the ability of *P. protegens* H78 to utilize mannitol, succinate, or phenylethanol as a carbon source. Correspondingly, we compared the bacterial growth measured for the H78 strain and its *relA/spoT* mutant in M9 medium supplemented with 5 g l^{-1} mannitol, 3 g l^{-1} succinate, or 1 ml l^{-1} phenylethanol as the sole carbon source. As shown in Fig. 6a, the growth of the *relA/spoT* mutant was strongly inhibited until 50 h in M9 medium with mannitol as the sole carbon source. However, after 60 h, the *relA/spoT* mutant became significantly superior to the wild-type H78 strain in bacterial growth (Fig. 6a). When succinate was used as the sole carbon source, an obvious upregulation of growth was observed in the *relA/spoT* mutant (Fig. 6b). In addition, in the M9 medium with phenylethanol as the sole carbon source, bacterial growth of the *relA/spoT* mutant was almost completely inhibited until 96 h. However, the growth of the *relA/spoT* mutant unexpectedly increased after 100 h of culture (Fig. 6c). In contrast, the growth of the wild-type H78 strain was steady after peaking at 24 h and began decreasing after 96 h (Fig. 6c). These results suggested that the utilization of different carbon sources is differentially controlled by the (p)ppGpp-mediated stringent response in *P. protegens* H78.

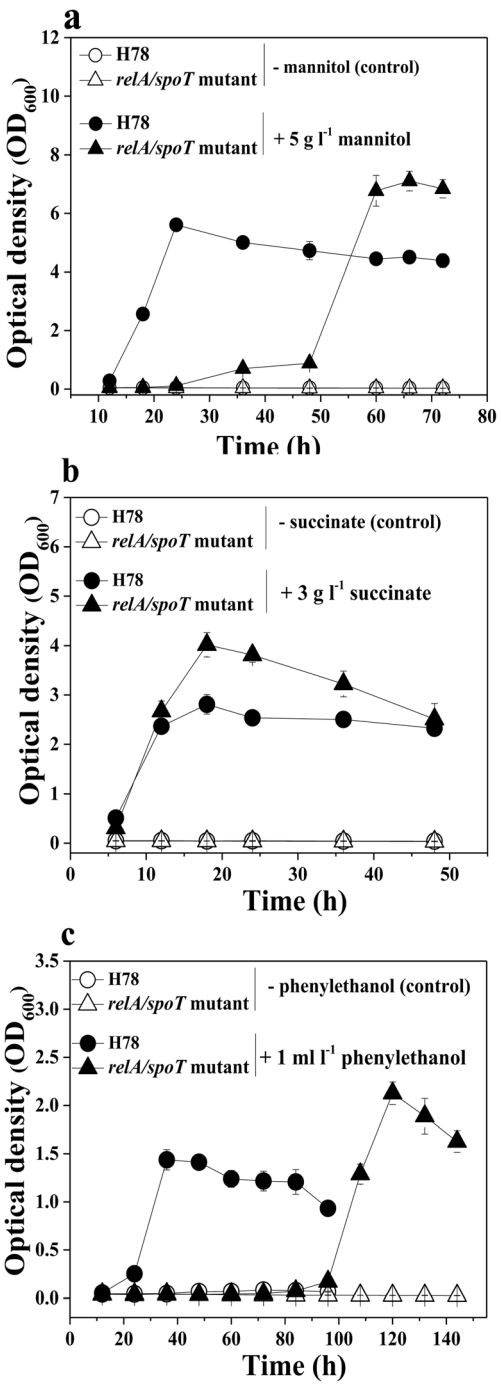

Fig. 6 Influence of *relA/spoT* on the ability of *P. protegens* H78 to utilize three carbon sources. The bacterial growth of *P. protegens* H78 and the *relA/spoT* mutant was assayed at 28 °C in M9 medium with mannitol (**a**), succinate (**b**), or phenylethanol (**c**) as the sole carbohydrate source

The *relA/spoT* mutant exhibits upregulated expression of multiple ion transport and protein secretion operons

