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Fructose promotes pyoluteorin biosynthesis via the CbrAB-CrcZ-Hfq/Crc pathway in the biocontrol strain Pseudomonas PA1201

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Abstract:	Biocontrol strain Pseudomonas PA1201 produces pyoluteorin (Plt), which is an antimicrobial secondary metabolite. Plt represents a promising candidate pesticide due to its broad-spectrum antifungal and antibacterial activity. Although PA1201 contains a complete genetic cluster for Plt biosynthesis, it fails to produce detectable level of Plt when grown in media typically used for Pseudomonas strains. In this study, minimum medium (MM) was found to favor Plt biosynthesis. Using the medium M, which contains all the salts of MM medium except for mannitol, as a basal medium, we compared 10 carbon sources for their ability to promote Plt biosynthesis. Fructose, mannitol, and glycerol promoted Plt biosynthesis, with fructose being the most effective carbon source. Glucose or succinic acid had no significant effect on Plt biosynthesis, but effectively antagonized fructose-dependent synthesis of Plt. Promoter-lacZ fusion reporter strains demonstrated that fructose acted through activation of the pltLABCDEFG (pltL) operon but had no effect on other genes of plt gene cluster; glucose or succinic acid antagonized fructose-dependent pltL induction. Mechanistically, fructose-mediated Plt synthesis involved carbon catabolism repression. The two-component system CbrA/CbrB and small RNA catabolite repression control Z (crcZ) were essential for fructose-induced Plt synthesis. The small RNA binding protein Hfq and Crc negatively regulated fructose-induced Plt. Taken together, this study provides a new model of fructose-dependent Plt production in PA1201 that can help improve Plt yield by biosynthetic approaches.
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	Yongxing He 中国, Lanzhou University heyx@lzu.edu.cn The main focus is on the regulatory mechanisms of secondary metabolite synthesis in agricultural microorganisms and adaptive mechanisms to the environment.
	Joyce E Loper Oregon State University Joyce.Loper@oregonstate.edu Dr. Loper’s more recent work has focused on the genetics of antifungal compound production by plant-associated bacteria, particularly Pseudomonas fluorescens. She is a world leader in the use of molecular biology for studying biological control. Not only

	has she cloned and characterized the structural genes for several important antibiotics such as pyoluteorin and other polyketide antibiotics, but also she has made major advances in our understanding of how production of antibiotics and other antimicrobial compounds are coordinately regulated.
Opposed Reviewers:	

Declaration of competing interest

The authors have no interests to declare.

Dear Editor-in-Chief,

We submitted a manuscript entitled “Fructose promotes pyoluteorin biosynthesis via the CbrAB-CrcZ-Hfq/Crc pathway in the biocontrol strain Pseudomonas PA1201” for consideration for publication.

The secondary metabolite pyoluteorin (Plt) represents a promising candidate pesticide due to its broad-spectrum antifungal and antibacterial activity. Although the *Pseudomonas* strain PA1201 contains a complete genetic cluster for Plt biosynthesis, it fails to produce detectable level of Plt when grown in media typically used for *Pseudomonas* strains. In this study, minimum medium (MM) was found to favor Plt biosynthesis. Using the medium M, which contains all the salts of MM medium except for mannitol, as a basal medium, we compared 10 carbon sources for their ability to promote Plt biosynthesis. Fructose, mannitol, and glycerol promoted Plt biosynthesis, with fructose being the most effective carbon source. Glucose or succinic acid had no significant effect on Plt biosynthesis, but effectively antagonized fructose-dependent synthesis of Plt. Promoter-lacZ fusion reporter strains demonstrated that fructose acted through activation of the pltLABCDEFG (pltL) operon but had no effect on other genes of plt gene cluster; glucose or succinic acid antagonized fructose-dependent pltL induction. Mechanistically, fructose-mediated Plt synthesis involved carbon catabolism repression. The two-component system CbrA/CbrB and small RNA catabolite repression control Z (crcZ) were essential for fructose-induced Plt synthesis. The small RNA binding protein Hfq and Crc negatively regulated fructose-induced Plt. Taken together, this study provides a new model of fructose-dependent Plt production in PA1201 that can help improve Plt yield by biosynthetic approaches.

All authors have reviewed the manuscript and approved it for publication. This manuscript has not been previously published in whole or in part, nor is it being considered for publication elsewhere. None of the authors of this manuscript have a financial interest related to this work.

Thank you very much for your attention to our manuscript.

Ya-Wen He, Ph.D, Professor

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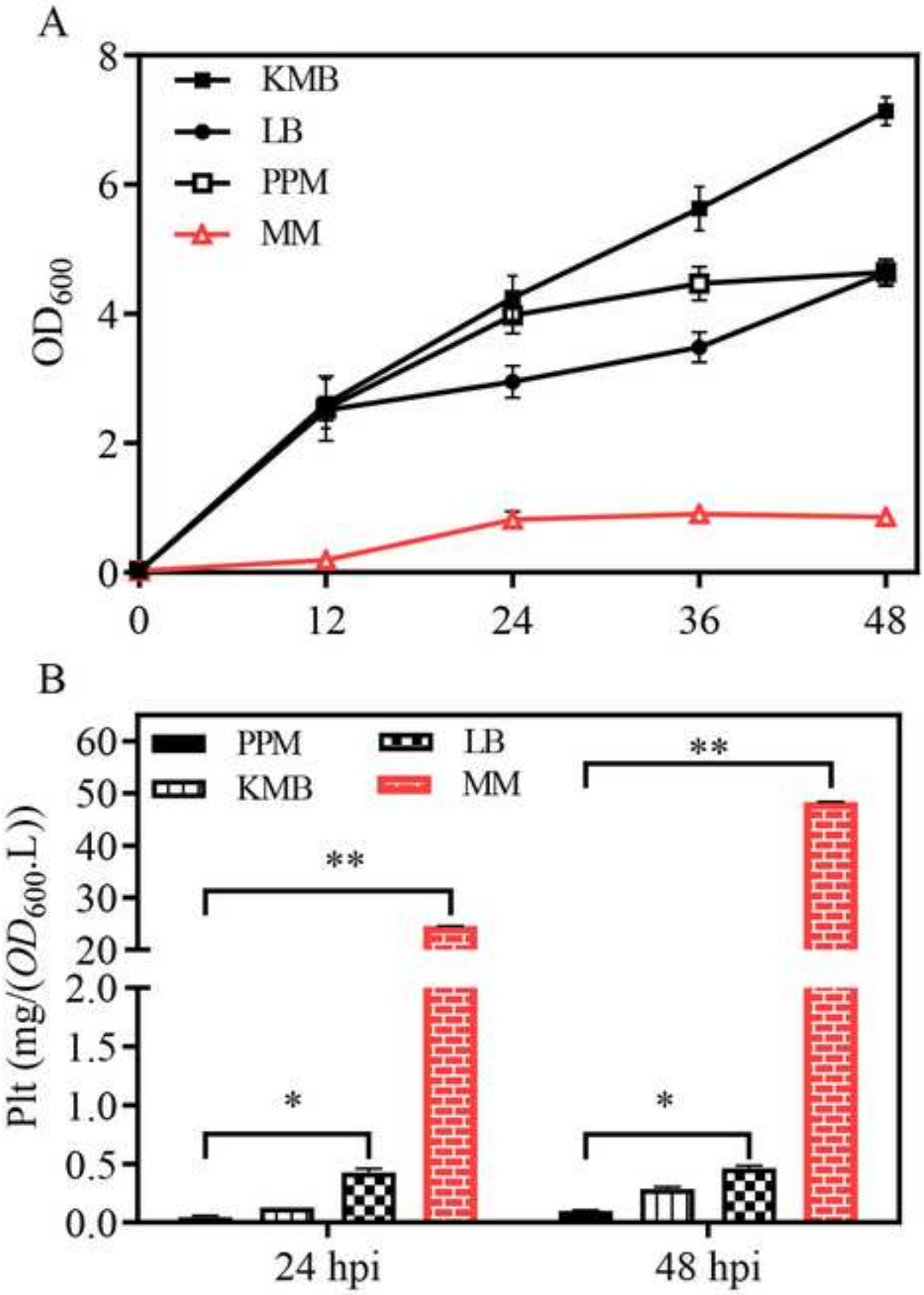

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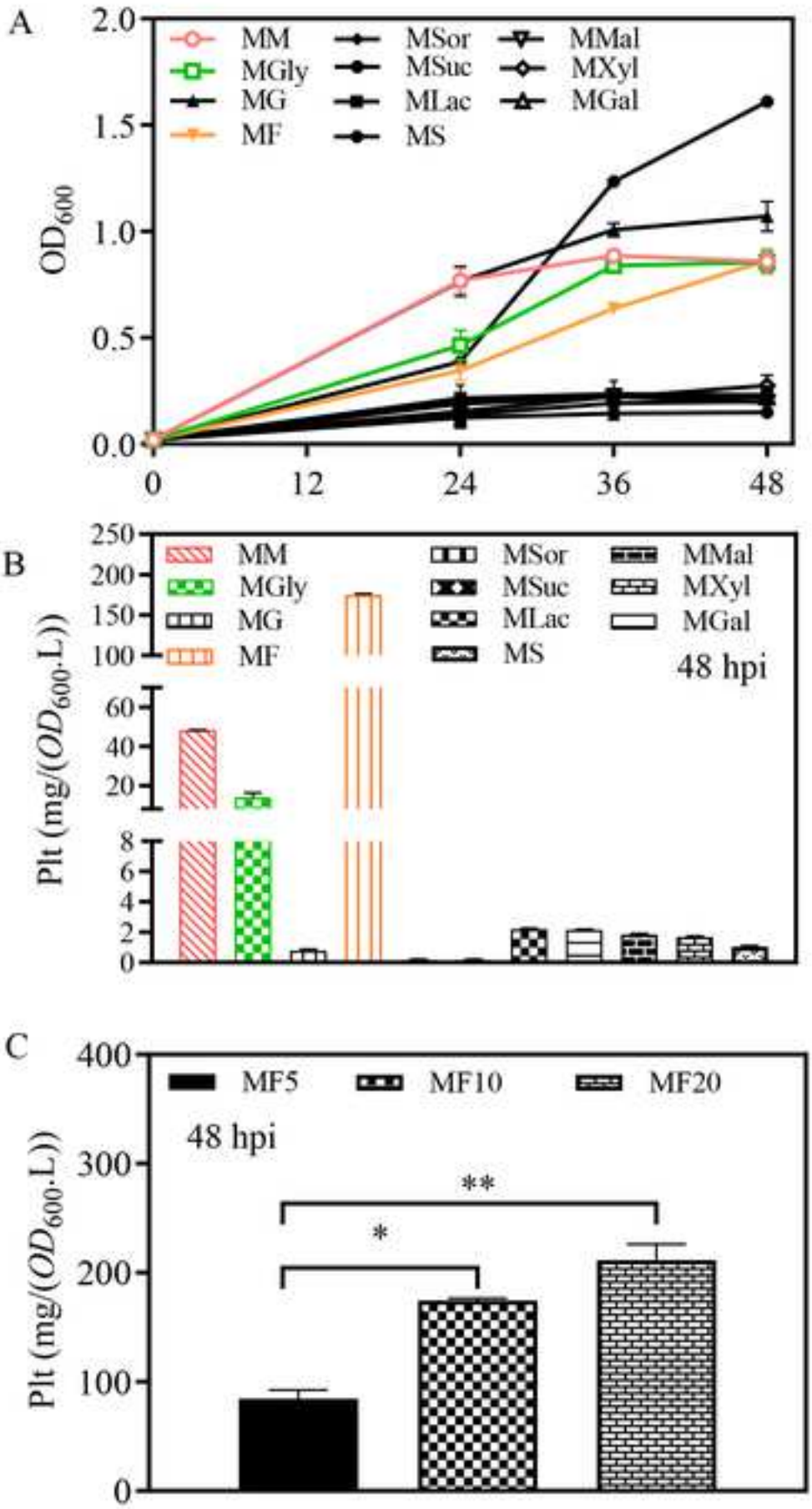

