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Paraburkholderia phymatum STM815 σ^{54} Controls Utilization of Dicarboxylates, Motility, and T6SS-b Expression

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Abstract: Rhizobia have two major life styles, one as free-living bacteria in the soil, and the other as bacteroids within the root/stem nodules of host legumes where they convert atmospheric nitrogen into ammonia. In the soil, rhizobia have to cope with changing and sometimes stressful environmental conditions, such as nitrogen limitation. In the beta-rhizobial strain *Paraburkholderia phymatum* STM815, the alternative sigma factor σ^{54} (or RpoN) has recently been shown to control nitrogenase activity during symbiosis with *Phaseolus vulgaris*. In this study, we determined *P. phymatum*'s σ^{54} regulon under nitrogen-limited free-living conditions. Among the genes significantly downregulated in the absence of σ^{54}, we found a C_4-dicarboxylate carrier protein (Bphy_0225), a flagellar biosynthesis cluster (Bphy_2926-64), and one of the two type VI secretion systems (T6SS-b) present in the *P. phymatum* STM815 genome (Bphy_5978-97). A defined σ^{54} mutant was unable to grow on C_4 dicarboxylates as sole carbon source and was less motile compared to the wild-type strain. Both defects could be complemented by introducing *rpoN in trans*. Using promoter reporter gene fusions, we also confirmed that the expression of the T6SS-b cluster is regulated by σ^{54}. Accordingly, we show that σ^{54} affects in vitro competitiveness of *P. phymatum* STM815 against *Paraburkholderia diazotrophica*.

Keywords: rhizobia; σ factor; RpoN; RNA-Sequencing; nitrogen; motility; type VI secretion system (T6SS)

1. Introduction

Symbioses between legumes and rhizobia increase soil fertility and crop yield by means of biological nitrogen fixation [1]. Rhizobia are soil bacteria, which adapt to different environmental stresses and eventually nodulate the roots or the stems of compatible legume plants. In the symbiotic organ—the nodule—bacteria live inside the host plant cells and eventually differentiate into nitrogen-fixing bacteroids. Bacteroids convert atmospheric nitrogen (N_2) into ammonia that is assimilated and used as a nitrogen source by the plant [2,3]. To support this energetically expensive reaction (16 molecules of ATP and 8 low potential electrons per N_2 reduced), the legume provides the bacteroids with energy in form of reduced carbon compounds. C_4-dicarboxylates such as succinate, fumarate, and malate have been shown to be the primary carbon source used by bacteroids and are oxidized to CO_2 in the tricarboxylic acid cycle [4].

Rhizobia are phylogenetically diverse and belong to the alpha-proteobacterial (alpha-rhizobia) and the beta-proteobacterial group (beta-rhizobia). Beta-rhizobial strains such as *Burkholderia* and *Cupriavidus* were first isolated from nodules in 2001 [5,6], and most of the legume nodulating *Burkholderia*

strains were recently re-classified into the new *Paraburkholderia* genus [7,8]. Symbiotic *Paraburkholderia* species have been mainly isolated from nodules of *Mimosa* plants in South America and South East Asia [9–12], but also from South African Fynbos [13–16]. Geographical position, environment, host plant, and coevolution with the symbiont have been shown to affect the presence and dominance of certain rhizobial species in soil and in nodules [17,18]. *Paraburkholderia phymatum* STM815 is an interesting strain since it is able to nodulate mimosoid as well as papilionoid legumes. Furthermore, it has been shown by several groups to be highly competitive in infecting the roots of mimosoid and papilionoid legumes [17,19–21]. A comparison of the phenotypic traits of several *Paraburkholderia* strains showed that *P. phymatum* STM815 produces large amounts of exopolysaccharides (EPS), is very motile, and able to outcompete other *Paraburkholderia* strains in vitro [20]. Our group has also shown that *P. phymatum* STM815 harbors two type VI secretion systems (T6SS) in its genome, which contribute to the competitive ability of this strain in vitro and in infecting plants [22]. These characteristics partly explain the success of this strain in competing with other rhizobia in the soil. However, the regulatory networks underlying the high competitiveness of *P. phymatum* STM815 in infecting several legumes are still unknown.

The σ factor σ^{54} is structurally distinct from the σ^{70}-type sigma factors, recognizes different promoter elements located at position −24 and −12 upstream of the transcription start site [23], and requires an enhancer-binding protein (EBP) to activate transcription of target genes. Usually bacteria encode multiple different EBPs in their genome, with each of them controlling different traits required to adapt to specific ecological niches [24]. We recently showed that the alternative σ factor σ^{54} is a key regulator of *P. phymatum* STM815 symbiotic nitrogen fixation inside root nodules of *Phaseolus vulgaris* (common bean) [25]. In fact, a σ^{54} mutant did not form an efficient symbiosis with *P. vulgaris* and was impaired in reducing N_2 to ammonium. By using RNA-Seq and metabolomics on bean root nodules formed by *P. phymatum* STM815 wild type and a σ^{54} mutant [26], we found that in addition to the symbiotic genes, σ^{54} also controls several genes potentially important for *P. phymatum* STM815 to persist in soil.