(i) *Phosphate (pst)*, *cysteine (cys)*, and *urea (urt)* transport operons

The transcriptomic profiles of *P. protegens* H78 and the *relA/spoT* mutant demonstrated that the mRNA levels of the phosphate-specific transport operon *pstSCAB-phoU* and the PhoR/PhoB TCS genes were enhanced by more than 25-fold in the *relA/spoT* mutant compared with those in the H78 strain (Supplemental Fig. S4a). The *cysTWA* ABC transporter operon and other related genes (*cysDN* and *cysI*), which are involved in the transport and metabolism of cysteine as a sulphur source, were significantly upregulated owing to the *relA/spoT* mutation (Supplemental Fig. S4b). Similarly, a strong inhibitory effect of *relA/spoT* on the *urtABCDE* operon involved in urea transport and utilization was observed (Supplemental Fig. S4c). In addition, some urease metabolic genes were also negatively regulated by *relA/spoT* in H78 (Supplemental Fig. S4c).

(ii) Three TonB-dependent transport systems: ExbB/ExbD/$TonB_{4–6}$

Like most bacteria, *P. protegens* H78 possesses multiple TonB-dependent transporters, ExbB/ExbD/TonBs, which are involved in the binding and transport of siderophores, vitamin B12, nickel complexes, and carbohydrates (Noinaj et al. 2010). Transcriptomic data showed that the mRNA levels of the fourth to sixth TonB transporter gene clusters *exbB/exbD/*$tonB_{4–6}$ and their individual flanking genes were much higher in the *relA/spoT* mutant than in the parental strain H78 (Supplemental Fig. S4d).

(iii) Bacterial secretion system Gsp (general secretion pathway)-T2SS

In gram-negative bacteria, a large number of secretion systems, including types I to VI secretion systems (T1SS to T6SS), deliver proteins out of the cells. The secreted proteins include toxins, virulence or biocontrol factors, cytochromes, and exoenzymes (Abby et al. 2016). In *P. protegens* H78, the expression of Gsp-T2SS gene cluster was significantly enhanced by the *relA/spoT* deletion (Supplemental Fig. S4e). In addition, five genes (*impB2*, *impC2*, *hcp*, *tssE*, and *clpV*) within the gene cluster (H78_06283 to H78_06304), which is homologous to *P. aeruginosa* H1-T6SS (Wei et al. 2013), were moderately inhibited by RelA/SpoT (Supplemental Table S4).

Regulatory effect of *relA/spoT* mutation on phosphate, cysteine, and urea utilization

The repressive activity of *relA/spoT* on the *pst*, *cys*, and *urt* operons led us to further assess the influence of *relA/spoT* on the ability of *P. protegens* H78 to take up and utilize phosphate, cysteine, and urea. H78 and its *relA/spoT* mutant strains were grown at 28 °C in minimal medium with KH_2PO_4, cysteine, and urea as the sole sources of phosphate, sulphur, and nitrogen, respectively. Bacterial growth was monitored at different time points, and the results were displayed in Fig. 7. In the minimal medium with 2 mM KH_2PO_4, the bacterial growth of the *relA/spoT* mutant was