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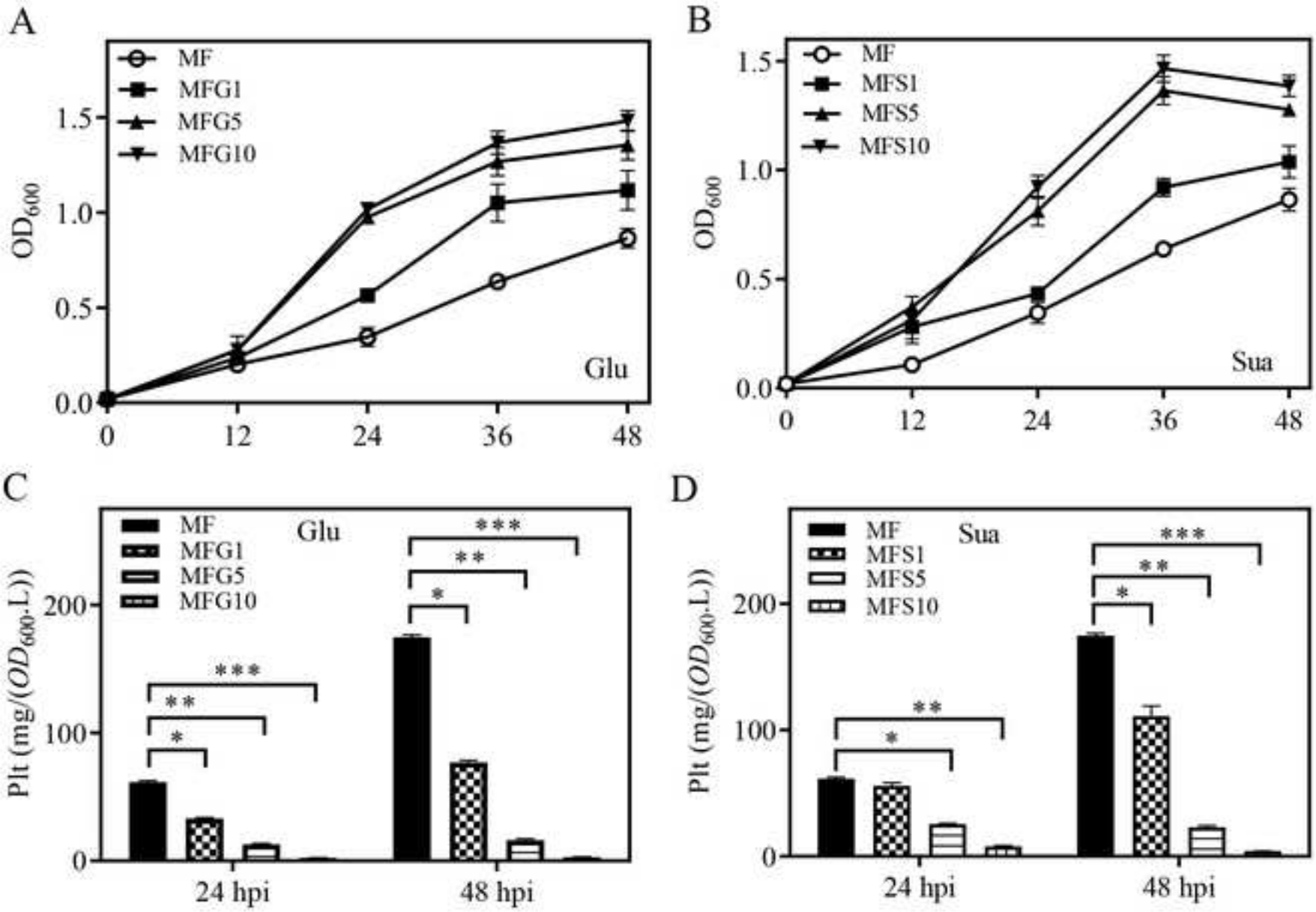

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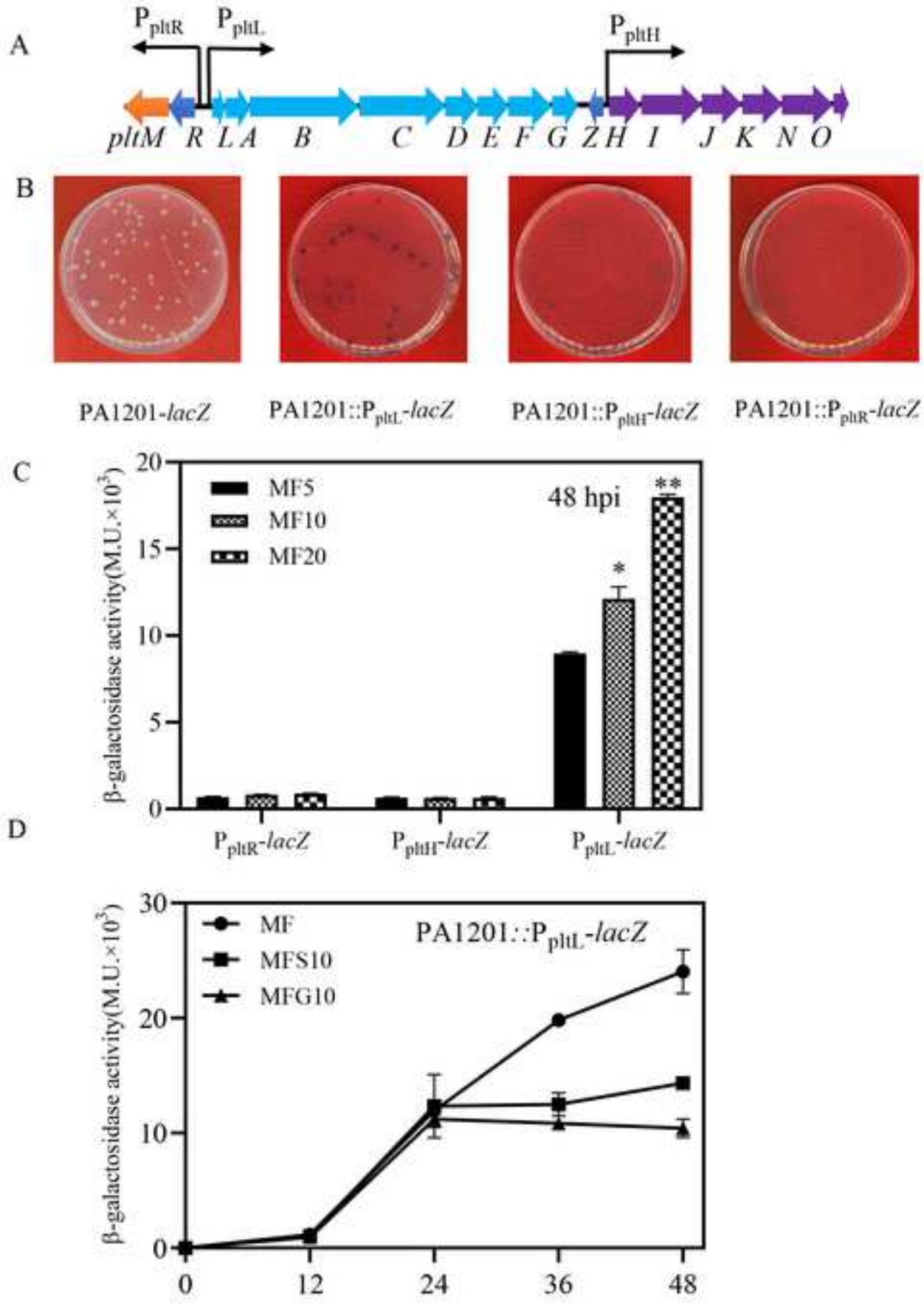

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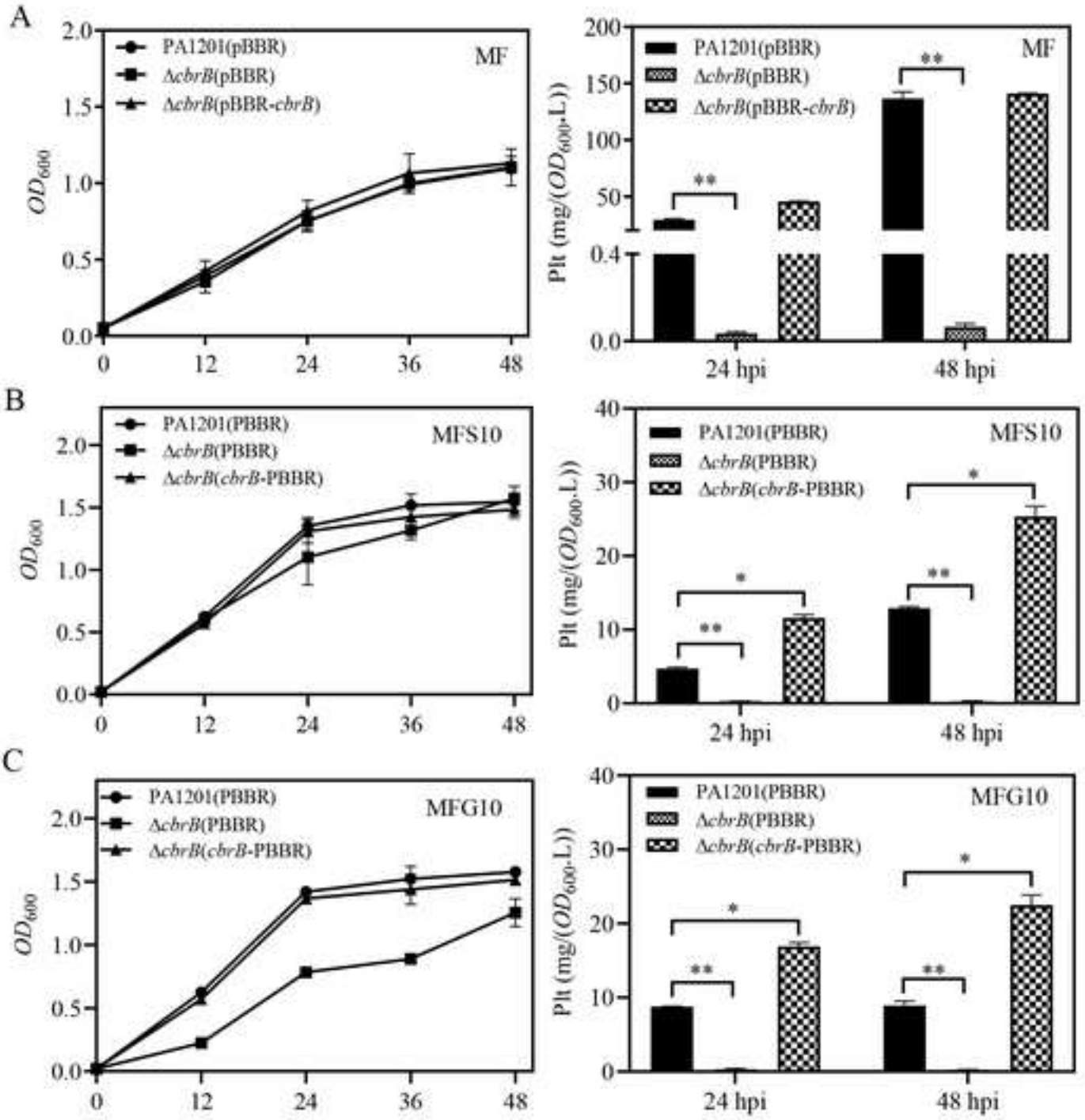