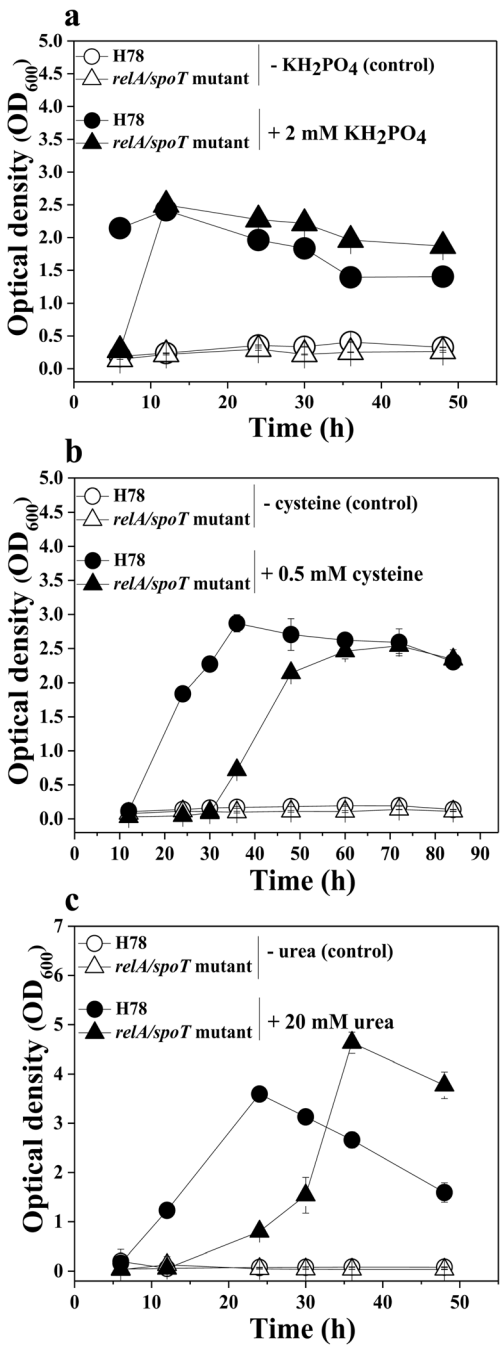

Fig. 7 Regulatory effect of *relA/spoT* on phosphate, cysteine, and urea utilization. Cell growth of *P. protegens* H78 and the *relA/spoT* mutant was monitored at 28 °C in minimal medium with the addition of KH_2PO_4 (**a**), cysteine (**b**), and urea (**c**) as the sole sources of phosphor, sulphur and nitrogen, respectively

superior to that of the wild-type H78 after 12 h of culture (Fig. 7a), demonstrating that the (p)ppGpp-mediated stringent response moderately inhibits phosphate utilization in *P. protegens* H78.

In the M9 medium with 0.5 mM cysteine as the sole source of sulphur, the *relA/spoT* mutant showed obviously inferior bacterial growth compared with the H78 strain until 60 h (Fig. 7b). This result seemed to be inconsistent with the repressive effect of *relA/spoT* on the cysteine transport operon *cysTWA*. This may be because cysteine utilization have been influenced by other operons in addition to *cysTWA*. In addition, in the minimal medium supplemented with 20 mM urea as the sole source of nitrogen, the bacterial growth of the *relA/spoT* mutant was significantly inhibited compared with that of the wild-type H78 strain before 30 h of culture. Conversely, the *relA/spoT* mutant showed obvious superiority to its parental strain H78 in terms of growth after 36 h of culture (Fig. 7c).

Reduction of antibiotic resistance by the *relA/spoT* mutation

Three common antibiotics, gentamicin, kanamycin, and tetracycline, were chosen to compare the antibiotic resistance levels of *P. protegens* H78 and its *relA/spoT* mutant. Bacterial growth was monitored in LB with or without increasing concentrations of antibiotics, and the results are summarized in Fig. 8. Without antibiotics in LB, the bacterial growth of the *relA/spoT* mutant was significantly superior to that of the wild-type strain H78. However, when grown in LB medium with increasing concentrations (2–12 μg ml^{-1}) of gentamicin, kanamycin, or tetracycline, the bacterial growth of the *relA/spoT* mutant was significantly inhibited compared with that of H78 (Fig. 8a–c). The results from the CFU assay indicated that the number of live cells was reduced more significantly (relative to OD_{600}) by the *relA/spoT* mutation in the medium with the adopted concentration of antibiotics (Fig. 8d). These results confirmed that antibiotic resistance is positively regulated by the (p)ppGpp-mediated stringent response in *P. protegens* H78.

The RelA/SpoT regulon is involved in flagellum, pilus, and peptidoglycan biosynthesis and chemotaxis

As shown in Supplemental Fig. S5, three gene clusters required for flagellar biogenesis and assembly, H78_01720 to H78_01729, H78_01754 to H78_01762, and H78_04786 to H78_04789, were upregulated by approximately 2-fold at the transcript level in the *relA/spoT* mutant compared with the H78 strain (Supplemental Fig. S5a). Similarly, the *relA/spoT* mutation caused a 2-fold increase in the expression of the *che* chemotaxis operon (Supplemental Fig. S5b). These results suggested that *relA/spoT* may negatively control flagellar biosynthesis and chemotaxis. In contrast, two exopolysaccharide biosynthetic operons, *psl* and *pel*, were significantly downregulated by approximately 2-fold at the transcript level by the *relA/spoT* mutation (Supplemental Fig. S5c). Similarly, the type I chaperone-usher pilus biogenesis operon, *csu*, displayed a nearly 2-fold decrease at the transcript level in the *relA/spoT* mutant relative to the wild-type H78 (Supplemental Fig. S5d). These results implied that the (p)ppGpp-mediated stringent response is involved in flagellum and pilus biogenesis, exopolysaccharide production, biofilm formation, and motility in *P. protegens* H78.

The RelA/SpoT regulon includes multiple transcriptional factors/regulators and three sRNAs (RsmY, RsmZ, and CrcZ)

Transcriptomic data showed that in the *relA/spoT* regulon of *P. protegens* H78, 144 genes encode regulatory systems or factors, including numerous transcriptional factors and regulators, two-component regulatory systems or elements, and three sRNAs (RsmY, RsmZ, and CrcZ). Among the 144 regulators, 93 regulatory genes were significantly downregulated, and 51 genes were upregulated in the *relA/spoT* mutant relative to the wild-type H78 (Supplemental Tables 6 and 7). Among the positive targets of *relA/spoT*, ten pairs of FecI/FecR family sigma factors/regulators, such as FecR/FecI (H78_01021/H78_01020) and HasI/HasS, are required for the regulation of iron utilization (Supplemental Table S5). Moreover, four pathway-specific transcriptional regulators (*pltR*, *pltZ*, *phlG*, and *pchR*) related to the biosynthesis of antibiotics and iron carriers are positively regulated by *relA/spoT*. The global regulation of *relA/spoT* appears to be mediated by multiple pathway-specific regulators.

Two important sRNAs, RsmY and RsmZ, which, together with another sRNA, RsmX, form a vital node of the Gac/Rsm cascade, were subject to strong positive regulation by *relA/spoT*. In the present transcriptomic sequencing study, most of the sRNAs were not sequenced because they are too short. However, RsmY and RsmZ transcripts were detected in the transcriptomic profiles of wild-type strain H78. These two sRNAs completely disappeared at the transcript level in the *relA/spoT* mutant (Supplemental Table S6). Therefore, the RelA/SpoT-mediated stringent response may activate the expression of the RsmXYZ sRNAs in the Gac/Rsm cascade and further activate the production of secondary metabolites, including antibiotics. Moreover, the transcript levels of *gacS* and *gacA* were unchanged between the *relA/spoT* mutant and its parental strain H78.

Among the negative targets of *relA/spoT*, the largest class of regulators is TCSs, including *glnG/glnL*, *pedS1/R1*, *pedS2/R2*, *rpeA/B*, *colS/R*, and *phoR/B* (Supplemental Table S7). In addition, the global transcriptional regulator Fis gene exhibited 2.5-fold upregulation at the transcript level in the *relA/spoT* mutant relative to the wild-type H78 (Supplemental Table S7).