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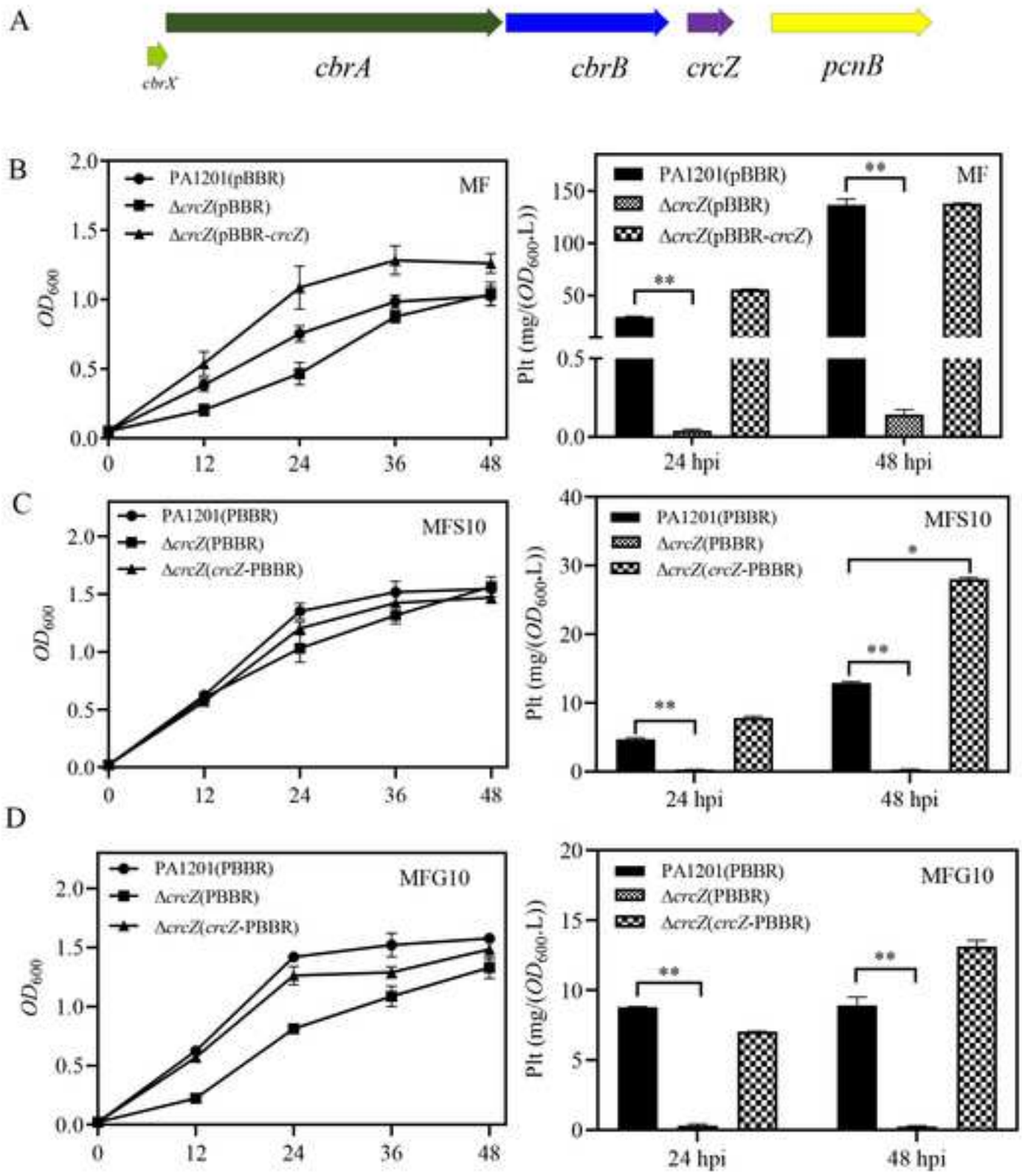

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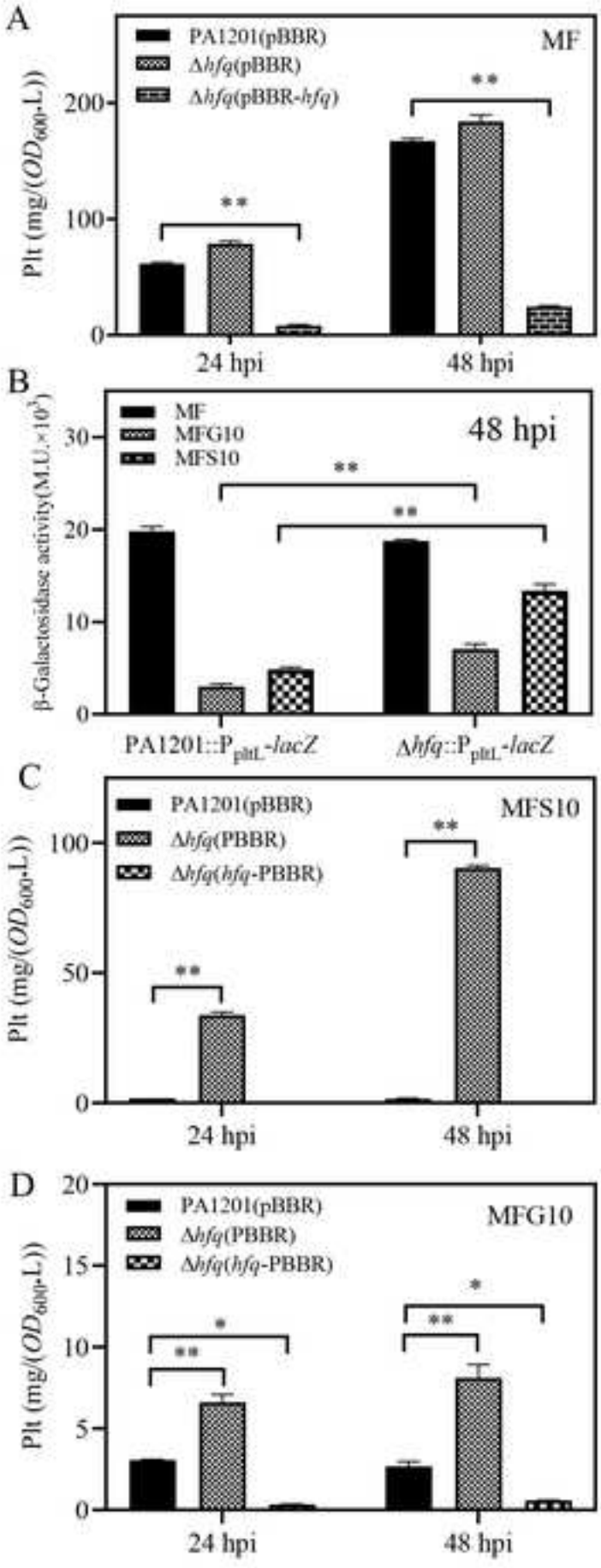

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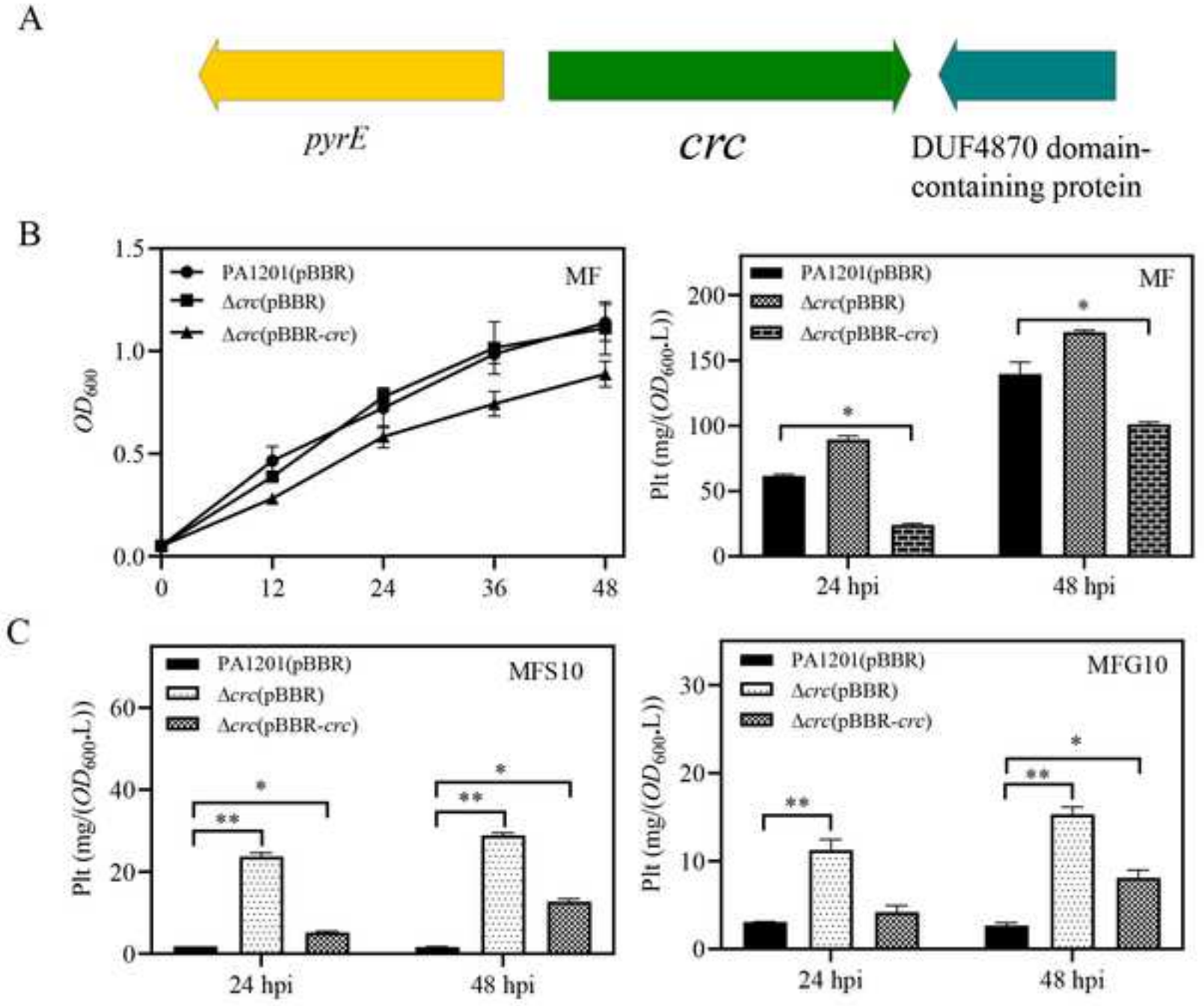

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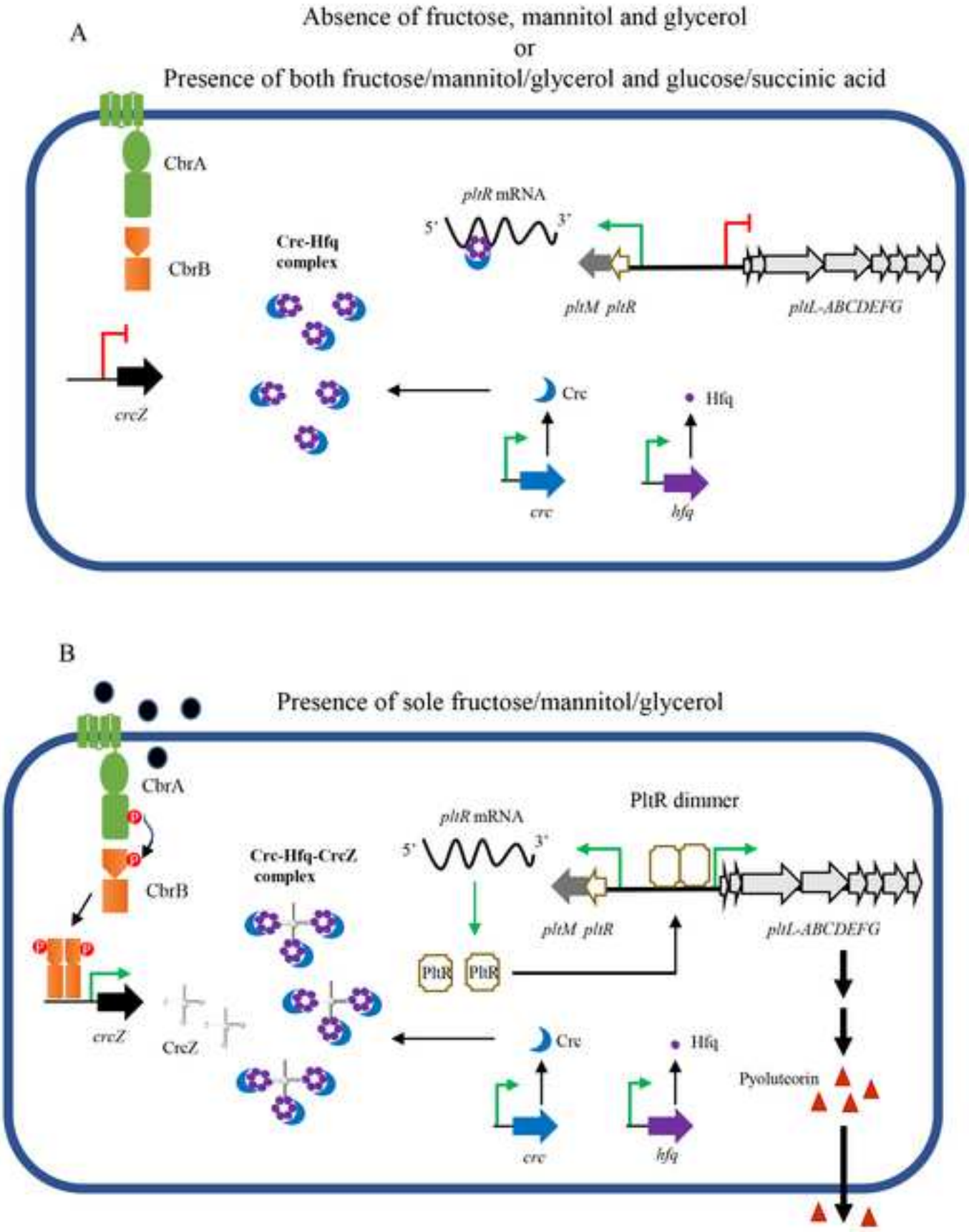

Fructose promotes pyoluteorin biosynthesis via the CbrAB-CrcZ-Hfq/Crc pathway in the biocontrol strain *Pseudomonas* PA1201

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Running title: Fructose promotes pyoluteorin biosynthesis by repressing carbon catabolism

Abstract

Biocontrol strain *Pseudomonas* PA1201 produces pyoluteorin (Plt), which is an antimicrobial secondary metabolite. Plt represents a promising candidate pesticide due to its broad-spectrum antifungal and antibacterial activity. Although PA1201 contains a complete genetic cluster for Plt biosynthesis, it fails to produce detectable level of Plt when grown in media typically used for *Pseudomonas* strains. In this study, minimum medium (MM) was found to favor Plt biosynthesis. Using the medium M, which contains all the salts of MM medium except for mannitol, as a basal medium, we compared 10 carbon sources for their ability to promote Plt biosynthesis. Fructose, mannitol, and glycerol promoted Plt biosynthesis, with fructose being the most effective carbon source. Glucose or succinic acid had no significant effect on Plt biosynthesis, but effectively antagonized fructose-dependent synthesis of Plt. Promoter-*lacZ* fusion reporter strains demonstrated that fructose acted through activation of the *pltLABCDEFG* (*pltL*) operon but had no effect on other genes of *plt* gene cluster; glucose or succinic acid antagonized fructose-dependent *pltL* induction. Mechanistically, fructose-mediated Plt synthesis involved carbon catabolism repression. The two-component system CbrA/CbrB and small RNA catabolite repression control Z (crcZ) were essential for fructose-induced Plt synthesis. The small RNA binding protein Hfq and Crc negatively regulated fructose-induced Plt. Taken together, this study provides a new model of fructose-dependent Plt production in PA1201 that can help improve Plt yield by biosynthetic approaches.

Keywords: *Pseudomonas*; biocontrol; pyoluteorin; fructose; carbon catabolism repression

1. Introduction

Pyoluteorin (Plt) is an aromatic polyketide metabolite produced by diverse *Pseudomonas* strains and composed of a resorcinol ring and a dichloropyrrole [1-4]. Plt is best known for its toxicity against *Pythium ultimum*, an important soil-borne plant pathogen that causes damping-off of over 300 diverse plant species, including cucumber and other cucurbits [3-5]. Plt also inhibits bacteria and fungi that impact on human health or crop production such as *Mycobacterium tuberculosis hominis* and *Phytophthora infestans*, respectively [2]. More recently, Plt was demonstrated to inhibit the fungal forest pathogen *Heterobasidion spp*, which causes destructive root and butt rots in coniferous forests of the Northern Hemisphere [6]. The presence of one or more electron-withdrawing groups on Plt's pyrrole is required for its antibacterial activity [7]. In parallel to these antibiotic properties, Plt has become a lead candidate compound for drug discovery against human triple-negative breast cancer and non-small cell lung cancer [8,9]. Thus, since the 1980's, Plt biosynthesis has attracted researchers' attention.

Pseudomonas aeruginosa M18, and *P. protegens* Pf-5 and H78 are three well-studied Plt-producers [10-12]. In *P. protegens* Pf-5, Plt production is associated with the gene cluster *pltMRLABCDEFGZIJKNOP* [11]. *pltLABCDEFG* encodes the enzymes responsible for polyketide synthesis (PKS) and non-ribosomal peptide synthesis (NRPS), two components essentials for plt synthesis [13], while the *pltIJKNOP* operon encodes an ATP-binding cassette (ABC) transporter thought to be involved in Plt efflux [14]. Moreover, Plt synthesis involves two transcription factors: PltR and PltZ [11,13]. PltM was elegantly demonstrated to catalyze the mono- and dichlorination of phloroglucinol, a compound that serves as potent transcriptional regulator of pyolyteorin biosynthesis, without the need for a biosynthetic intermediate [15].

In *P. aeruginosa*, Plt biosynthesis is strictly regulated by a complex protein network. PltR, a LysR family regulator, binds *pltL* promoter to activate *pltL* expression [10]. The TetR family regulator PltZ recognizes a semi-palindromic sequence in the promoter

region of the *pltIJKNOP* operon [16]. While PltR is required for Plt autoinduction, it is not sufficient, and the direct binding of PltZ to pyoluteorin should concur [11]. In addition, Plt biosynthesis is regulated by a range of pathways, such as the Gac/Rsm network and quorum sensing (QS) systems [17-21].

Compared to intrinsic or QS regulation, the effects of environmental cues and medium nutrients on Plt production are relatively less studied. Carbon catabolism repression (CCR) is a main regulator of bacterial growth and metabolite biosynthesis [22]. In media containing multiple carbon sources, most bacteria use a unique source. CCR contributes to this exclusivity by repressing the genes involved in the metabolism and utilization of the other sources [23]. In *P. aeruginosa*, this regulation involves the CCR-related protein catabolite repression control (Crc), and the small RNA (sRNA) binding protein Hfq [24]. Crc-bound Hfq binds to the A-rich motifs on target mRNA near to ribosome binding site, thereby preventing their translation [25]. The transcription of regulatory sRNAs, including *CrcZ*, is activated by the two-component signaling system CbrA/CbrB [26,27]. Thus, Hfq binds to sRNA *CrcZ* and Crc protein to form a regulatory complex [22,28-30].

In this study, we tested whether the carbon source in the growth medium and CCR influenced Plt synthesis. Such clues may serve to improve Plt yield via biosynthesis in the biocontrol strain *Pseudomonas* PA1201.

2. Materials and Methods

2.1 Bacterial strains, media, and growth conditions

The bacterial strains and plasmids used in this study are described in Table S1 and S2. *Escherichia coli* strains were grown aerobically at 37°C in lysogeny broth medium (LB; 5 g/L yeast extract, 10 g/L peptone and 10 g/L NaCl). When required, 20 μg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) was used for blue/white colony screening. The following media with difference carbon sources were used for PA1201 culture: KMB (King's Medium B, 20 g/L tryptone, 0.392 g/L K_2HPO_4, 15 mL/L glycerol and 0.732 g/L $MgSO_4$, pH 7.5); PPM (pigment-producing medium, 22

g/L tryptone, 20 g/L glucose and 5 g/L KNO_3, pH 7.5); LB; MM (minimal medium, 4.5 g/L KH_2PO_4, 10.5 g/L K_2HPO_4, 2.0 g/L mannitol, 2.0 g/L $(NH_4)_2SO_4$, 0.16 g/L $MgSO_4$, 5 mg/L $FeSO_4 \cdot 7H_2O$, 11 mg/L $CaCl_2 \cdot 2H_2O$ and 2 mg/L $MnCl_2 \cdot 4H_2O$); M (MM medium without mannitol). All the strains were grown at 28°C in Erlenmeyer flasks (250 mL) at 220 rpm in a rotary shaker (ZQWY-200N, SHzhichu, China). Antibiotics were added at the following concentrations when needed: 100 μg/mL spectinomycin (Spe); 50 μg/mL kanamycin (Kan); and 20 μg/mL tetracycline (Tet). All chemicals were purchased from Sangon Biotech (Shanghai).