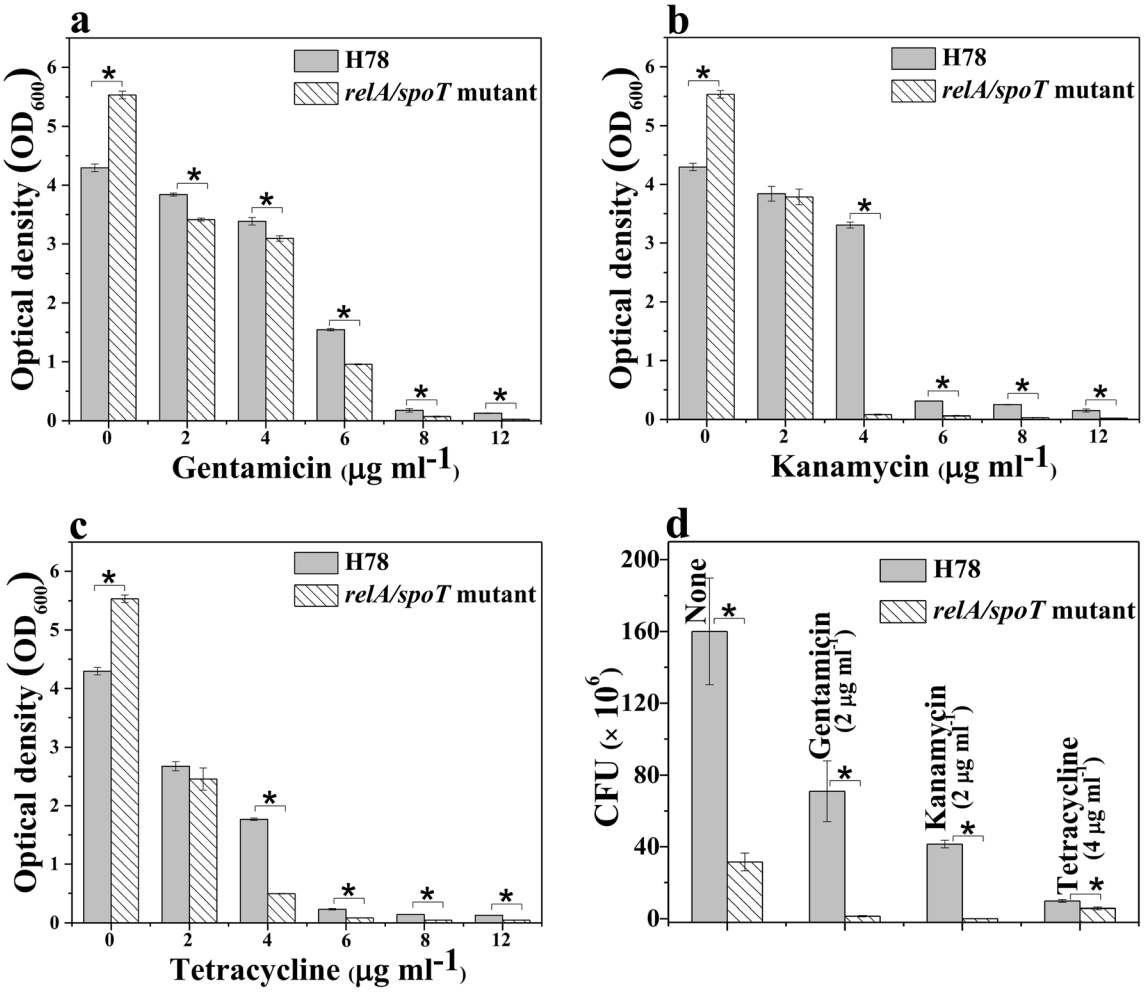

Fig. 8 Effect of *relA/spoT* on antibiotic resistance in *P. protegens* H78. **a**–**c** The bacterial growth (OD_{600}) of *P. protegens* H78 and the *relA/spoT* mutant was assessed after 12 h of culture at 28 °C in LB medium with increasing concentrations (0 to 12 μg ml^{-1}) of gentamicin (**a**), kanamycin (**b**), or tetracycline (**c**). **d** Viable counts (CFU ml^{-1}) of H78 and its *relA/spoT* mutant after 12 h of culture in LB medium supplemented with gentamicin (2 μg ml^{-1}), kanamycin (2 μg ml^{-1}), or tetracycline (4 μg ml^{-1}). *$P < 0.05$, significant difference

The CrcZ sRNA gene in the CbrA/B-CrcYZ-Crc signal transduction cascade was upregulated by 2.5-fold because of the *relA/spoT* mutation (Supplemental Table S7).

Discussion

The (p)ppGpp-mediated stringent response is widely distributed in bacteria and utilized to sense and respond to starvation and stresses for survival in adverse environments (Hauryliuk et al. 2015). In this study, we investigated the genome-wide regulation of RelA/SpoT in *P. protegens* H78 grown to the mid- to late log phase with an OD_{600} of 5.0–6.0 in KMB at 28 °C. Systematic phenotype assays were conducted to assess the influence of *relA/spoT* deletion on the physiology and metabolism of *P. protegens* H78. A global regulatory model of the (p)ppGpp-mediated stringent response in *P. protegens* H78 is summarized in Fig. 9. In *P. protegens* H78, the stringent response plays an important role in inhibiting bacterial growth and maintaining cell survival. The (p)ppGpp-mediated stringent response globally inhibits bacterial primary metabolism, including DNA replication, transcription, and translation; amino acid metabolism; carbohydrate and energy metabolism; ion transport and secretion systems; and flagellar biogenesis, assembly, and chemotaxis. However, secondary metabolism, including the biosynthesis of antibiotics, iron carriers, and exopolysaccharides, is globally activated by the (p)ppGpp-mediated stringent response. Moreover, the global regulation of the stringent response system may be mediated by numerous transcriptional factors/regulators, TCSs, and sRNAs.