2.2 Quantitative analysis of Plt level in PA1201 cultures

A total of 500 μL of the appropriate culture was collected and extracted with 1 mL of ethyl acetate. The organic phase was subsequently collected and evaporated. The residues were dissolved in 100 μL of methanol for analysis by HPLC (Agilent Technologies 1260 Infinity). A 5-μL sample was injected into a C18 reverse-phase column (Zorbax XDB; 5 μm, 4.6 × 150 mm) with a flow rate of 1 ml/min with the following steps: solvent A was water plus 0.1% (vol/vol) acetic acid, while solvent B was acetonitrile plus 0.1% (vol/vol) acetic acid. The column was preequilibrated in 90% solvent A–10% solvent B and was eluted using a linear gradient. After separation of an injected sample, the column was equilibrated in 90% solvent A–10% solvent B for 4.9 min prior to the next injection. Under these chromatographic conditions, pyoluteorin was eluted at 11.05 min. Quantification was performed by integrating the peak area under the wavelength at 300 nm and Plt concentration using the standard curve obtained with a commercial pyoluteorin. Due to the different growth rate of PA1201 strains in different media, Plt level was defined as mg/(OD_{600}.L) to normalize Plt production of the same population.

2.3 Construction of *lacZ*-dependent reporter strains for transcriptional assay

The method for constructing promoter-*lacZ* fusion reporter strains in PA1201 was previously described by Sun et al [31]. Briefly, the promoter region of a target gene (approximately 500 bp upstream of the start codon) was amplified by PCR. The primers

used for the different reporter strains are listed in Table S3. The PCR products were then cloned into the vector mini-CTX-lacZ. The recombinant plasmids were integrated into the chromosomes of the PA1201-derived strains at the *attP* site, according to the protocol described by Becher and Schweizer [32].

2.4 Measurement of the β-Galactosidase activity in reporter strains

The reporter strains were grown in different medium for 12 to 48 h at 28°C. A total of 100 μL of culture was then collected, centrifuged at 12,000 rpm (Thermo Scientific, Legend Micro 17R) for 5 min, and suspended in 1 mL Z buffer (0.2148 g/L $Na_2HPO_4•12H_2O$, 62.4 mg/L $NaH_2PO_4•2H_2O$, 7.455 mg/L KCl, 1.2037 mg/L $MgSO_4$, pH 7.0) following the addition of 40 μL of chloroform and 40 μL of 0.1% (w/v) SDS solution cell lysis. Next, 200 μL of ONPG (o-nirophenyl-beta-β-D-galactopyranoside) was added to the cell extract and incubated at 28°C. When the reaction mixtures became yellow, the reaction was terminated by adding 500 μL of 1 M Na_2CO_3 solution and the reaction time (T) was recorded. The mixture was centrifuged at 12,000 rpm for 10 minutes. The optical density at a wavelength of 420 nm was measured on the collected 600-μL supernatant. The β-galactosidase activity was calculated according to the following formula: Miller Units = 2 [1000 OD_{420}]/[OD_{600} T].

2.5 Gene deletion and functional complementation analysis

The method used for in-frame gene deletion was previously described elsewhere [31]. Briefly, the upstream and downstream regions of the gene to be deleted were fused by overlap extension PCR. The fusion product was then subcloned into the suicide vector pK18mobsacB carrying the sucrose-sensitive *sacB* gene. The resulting recombinant plasmid was introduced into PA1201 through mating, and the plasmid was subsequently integrated within the target gene by homologous recombination. The resulting strain was then plated on LB agar plate with 50 μg/mL Spectinomycin (Spe) and 5% (w/v) sucrose for a second single crossover homologous recombination event, resulting in allelic exchange. The resulting mutant was verified by PCR and subsequent DNA sequencing. The primers used for the PCR and subsequent screening are listed in Table

S3.

For complementation analysis, the target gene was amplified by PCR and cloned into the PBBR-1-MCS plasmid. The different constructs were then transferred into PA1201 through triparental mating. Triparental mating between PA1201 and *E. coli* was carried out with the helper strain *E. coli* (pRK2013). The primers used for this process are shown in Table S3.

2.6 Statistical Analysis

All experiments were performed at least in triplicate independently. The ANOVA tests for all experimental datasets were performed using the JMP software program (version 5.0). The significant effects of the different treatments were assessed by F values. The differences with significant F tests underwent further analysis by separation of means with Fisher's protected least significant difference test using $p < 0.05$.

3. Results

3.1 Nutrient-poor MM medium favors Plt production in PA1201

First, we compared the effect of different media on Plt production by *Pseudomonas* PA1201. PA1201 was inoculated to and grown in four types of media, *i.e.*, KMB, LB, MM, and PPM for 48 h at 28°C. At the endpoint, Plt in the different cultures was quantified by HPLC, using a commercially available Plt sample as reference (Fig. S1). PA1201 cultures grew best in KMB, LB, and PPM media, reaching OD_{600} ranging from 7.3, 4.8 and 4.7, whereas MM medium supported PA1201 growth poorly, with an OD_{600} of 0.9 at 48 h post inoculation (hpi) (Fig. 1A). However, MM medium yielded the highest concentration of Plt, with 24.5 mg/(OD_{600}.L) at 24 hpi and 48.3 mg/(OD_{600}.L) at 48 hpi; Plt concentration in KMB, LB, and PPM medium were less than 0.5 mg/(OD_{600}.L) (Fig. 1B).

MM is a nutrient-poor medium with mannitol as the major carbon source. To further determine the effect of medium composition on Plt production, two media, 1/3 KMB medium, containing one third of all KMB components, and KMBM, containing all

KMB components supplemented with 10 g/L mannitol, were prepared. No improvement in Plt yield was observed in 1/3 KMB or KMBM (Fig. S2), suggesting that Plt biosynthesis in MM was not improved by nutrient limitation or the unique availability of mannitol as carbon source, but rather, it was involved other specific regulators.

3.2 Fructose is the optimal carbon source for Plt biosynthesis

Carbon sources are key to bacterial growth and metabolite production. To determine the effects of different carbohydrates on PA1201 growth and Plt biosynthesis, a M medium with the same composition as MM medium except for mannitol was used as basis. Mannitol, glycerol, glucose, fructose, sorbitol, galactose, sucrose, lactose, maltose, xylose, and succinic acid was respectively added to M medium at a final concentration of 10 mM to generate the media MM, MGly, MG, MF, MSor, MGal, MSuc, MLac, MMal, MXyl, and MS. Succinic acid and glucose significantly increased PA1201 growth (Fig. 2A), with succinic acid being the most effective. Regardless, high level of Plt was only observed with mannitol, fructose, or glycerol supplementation (Fig. 2B), with fructose being the most effective carbon source, reaching 174.6 mg/(OD_{600}.L) at 48 hpi at 10 mM and displaying a dose-dependent effect at concentrations ranging from 5 mM to 20 mM (Fig. 2C).

3.3 Glucose or succinic acid antagonizes fructose-promoted Plt biosynthesis

In keeping with a previous report [33], we found that glucose and succinic acid were the preferred carbon sources for *Pseudomonas* PA1201 growth (Fig. 2A). To attempt combining the growth-promoting effect of glucose or succinic acid with the Plt-promoting effect of fructose, and further improve Plt yield, glucose or succinic acid was respectively added into the MF medium at final concentrations of 1, 5, and 10 mM, which generated respectively, the media MFG1, MFG5, MFG10, MFS1, MFS5, and MFS10. Addition of glucose or succinic acid to MF medium significantly promoted PA1201 growth (Fig. 3A, B) but decreased Plt levels in a dose-dependent manner (Fig. 3C, D). These results suggested an antagonistic effect between fructose and glucose, or

fructose and succinic acid for Plt biosynthesis.

3.4 Both the promoting effect of fructose and the antagonizing effect of glucose or succinic acid on Plt biosynthesis are mediated by the operon *pltL*

In PA1201, Plt biosynthesis relies on the gene cluster *pltMLRABCDEFGHIJKNO*, composed of at least three operons, *i.e.*, *pltR*, *pltL* and *pltH* (Fig. 4A). To monitor the activity of these operons upon exposure to different carbon sources, three reporter strains, PA1201::P_{pltL}-*lacZ*, PA1201::P_{pltH}-*lacZ*, and PA1201::P_{pltR}-*lacZ*, were generated as previously described [31]. In MF agar plates supplemented with X-gal, PA1201::P_{pltH}-*lacZ* and PA1201::P_{pltR}-*lacZ* colonies exhibited a light blue color, while the PA1201::P_{pltL}-*lacZ* colonies exhibited a dark blue color, provoked by the degradation of X-gal substrate by the reporter enzyme β-galactosidase, encoded by *lacZ* under the control of *PltL* (Fig. 4B). The quantification of the β-galactosidase activity confirmed that the promoter P_{pltL} was activated to a higher level than P_{pltH} or P_{pltR} in presence of 10 mM fructose (Fig. 4C). Fructose upregulated P_{pltL} activity in a dose-dependent manner (Fig. 4C), while increasing fructose concentration did not modify P_{pltR} or P_{pltH} activity (Fig. 4C). These results suggested that the effect of fructose on Plt production is mediated by the *pltL* operon.When 10 mM glucose (MFG10) or 10 mM succinic acid (MFS10) were added to MF liquid medium, P_{pltL} activity was significantly lowered compared with that observed on MF liquid medium (Fig. 4D), suggesting that glucose or succinic acid antagonized the effect of fructose on operon *pltL,* and consequently, on Plt biosynthesis.

3.5 The two-component signal system CbrA/CbrB is essential for fructose-dependent induction of Plt biosynthesis

The CbrA/CbrB system is unique to bacteria of the *Pseudomonaceae* family. It integrates various signals and regulates multiple physiological processes involved in bacterial adaptation to varying environments [34]. To investigate the possible role of CbrA/CbrB in fructose-dependent Plt induction, strains either deleted for *cbrB* [Δ*cbrB*(pBBR)] or deleted and complemented wi[[th overexpressed CbrB

[Δ*cbrB*(pBBR-*cbrB*)] were generated and cultured in MF medium. These genetic alterations did not alter PA1201 growth in MF medium (Fig. 5A). Nonetheless, Plt was not detected in Δ*cbrB*(pBBR) cultures at 24 or 48 hpi, whereas *cbrB* overexpression restored Plt expression to wild-type level in the Δ*cbrB*(pBBR-*cbrB*) strain (Fig. 5A). These results suggested that the CbrA/CbrB system is required for fructose-promoted Plt biosynthesis.