In *P. protegens* H78, the (p)ppGpp-mediated stringent response system is involved in regulating the entire process of genetic information transfer: DNA replication and cell division (*grpE-dnaK-dnaJ*, *fts-mur-mra*, *gyrB*, *ssb*, *hupA*, *ftsX*, *recO*, and H78_01844), transcription (*rpo*, *rpoC*, and multiple ECF sigma factors/regulators), and translation (*rplQ*, *rpmE*, *rpsT*, *rsmB*, *rsmE*, *tsf*, *tuf*, *pth*, and *fmt*). The negative

Fig. 9 Proposed model for the genome-wide regulation of the (p)ppGpp-mediated stringent response regulatory system in *P. protegens* H78. Arrows indicate positive regulation; lines with flat ends show negative regulation

regulatory effect of the (p)ppGpp-mediated stringent response on DNA replication and cell division corresponds to the upregulation of bacterial growth in the *relA/spoT* mutant of H78. The (p)ppGpp-mediated stringent response, as a major factor in growth control (Potrykus et al. 2011), is involved in growth and cell cycle arrest (Bokinsky et al. 2013; Ferullo and Lovett 2008), morphological differentiation (Hesketh et al. 2007), and cell persistence (Hauryliuk et al. 2015). In addition, the gene clusters for amino acid, carbohydrate and energy, and inorganic ion transport and metabolism constitute the largest functional groups of the RelA/SpoT regulon. These gene clusters related to nutritional utilization are negatively regulated by *relA/spoT*, confirming that the (p)ppGpp-mediated stringent response is an important strategy by which bacteria metabolically adapt to nutritional starvation. In this study, *P. protegens* H78 and its *relA/spoT* mutant were grown in the rich nutritional medium KMB for RNA-seq analysis. The nutritional level started to decline gradually during mid- to late log phase, at which time the cultures were sampled for RNA-seq. The decrease in nutritional level triggers the bacterial stringent response to globally downregulate bacterial growth and metabolism to save energy and survive the upcoming nutritional depletion.

An interesting phenomenon worth mentioning is that the *relA/spoT* dual mutant of H78 resumed growth after 50 h of lag phase in M9 medium with mannitol as carbon source (Fig. 6a). Moreover, the lag phase of the *relA/spoT* mutant still existed when the growth-resuming culture of the *relA/spoT* mutant after lag phase was re-inoculated into the fresh M9 medium with mannitol as carbon source (Supplemental Fig. S6). It was reported that the *relA/spoT* dual mutant of *E. coli* easily acquires suppressors in *rpoB* and *rpoC* genes which suppress amino acid auxotrophy phenotype of the *relA/spoT* mutant in minimal medium (Conrad et al. 2010; Murphy and Cashel 2003). The *rpoC* mutation can increase growth rate of *E. coli* in M9 minimal media with glycerol, glucose, or lactate carbon source (Conrad et al. 2010). The growth resumption of the *relA/spoT* mutant after lag phase may be due to appearance of suppressors. However, DNA sequencing and analysis results showed that no any mutation was found in three genes, including *rpoB*, *rpoC*, and *rpoZ*, in the *relA/spoT* dual mutant of *P. protegens* H78 (Supplemental materials).

In contrast with primary metabolism, secondary metabolism, including antibiotic and iron carrier biosynthesis, is globally activated by the (p)ppGpp-mediated stringent response in *P. protegens* H78. Antibiotics and iron carriers (siderophores and haemophores) mainly contribute to the ecological competency of bacteria. Most antibiotic operons, including *plt*, *prn*, *ofa*, and *fit*, are positively regulated by *relA/spoT*. However, the DAPG biosynthetic operon *phlACBDE* is an exception and its transcript level is not influenced by *relA/spoT*. In contrast, DAPG production is entirely inhibited by

the *relA/spoT* mutation (Fig. 4). In another report, DAPG production was also found to be positively regulated by *relA/spoT* in *P. protegens* CHA0 (Takeuchi et al. 2012). In this study, the downregulation of the transcript level in both the transcriptional repressor gene *phlF* and the DAPG hydrolase gene *phlG* seems contradictory with the inhibition of DAPG biosynthesis in the *relA/spoT* mutant. We speculate that (p)ppGpp may exert indirect posttranscriptional regulation of *phlACBDE* expression through other underlying posttranscriptional regulatory pathways, such as the Gac/Rsm cascade. This has been implied by the result that the *phlA'-'lacZ* in-frame fusion expression, which indicated total regulation at both the transcriptional and posttranscriptional levels, was substantially reduced by *relA/spoT* deficiency (Fig. 4g). Therefore, it can be reasonably deduced that the (p)ppGpp-mediated stringent response globally activates antibiotic biosynthesis directly or indirectly through other regulatory factors or systems.