Similarly, in the MFS10 or MFG10 medium, containing respectively the antagonist glucose or succinic acid, Plt synthesis was not detectable with Δ*cbrB*(pBBR), but was restored beyond wild-type level with Δ*cbrB*(pBBR-*cbrB*), albeit Plt levels in these media remained below those in MF for all strains (Fig. 5B, C). These observations suggested that the inhibition exerted by glucose or succinic acid was slightly overcome by CbrB overexpression, implying that CbrA/CbrB may participate to the antagonization of fructose-promoted Plt biosynthesis by these nutrients.

3.6 The sRNA *CrcZ* is essential for fructose-dependent induction of Plt biosynthesis

The *crcZ* gene, encoding *crcZ* sRNA, is located immediately downstream of *cbrB* (Fig. 6A). It has been shown that CbrB could bind the regulatory regions of *crcZ* and activate its transcription from RpoN-dependent promoters [35]. To investigate whether *crcZ* is required for fructose-dependent Plt biosynthesis, PA1201(pBBR), Δ*crcZ*(pBBR) and Δ*crcZ*(pBBR-*crcZ*) strains were constructed and grown in MF medium. In Δ*crcZ*(pBBR) cultures, Plt level was strongly diminished at 24- and 48-hpi compared to PA1201(pBBR) cultures, whereas in Δ*crcZ*(pBBR) cultures, Plt production was restored to levels obtained in PA1201(pBBR) control cultures (Fig. 6B).

In MFS10 or MFG10 medium, the Plt level produced by the Δ*crcZ*(pBBR) strain was significantly lower than that produced by PA1201(pBBR); c*rcZ* overexpression in Δ*crcZ*(pBBR-*crcZ*) increased significantly Plt level at 48 hpi in MFS10 medium, exceeding wild-type Plt level, and to a lesser extend in MFG10 medium (Fig. 6C, D). These findings suggested that *crcZ* partly mediates the antagonistic effects of glucose

and succinate on fructose-induced Plt biosynthesis.

3.7 Hfq is involved in fructose promoting Plt biosynthesis and mediates the antagonistic effects of succinic acid and glucose on fructose-induced Plt biosynthesis

Hfq is a pleiotropic regulator notably involved in CCR in *Pseudomonas* and related bacterial species [36]. To investigate the possible roles of *hfq* in fructose-induced Plt biosynthesis, strains deleted for *hfq* (Δ*hfq*) or overexpressing *hfq* [Δ*hfq*(pBBR-*hfq*)] were generated in PA1201 and grown in MF medium. Deletion of *hfq* had no significant impact on Plt level at 24- or 48-hpi (Fig. 7A). Consistently, *pltL* promoter-dependent β-galactosidase activity in the reporter strain PA1201::P_{pltL}-*lacZ* at 48 hpi was not different from that in Δ*hfq*::P_{pltL}-*lacZ* (Fig. 7B). However, overexpression of *hfq* in Δ*hfq*(pBBR-*hfq*) reduced Plt biosynthesis to a level much lower than that in wild-type PA1201 (Fig. 7A), indicating an inhibitory effect of Hfq on fructose-induced Ptl synthesis.

In MFS10 medium, Plt production by the Δ*hfq* mutant reached 39.5 mg/(OD_{600}.L) at 48 hpi, which was significantly higher than the production achieved by wild-type PA1201 [8.4 mg/(OD_{600}.L); Fig. 7C]. This result indicated that the inhibition of fructose-induced Plt synthesis by succinic acid required Hfq. Overexpression of *hfq* in Δ*hfq* restored Plt inhibition to wild-type level (Fig. 7C). Consistently, P_{pltL}-dependent β-galactosidase activity in the reporter strain Δ*hfq*::P_{pltL}-*lacZ* was significantly higher than that in PA1201::P_{pltL}-*lacZ* when cultured in MFS10 (Fig. 7B). Similar trends in Plt level and P_{pltL}-dependent β-galactosidase activity were observed in MFG10 medium (Figs. 7B–D). These findings suggest that Hfq mediates the antagonistic effects of succinate and glucose on fructose-dependent Plt biosynthesis.

3.8 Crc protein is involved in fructose promoting Plt biosynthesis and mediates the antagonistic effects of succinic acid and glucose on fructose-induced Plt biosynthesis

The Crc protein can stabilize Hfq binding to the A-rich motifs of target mRNAs to form

tripartite Hfq–RNA–Crc complexes [37]. In PA1201, the 780-bp *crc* gene is flanked by the *pyrE* gene, encoding an orotate phosphoribosyltransferase, and the gene encoding DUF4870 domain-containing protein (Fig. 8A). To investigate the possible role of Crc in fructose-promoted Plt biosynthesis, strains deleted for *crc* [Δ*crc*(pBBR)] or overexpressing *crc* [Δ*crc*(pBBR-*crc*)] were constructed in PA1201 and grown in MF. Plt levels in Δ*crc* was not significantly different from that in PA1201 at 24 and 48 hpi (Fig. 8B). In contrast, overexpression of *crc* in Δ*crc*(pBBR-*crc*) decreased Plt production below wild-type level. Further, no additive effect was observed on Plt biosynthesis in the double knockout strain Δ*hfq*Δ*crc*, which suggested that these two gene products acted in the same inhibitory pathway (Fig. S3).

In MFS10 or MFG10 medium, the Plt levels obtained with Δ*crc* at 48 hpi, which were respectively, 15.4 and 20.7 mg/(OD_{600}.L), were significantly higher than that obtained with PA1201 [2.7 and 7.0 mg/(OD_{600}.L), respectively]. *Crc* overexpression in Δ*crc*(pBBR-*crc*) strain restored the inhibition of Plt production observed in wild-type PA1201 cultured in MFG10 and MFS10 media (Fig. 8C). Thus, the antagonizing effect of glucose or succinate on fructose-dependent Plt biosynthesis is mediated by Crc.

4. Discussion

The natural metabolite Plt can effectively inhibit the growth of a variety of plant pathogenic bacteria and fungi, especially oomycetes causing crop diseases that have high economic impacts [38]. This antimicrobial property makes Plt a promising candidate for the development of new biopesticides. However, Plt yield in wild-type *Pseudomonas* strains is far too low to meet industrial demands. The type of carbon source and its availability was shown to affect the production of bacterial antimicrobials in various bacterial genera [39]. The environment and nutrients have been identified as influential factors for Plt production in *Pseudomonas*. For example, the co-production of approximately 150 mg/L of Plt and 500 mg/L of 2,4-diacetylphloroglucinol, another antimicrobial metabolite, was achieved by flask cultivation in a medium containing

approximately 2% ethanol [40]; Duffy et al. found that Plt production was stimulated by Zn^{2+}, Co^{2+}, and glycerol, but repressed by glucose; adding glucose to NBY medium could inhibit Plt production by *Pseudomonas* fluorescens CHA0, while CHA0 produced more Plt using mannitol and glycerol as sole carbon sources [41]. In this study, we investigated the effects of 11 carbon sources on bacterial growth and Plt production in PA1201. Our results showed that the nutrient-poor MM medium favored Plt production. Addition of fructose, mannitol, or glycerol promoted Plt biosynthesis, whereas addition of glucose or succinic acid enhanced bacterial growth but has no significant effect on Plt biosynthesis. Importantly, glucose or succinic acid antagonized fructose-dependent Plt production. Based on these results, we developed the medium MF and obtained a Plt yield of 190.26 mg/L in wild-type PA1201. These findings provide new clues to improve Plt titer through medium optimization.

Plt biosynthetic and efflux mechanisms have been well established [11,16,20,21,42]. The regulatory network for Plt biosynthesis, including transcriptional factors, two-component systems, and QS systems, has also been elucidated in several *Pseudomonas* strains [17,43-48]. This network has been used as target to enhance Plt production by engineering. For example, Plt production in *P. protegens* H78 was substantially enhanced from 15 to 214 mg/L by deletion of the *rsmE* gene involved in the Gac/Rsm-RsmE cascade, *lon* ATP-dependent protease gene, inhibitor gene *pltZ*, and inhibitory sequence in *pltR* operator region, followed by overexpression of Plt ABC-type transporter operon *pltIJKNOP* [12]. A derivative of *P. protegens* Pf-5, in which 23 types of rare codons in pltR were substituted with preferred synonymous codons, produced 15-time higher levels of pyoluteorin than wild-type Pf-5 [49].

CCR is a general mechanism that facilitates the catabolism (assimilation) of carbon from different sources, supports efficient growth, and represses the catabolism of other potentially usable carbon sources that are less energetically efficient [50]. Thus, CCR allows bacterial cells to preferentially assimilate a single carbon compound among multiple carbon sources. In addition, CCR potentially control antibiotic biosynthesis

indirectly in *Pseudomonas* spp. [51]. The CCR regulatory cascade is composed of three layers: the two-component system CbrA/CbrB, the CrcZ/Y sRNAs, and the translational repressor Crc [28]. Our results clearly demonstrated that fructose promotes Plt production directly by increasing the transcription of *pltL* operon. CCR-associated regulators are required for fructose-dependent *pltL* expression and Plt production. A more complete understanding of the molecular mechanisms underlying the regulation of *pltL* expression by CCR in PA1201 can help optimizing Plt production and its industrial application.

The two-component CbrA/CbrB system is involved in nutritional adaptation and was first described in *P. aeruginosa* as a regulator of hierarchical utilization of various carbon sources [26]. To date, no orthologous system has been described in other species, and its activating signals remain elusive, although some authors suggested that it could include the C:N balance [34]. In this study, we found that at least three carbohydrates, fructose, mannitol, and glycerol, could promote Plt production. Glucose or succinic acid antagonized fructose-dependent Plt production. Thus, these carbohydrates are unlikely the direct activators of the CbrA/CbrB system and further investigation is necessary to clarify the underlying mechanisms of this regulation. From the current results, we proposed a working model to explain how different carbohydrates affect Plt production in PA1201 cells (Fig. 9). In absence of fructose, mannitol, or glycerol, or in presence of both fructose and glucose or fructose and succinic acid, the CbrA/CbrB system is not activated, no sRNA CrcZ is expressed, and Hfq and Crc form a two-protein complex. This complex binds *pltR* mRNA, inhibiting PltR protein production, thereby impeding the initiation of *pltL* expression. In sole presence of fructose, mannitol or glycerol, the CbrA/CbrB system is activated and phosphorylated CbrB binds *crcZ* promoter to initiate the transcription of *CrcZ* sRNA. Hfq and Crc proteins bind *CrcZ* to form a three-partite complex. This complex loses the capacity to bind *pltR* mRNA, enabling PltR protein translation. PltR dimers activates the promoter of *pltL* operon, which in turn, initiates Plt biosynthesis (Fig. 9).

Credit authorship contribution statement

Ying Cui: Methodology, Investigation, Writing – original draft. Kai Song: Data curation, Formal analysis. Zi-Jing Jin: Conceptualization, Methodology. Learn-Han Lee: Writing – review. Chitti Thawai: Data curation, Conceptualization. Ya-Wen He: Supervision, Writing – review & editing, Funding acquisition, Project administration.

Declaration of competing interest

The authors have no interests to declare.

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Reference

[1] Takeda R. Pseudomonas pigments: I. Pyoluterin, a new chlorinecontaining pigment produced by *Pseudomonas aeruginosa*. Hako Kogaku Zasshi 1958;36: 281-90.

[2] Gross H, Loper JE. Genomics of secondary metabolite production by *Pseudomonas spp*. Natural product reports 2009;26(11):1408-1446. https://doi.org/10.1039/B817075B.

[3] Hu HB, Xu YQ, Feng C, Zhang XH, Hur BK. Isolation and characterization of a new fluorescent *Pseudomonas* strain that produces both phenazine 1-carboxylic acid and pyoluteorin. Journal of Microbiology and Biotechnology 2005;15(1): 86-90. https://doi.org/10.1016/j.bmc.2015.06.021.

[4] Howell CR, Stipanovic RD. Suppression of *Pythium ultimum*-induced damping-off of cotton seedlings by *Pseudomonas fluorescens* and its antibiotic,pyoluteorin. Phytopathology 1980;70:712-715. https://doi.org/10.1094/Phyto-70-712.

[5] Kamoun S, Furzer O, Jones JDG, Judelson HS, Ali GS, Dalio R, et al. The Top 10 oomycete pathogens in molecular plant pathology. Molecular plant pathology 2015;16(4):413-434. https://doi.org/10.1111/mpp.12190.

[6] Pellicciaro M, Padoan E, Lione G, Celi L, Gonthier P. Pyoluteorin Produced by the Biocontrol Agent *Pseudomonas protegens* Is Involved in the Inhibition of *Heterobasidion* Species Present in Europe. Pathogens 2022;11(4):391. https://doi.org/10.3390/pathogens11040391.

[7] Cascioferro S, Raimondi MV, Cusimano MG, Raffa D, Maggio B, Daidone G, et al. Pharmaceutical potential of synthetic and natural pyrrolomycins. Molecules 2015;20(12):21658-21671. https://doi.org/10.3390/molecules201219797.

[8] Ding T, Yang LJ, Zhang WD, Shen YH. Pyoluteorin induces cell cycle arrest and apoptosis in human triple-negative breast cancer cells MDA-MB-231. Journal of Pharmacy and Pharmacology 2020;72(7):969-978. https://doi.org/10.1111/jphp.13262.

[9] Fan D, Yu S, Yang Y, Qu S. Pyoluteorin Induces Apoptosis and Autophagy in NSCLC Cells. Biological and Pharmaceutical Bulletin 2021;44(7):976-983. https://doi.org/10.1248/bpb.b21-00120.

[10] Li S, Huang XQ, Wang G, Xu YQ. Transcriptional activation of pyoluteorin operon mediated by the LysR-type regulator PltR bound at a 22 bp lys box in *Pseudomonas aeruginosa* M18. PloS one 2012;7(6):e39538. https://doi.org/10.1371/journal.pone.0039538.

[11] Yan Q, Liu M, Kidarsa T, Johnson CP, Loper JE. Two Pathway-Specific Transcriptional Regulators, PltR and PltZ, Coordinate Autoinduction of Pyoluteorin in *Pseudomonas protegens* Pf-5. Microorganisms 2021;9(7):1489. https://doi.org/10.3390/microorganisms9071489.

[12] Shi HM, Huang XQ, Wang Z, Guan YJ, Zhang XH. Improvement of pyoluteorin production in *Pseudomonas protegens* H78 through engineering its biosynthetic and regulatory pathways. Applied microbiology and biotechnology 2019;103(8): 3465-3476. https://doi.org/10.1007/s00253-019-09732-z.

[13] Nowak-Thompson B, Chaney N, Wing JS, Gould SJ, Loper JE. Characterization of the pyoluteorin biosynthetic gene cluster of *Pseudomonas fluorescens* Pf-5. Journal of bacteriology 1999;181(7):2166-2174. https://doi.org/10.1128/JB.181.7.2166-2174.1999.

[14] Brodhagen M, Paulsen I, Loper JE. Reciprocal regulation of pyoluteorin production with membrane transporter gene expression in *Pseudomonas*

fluorescens Pf-5. Applied and Environmental Microbiology 2005;71(11):6900-6909. https://doi.org/10.1128/AEM.71.11.6900-6909.2005.

[15] Yan Q, Philmus B, Chang JH, Loper JE. Novel mechanism of metabolic co-regulation coordinates the biosynthesis of secondary metabolites in *Pseudomonas protegens*. Elife 2017;6:e22835. https://doi.org/10.7554/eLife.22835.

[16] Guo DD, Luo LM, Ma HL, Zhang SP, Xu H, Zhang H, et al. The Regulator PltZ Regulates a Putative ABC Transporter System PltIJKNOP of *Pseudomonas aeruginosa* ATCC 27853 in Response to the Antimicrobial 2, 4-Diacetylphloroglucinol. Frontiers in microbiology 2020;11:1423. https://doi.org/10.3389/fmicb.2020.01423.

[17] Wang Z, Huang XQ, Liu YJ, Yang GH, Liu Y, Zhang XH. GacS/GacA activates pyoluteorin biosynthesis through Gac/Rsm‐RsmE cascade and RsmA/RsmE-driven feedback loop in *Pseudomonas protegens* H78. Molecular microbiology 2017;105(6):968-985. https://doi.org/10.1111/mmi.13749.

[18] Huang XQ, Zhang XH, Xu YQ. PltR expression modulated by the global regulators GacA, RsmA, LasI and RhlI in *Pseudomonas* sp. M18. Research in Microbiology 2008;159(2):128-136. https://doi.org/10.1016/j.resmic.2007.10.006.

[19] Chen Y, Wang XL, Huang XQ, Zhang XH, Xu YQ. Las-like quorum-sensing system negatively regulates both pyoluteorin and phenazine-1-carboxylic acid production in *Pseudomonas sp.* M18. Science in China Series C: Life Sciences 2008;51(2):174-181. https://doi.org/10.1007/s11427-008-0026-8.

[20] Lu JS, Huang XQ, Li K, Li SN, Zhang MY, Wang Y, et al. LysR family transcriptional regulator PqsR as repressor of pyoluteorin biosynthesis and activator of phenazine-1-carboxylic acid biosynthesis in *Pseudomonas sp.* M18. Journal of biotechnology 2009;143(1):1-9. https://doi.org/10.1016/j.jbiotec.2009.06.008.

[21] Yan A, Huang X, Liu HM, Dong DX, Zhang DB, Zhang XH, et al. An rhl-like quorum-sensing system negatively regulates pyoluteorin production in *Pseudomonas sp.* M18. Microbiology 2007;153(1):16-28. https://doi.org/10.1099/mic.0.29211-0.

[22] McGill S L, Yung Y, Hunt K A, Henson MA, Hanley L, Carlson RP. Pseudomonas aeruginosa reverse diauxie is a multidimensional, optimized, resource utilization strategy. Scientific reports 2021;11(1):1-16. https://doi.org/10.1038/s41598-020-80522-8.

[23] Amador C I, Canosa I, Govantes F, Santero E. Lack of CbrB in *Pseudomonas putida* affects not only amino acids metabolism but also different stress responses and biofilm development. Environmental Microbiology 2010;12(6):1748-1761. https://doi.org/10.1111/j.1462-2920.2010.02254.x.

[24] Bharwad K, Rajkumar S. Rewiring the functional complexity between Crc, Hfq and sRNAs to regulate carbon catabolite repression in *Pseudomonas*. World Journal of Microbiology and Biotechnology 2019;35(9):1-12.https://doi.org/10.1007/s11274-019-2717-7.

[25] Moreno R, Hernández‐Arranz S, La Rosa R, Yuste L, Madhushani A, Shingler V et al. The Crc and Hfq proteins of *P seudomonas putida* cooperate in catabolite repression and formation of ribonucleic acid complexes with specific target motifs. Environmental microbiology 2015;17(1):105-118. https://doi.org/10.1111/1462-2920.12499.

[26] Nishijyo T, Haas D, Itoh Y. The CbrA–CbrB two‐component regulatory system

controls the utilization of multiple carbon and nitrogen sources in *Pseudomonas aeruginosa*. Molecular microbiology 2001;40(4):917-931. https://doi.org/10.1046/j.1365-2958.2001.02435.x.
[27] Sonnleitner E, Romeo A, Blaesi U. Small regulatory RNAs in *Pseudomonas aeruginosa*. RNA biology 2012;9(4):364-371. https://doi.org/10.4161/rna.19231.
[28] Rojo F. Carbon catabolite repression in *Pseudomonas*: optimizing metabolic versatility and interactions with the environment. FEMS microbiology reviews 2010;34(5):658-684. https://doi.org/10.1111/j.1574-6976.2010.00218.x.
[29] Sonnleitner E, Bläsi U. Regulation of Hfq by the RNA CrcZ in *Pseudomonas aeruginosa* carbon catabolite repression. PLoS Genetics 2014;10(6):e1004440. https://doi.org/10.1371/journal.pgen.1004440.
[30] Santiago‐Frangos A, Woodson S A. Hfq chaperone brings speed dating to bacterial sRNA. Wiley Interdisciplinary Reviews: RNA 2018;9(4):e1475. https://doi.org/10.1002/wrna.1475.
[31] Sun S, Zhou L, Jin KM, Jiang HX, He YW. Quorum sensing systems differentially regulate the production of phenazine-1-carboxylic acid in the rhizobacterium *Pseudomonas aeruginosa* PA1201. Scientific reports 2016;6(1):1-14. https://doi.org/10.1038/srep30352.
[32] A Becher, HP Schweizer. Integration-proficient *Pseudomonas aeruginosa* vectors for isolation of single-copy chromosomal *lacZ* and *lux* gene fusions. Biotechniques 2000;29(5):948-950. https://doi.org/10.2144/00295bm04.
[33] Sonnleitner E, Abdou L, Haas D. Small RNA as global regulator of carbon catabolite repression in *Pseudomonas aeruginosa*. Proc Natl Acad Sci U S A 2009; 106(51):21866-71. https://doi.org/10.1073/pnas.0910308106.
[34] Monteagudo-Cascales E, Santero E, Inés Canosa. The Regulatory Hierarchy Following Signal Integration by the CbrAB Two-Component System: Diversity of Responses and Functions. Genes 2022;13(2):375-. https://doi.org/10.3390/genes13020375.
[35] Martínez-Valenzuela M, Guzmán J, Moreno S, Ahumada-Manuel CL, Espín G, Núñez C. Expression of the sRNAs CrcZ and CrcY modulate the strength of carbon catabolite repression under diazotrophic or non-diazotrophic growing conditions in *Azotobacter vinelandii*. PLoS ONE 2018;13(12). https://doi.org/10.1371/journal.pone.0208975.
[36] Pei XY, Dendooven T, Sonnleitner E, Chen S, Bläsi U, Luisi BF. Architectural principles for Hfq/Crc-mediated regulation of gene expression. Elife 2019;8:e43158. https://doi.org/10.7554/eLife.43158.
[37] Sonnleitner E, Wulf A, Campagne S, Pei XY, Wolfinger MT, Forlani G, et al. Interplay between the catabolite repression control protein Crc, Hfq and RNA in Hfq-dependent translational regulation in *Pseudomonas aeruginosa*. Nucleic acids research 2018;46(3):1470-1485. https://doi.org/10.1093/nar/gkx1245.
[38] Vinay JU, Naik MK, Rangeshwaran R, Chennappa G, Shaikh SS, Sayyed RZ. Detection of antimicrobial traits in *fluorescent pseudomonads* and molecular characterization of an antibiotic pyoluteorin. 3 Biotech 2016;6(2): 227. https://doi.org/10.1007/s13205-016-0538-z.
[39] Matuszewska M, Macig T, Rajewska M, Wierzbicka A, Jafra S. The carbon source-dependent pattern of antimicrobial activity and gene expression in *Pseudomonas donghuensis* P482. Scientific Reports 2021;11(1):10994. https://doi.org/10.1038/s41598-021-90488-w.
[40] Yuan Z, Song C, Matsufuji M, Nakata K, Yoshimoto A. High production of pyoluteorin and 2,4-diacetylphloroglucinol by *Pseudomonas fluorescens* S272

grown on ethanol as a sole carbon source. Journal of Fermentation & Bioengineering 1998;86(6):559-563. https://doi.org/10.1016/S0922-338X(99)80006-3.