Here, it is interesting why *P. protegens* needs to be activated by the (p)ppGpp-mediated stringent response to produce antibiotics at the expense of energy consumption. *Pseudomonas* spp. inhabit diverse environments, including soil, water, the plant rhizosphere, and animals. Their relatively small core genomes and their flexible and adaptable accessory genomes may provide *Pseudomonas* spp. with remarkable metabolic and physiologic versatility and an astonishing ecological adaptability (Silby et al. 2011). Each species of *Pseudomonas* can produce a specific spectrum of secondary metabolites, including antibiotics, contributing to its specific lifestyle and ecological competitiveness in its natural habitat. *P. protegens*, as a representative of PGPR, can produce a set of antibiotics, including Plt, DAPG, and Prn (Gross and Loper 2009). Under nutritional starvation and other environmental stresses, *P. protegens* H78 is induced by the (p)ppGpp-mediated stringent response to downregulate primary metabolism for conserving energy and surviving in nutritional starvation and activate secondary metabolism, including antibiotic biosynthesis, for acquiring a competitive advantage over other competitors (such as plant pathogens) and predators in natural habitats.

Specifically, the (p)ppGpp-dependent transcriptomic profile in *P. protegens* H78 correlates the (p)ppGpp-mediated stringent response with the Gac/Rsm cascade. These two systems similarly activate secondary metabolism, including antibiotic biosynthesis. The Gac/Rsm cascade consists of the GacS/GacA TCS, the RsmY family sRNAs, and the RsmA family translational repressors in *Pseudomonas* spp. (Haas and Keel 2003). The expression levels of two sRNAs, RsmY and RsmZ, were strongly reduced in the *relA/spoT* mutant of H78 (Supplemental Table S6), suggesting that the (p)ppGpp-mediated stringent response may activate the expression of RsmYZ sRNAs. Another sRNA, RsmX, was not detected by RNA-seq in either *P. protegens* H78 or its *relA/spoT* mutant. In the Gac/Rsm cascade, the GacS/GacA TCS was not significantly influenced by *relA/spoT* at the transcript level in H78. In a previous report, RsmYZ expression in *P. protegens* CHA0 was drastically reduced, whereas the expression levels of *gacS* and *gacA* were relatively moderately downregulated, in the *relA/spoT* mutant compared with the wild-type CHA0. Moreover, the *gacA* mutation upregulated the expression of the (p)ppGpp synthetase gene *relA* in CHA0 (Takeuchi et al. 2012). However, the potential molecular mechanism by which (p)ppGpp regulates the Gac/Rsm cascade and vice versa remains unknown. This study may provide some information for further clarifying the overlapping activation pattern of secondary metabolism induced by the (p)ppGpp-mediated stringent response and the Gac/Rsm cascade in *Pseudomonas* and other related bacteria.

In conclusion, the (p)ppGpp-mediated stringent response system plays an important role in downregulating bacterial growth and improving cell survival in *P. protegens* H78. This system systematically downregulates primary metabolism, including DNA replication, transcription and translation, amino acid metabolism, and carbohydrate and energy metabolism, to save energy and survive starvation. In contrast, it globally activates the biosynthesis of secondary metabolites, including antibiotics, iron carriers, and exopolysaccharides, contributing to bacterial ecological competence. Further research should focus on clarifying the molecular regulatory mechanisms of (p)ppGpp on secondary metabolism, including antibiotic biosynthesis, that are directly related to biocontrol ability in the rhizobacterium *P. protegens* H78. We should also ascertain whether (p)ppGpp directly targets the RsmXYZ sRNAs or other factors and whether (p)ppGpp functions as a potential environmental cue to stimulate phosphorylation of the sensor kinase GacS and thus activate the Gac/Rsm cascade in *Pseudomonas* spp. The identification and characterization of direct target sites and regulatory mechanisms of (p)ppGpp in the Gac/Rsm cascade, as well as the clarification of the regulatory mechanism of the Gac/Rsm cascade on the expression of (p)ppGpp biosynthetic or regulatory genes, will help clarify the mutual regulatory relation between these two systems.

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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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