[41] Duffy B K, Geneviève D. Environmental factors modulating antibiotic and siderophore biosynthesis by *Pseudomonas fluorescens* biocontrol strains.Applied and Environmental Microbiology 1999;65(6):2429-2438. https://doi.org/10.1128/AEM.65.6.2429-2438.1999.

[42] Jaremko MJ, Lee DJ, Opella SJ, Burkart MD. Structure and substrate sequestration in the pyoluteorin type peptidyl carrier protein PltL. Journal of the American Chemical Society 2015;137(36):11546-11549. https://doi.org/10.1021/jacs.5b04525.

[43] Wang Z, Huang XQ, Jan M, Kong DY, Pan JW, Zhang XH. The global regulator Hfq exhibits far more extensive and intensive regulation than Crc in *Pseudomonas protegens* H78. Molecular Plant Pathology 2021;22(8):921-938. https://doi.org/10.1111/mpp.13070.

[44] Humair B, Wackwitz B, Haas D. GacA-Controlled activation of promoters for small RNA genes in *Pseudomonas fluorescens*. Applied and Environmental Microbiology 2010;76(5):1497-1506. https://doi.org/10.1128/AEM.02014-09.

[45] Huang XQ, Zhang XH, Xu YQ. Positive regulation of pyoluteorin biosynthesis in *Pseudomonas* sp. M18 by quorum-sensing regulator VqsR. Journal of Microbiology and Biotechnology 2008;18(5):828-836. https://doi.org/10.1016/j.mimet.2008.03.001.

[46] Liu YJ, Shi HM, Wang Z, Huang XQ, Zhang XH. Pleiotropic control of antibiotic biosynthesis, flagellar operon expression, biofilm formation, and carbon source utilization by RpoN in *Pseudomonas protegens* H78. Applied microbiology and biotechnology 2018;102(22):9719-9730. https://doi.org/10.1007/s00253-018-9282-0.

[47] Zhang QX, Ji YY, Xiao Q, Chng S, Tong YH, Chen XJ, et al. Role of Vfr in the regulation of antifungal compound production by *Pseudomonas fluorescens* FD6. Microbiological Research 2016;(188-189):106-112. https://doi.org/10.1016/j.micres.2016.04.013.

[48] Xie YL, Liu ZQ, Zhang GY, Mo XT, Ding XZ, Xia LQ, et al. A rifampicin-resistant (*rpoB*) mutation in *Pseudomonas protegens* Pf-5 strain leads to improved antifungal activity and elevated production of secondary metabolites. Research in Microbiology 2016;167(8):625-629. https://doi.org/10.1016/j.resmic.2016.05.001.

[49] Yan Q, Philmus B, Hesse C, Kohen M, Chang JH, Loper JE. The Rare Codon AGA Is Involved in Regulation of Pyoluteorin Biosynthesis in *Pseudomonas protegens* Pf-5. Frontiers in Microbiology 2016;7:497. https://doi.org/10.3389/fmicb.2016.00497.

[50] B Görke, J Stülke. Carbon catabolite repression in bacteria: many ways to make the most out of nutrients. Nature Reviews Microbiology 2008;6(8):613. https://doi.org/10.1038/nrmicro1932.

[51] Hassan MN, Afghan S, Hafeez FY. Biological control of red rot in sugarcane by native pyoluteorin-producing *Pseudomonas putida* strain NH-50 under field conditions and its potential modes of action. Pest Management Science 2011; 67(9):1147-1154. https://doi.org/10.1002/ps.2165.

Figure legends:

Figure 1. Plt production by PA1201 strain in different media. (A) Growth kinetics of PA1201 in KMB, LB, PPM and MM media. (B) Plt production at 24- and 48-hours post inoculation (hpi). The data are shown as the averages of three technical repeats with standard deviation. Statistical significance between groups, assessed by ANOVA followed by Fisher's protected least significant difference test, are shown as one asterisk ($p \leq 0.05$) or two asterisks ($p \leq 0.01$).

Figure 2. Fructose promotes Plt production in minimal medium (M). (A) Growth kinetics of PA1201 in minimal M medium (KH_2PO_4 4.5 g/L, K_2HPO_4 10.5 g/L, $(NH_4)_2SO_4$ 2.0 g/L, $MgSO_4.7H_2O$ 0.16 g/L, $FeSO_4.7H_2O$ 0.005 g/L,$CaCl_2.2H_2O$ 0.011 g/L, $MnCl_2.4H_2O$ 0.002 g/L), supplemented with 5 mM carbohydrates, as indicated: Fru: fructose; Mal: maltose; Glu: glucose; Sor:sorbitol; Suc: sucrose; Lac: lactose; Gal: galactose; Xyl: xylose; Succ: succinic acid. (B) Plt level in PA1201. (C) Plt level at 48 hpi in M medium supplemented with 5–20 mM fructose. The data are shown as the averages of three technical repeats with standard deviation. Statistical significance between groups, assessed by ANOVA followed by Fisher's protected least significant difference test, are shown as one asterisk ($p \leq 0.05$) or two asterisks ($p \leq 0.01$).

Figure 3. Glucose or succinic acid antagonizes fructose-induced Plt biosynthesis.(A) Growth kinetics of PA1201 in MF medium supplemented with 1–10 mM glucose, or (B), 1-10 mM succinic acid. (C) Plt levels at 24- and 48-hpi in MF medium supplemented with 1-10 mM, or (D), 1–10 mM succinic acid. The data are shown as the averages of three technical repeats with standard deviation. Statistical significance between groups, assessed by ANOVA followed by Fisher's protected least significant difference test, are shown as one asterisk ($p \leq 0.05$), two asterisks ($p \leq 0.01$), or three asterisks ($p \leq 0.001$)

Figure 4. Effects of fructose, glucose, and succinic acid on *pltL* expression. (A) Plt gene cluster and the three studied promoters. (B) Representative pictures showing the colonies carrying different reporter transgenes PA1201-*lacZ* (negative control),

PA1201::P_{pltL}-*lacZ*, PA1201::P_{pltH}-*lacZ* and PA1201::P_{pltR}-*lacZ* on the MF agar plate supplemented with 40 mg/L X-gal. (C) Effects of 5–20 mM fructose on P_{pltR}-, P_{pltH}- and P_{pltL}-dependent β-galactosidase activity in PA1201 at 48 hpi. (D) P_{pltL}-dependent β-galactosidase activity in PA1201 cultured in MF, MFS10, and MFG10 media. The data are shown as the averages of three technical repeats with standard deviation. Statistical significance between groups, assessed by ANOVA followed by Fisher's protected least significant difference test, are shown as one asterisk ($p \leq 0.05$) or two asterisks ($p \leq 0.01$).

Figure 5. CbrB positively regulates Plt biosynthesis in PA1201. (A) Growth kinetics and Plt production by the strains PA1201(pBBR), Δ*cbrB*(pBBR) and Δ*cbrB*(pBBR-*cbrB*) at 24- and 48-hpi in MF medium, in (B) MFS medium, and in (C) MFG medium. The data are shown as the averages of three technical repeats with standard deviation. Statistical significance between groups, assessed by ANOVA followed by Fisher's protected least significant difference test, are shown as one asterisk ($p \leq 0.05$) or two asterisks ($p \leq 0.01$).

Figure 6. sRNA CrcZ positively regulates Plt biosynthesis in PA1201. (A) *cbrA*, *cbrB*, and *crcZ* loci on PA1201 chromosome. (B) Growth kinetics and Plt production of the strains PA1201(pBBR), Δ*crcZ*(pBBR) and Δ*crcZ*(pBBR-*crcZ*) at 24- and 48-hpi in MF medium, in (C) MFS10 medium, or in (D) MFG10 medium. The data are shown as the averages of three technical repeats with standard deviation. Statistical significance between groups, assessed by ANOVA followed by Fisher's protected least significant difference test, are shown as one asterisk ($p \leq 0.05$) or two asterisks ($p \leq 0.01$).

Figure 7. Role of Hfq in Plt biosynthesis. (A) Plt production by PA1201(pBBR), Δ*hfq*(pBBR), and Δ*hfq*(pBBR-*hfq*) at 24- and 48-hpi in MF medium. (B) P_{pltL}-dependent β-galactosidase activity in strains PA1201 and Δ*hfq* cultured in MF, MFG10, or MFS10 medium. (C) Plt production by PA1201(pBBR), Δ*hfq*(pBBR) and Δ*hfq*(pBBR-*hfq*) at 24- and 48-hpi in MFS10 medium, or in (D) MFG10 medium. The

data are shown as the averages of three technical repeats with standard deviation. Statistical significance between groups, assessed by ANOVA followed by Fisher's protected least significant difference test, are shown as one asterisk ($p \leq 0.05$) or two asterisks ($p \leq 0.01$).

Figure 8. Role of Crc in Plt biosynthesis. (A) *crc* locus on PA1201 chromosome. (B)Growth kinetics and Plt production by PA1201(pBBR), Δ*crc*(pBBR), and Δ*crc*(pBBR-*crc*) at 24- and 48-hpi in MF medium, or in (C) MFS10 and MFG10 media. The data are shown as the averages of three technical repeats with standard deviation. Statistical significance between groups, assessed by ANOVA followed by Fisher's protected least significant difference test, are shown as one asterisk ($p \leq 0.05$) or two asterisks ($p \leq 0.01$).

Figure 9. Model of regulation of pyoluteorin biosynthesis by carbohydrates via carbon catabolite repression (CCR) mechanism. (A) Plt production is limited in the absence of fructose, mannitol and glycerol or in the presence of both fructose/mannitol/glycerol and glucose/succinic acid. (B) Plt production is induced in the presence of sole fructose/mannitol/glycerol.