In this study, we analyzed the regulon of σ^{54} (Bphy_0326) by growing the cells under free-living conditions in a nitrogen-limited medium. Among the top genes activated by σ^{54}, we found *dctA*, which codes for a C_4 dicarboxylate transporter, a flagellar gene cluster, and one of the two T6SS present in the genome of *P. phymatum* STM815 (T6SS-b). Indeed, we confirmed that the σ^{54} mutant was not able to grow on C_4 dicarboxylates, was impaired in swimming motility, and that both these defects were complemented by providing *rpoN in trans*. Moreover, the mutation in *rpoN* affected the expression of the T6SS-b gene cluster and rendered *P. phymatum* STM815 slightly less competitive against *Paraburkholderia diazotrophica* [27], suggesting that σ^{54} is involved in the control of interbacterial competition.

2. Materials and Methods

2.1. Bacterial Strains, Media, and Cultivation

The strains, plasmids, and primers employed in this study are listed in Table S1. *Escherichia coli* cells were routinely grown in Luria-Bertani liquid medium (LB), whereas *P. phymatum* STM815 strains were cultivated in LB salt-free liquid medium [20].

The bacterial cultures used for RNA-Seq were prepared by growing *P. phymatum* cells in a modified AB minimal medium [28], with 10 mM sodium citrate (Sigma-Aldrich) as carbon source. The nitrogen source in AB minimal medium $(NH_4)_2SO_4$ was replaced with either 30 mM NH_4Cl (Sigma-Aldrich, to obtain nitrogen-replete conditions) or 0.5 mM NH_4Cl (nitrogen-limited condition). Na_2SO_4 (Sigma-Aldrich) was added to AB minimal medium to obtain a final concentration of 15 mM of sulfate. Bacterial cultures were grown in 250 mL Erlenmeyer flasks containing 100 mL medium and incubated on a shaker (220 rpm) at 30 °C. *P. phymatum* STM815 wild-type and σ^{54} mutant cells were grown to exponential phase (OD_{600} = 0.4–0.5) in minimal medium under nitrogen-replete conditions, then washed twice in AB minimal medium without a nitrogen source and incubated further aerobically

for one hour under nitrogen-limited conditions. For each strain, three independent biological replicates were prepared and processed for RNA-Seq analysis.

To test the induction of the promoter fusions, the bacteria were grown at 30 °C in AB medium containing 10 mM glucose as C-source (ABG) in a 96-well plate (Falcon, Corning, USA) to exponential phase. The optical density and GFP fluorescence (excitation/emission wavelength equal to 488 nm/520 nm) were measured using a TECAN plate reader (TECAN Infinite M200 PRO, Tecan Trading AG, Switzerland).

The growth of the following *P. phymatum* STM815 strains—wild type (wt), σ^{54} mutant, and σ^{54} complemented—was tested in AB minimal medium with 15 mM of three different C_4-carbon sources: Fumaric acid (Sigma-Aldrich, ABF), malic acid (Sigma-Aldrich, ABM), and succinic acid (Sigma-Aldrich, ABS). For each strain and C-source tested, the growth of three independent biological replicates was measured.

2.2. Promoter Fusion Construction

To construct promoter fusions, the two promoters of interest were introduced into the vector pPROBE-NT (Table S1) [29]. To construct pPROBE-T6SS-b (p5978), the promotor of T6SS-b was amplified from *P. phymatum* STM815 genomic DNA (gDNA) with the primers Bphy_5978_Promotor_F_HindIII and Bphy_5978_Promotor_R_BamH1 (Table S1). The 657 bp long PCR product was restricted with the enzymes HindIII and BamHI and cloned into pPROBE-NT. The correct sequence was confirmed by sequencing at Microsynth (Balgach, Switzerland) using pCO13-R3 primer [30]. The plasmid was then conjugated into *P. phymatum* STM815 wild type and σ^{54} mutant, and the transconjugants were selected on ABS plates. To obtain the second promoter fusion, the promotor region of the T6SS-3 cluster (p6115) was amplified using primers p6115_SalI_For and p6115_EcoRI_rev (Table S1). The obtained PCR fragment (480 bp) was digested and cloned into pPROBE-NT between the SalI and EcoRI sites. Once the sequence of the construct had been confirmed through sequencing at Microsynth using pCO13-R3 primer [30], the plasmid was mobilized into *P. phymatum* STM815 wild type and σ^{54} mutant.

2.3. RNA-Sequencing and Data Processing

Total RNA was isolated from flash-frozen, pelleted cells of the wild type and the σ^{54} mutant using a modified hot acid phenol protocol [31]. Afterwards, gDNA was digested by DNase treatment and the quality of the total RNA as well as the complete removal of gDNA were checked by PCR [25,26]. The cDNA synthesis was initiated with 150 ng of total RNA and the library was prepared and purified with the Ovation® Complete Prokaryotic RNA-Seq DR Multiplex Systems (NuGEN, San Carlos, CA, USA) [25,26]. Next, the quality and quantity of the cDNA libraries were analyzed with a TapeStation System (Agilent Technologies, Santa Clara, CA, USA). The prepared cDNA libraries were single-end sequenced with a HiSeq2500 instrument (Illumina, San Diego, CA, USA) at the Functional Genomic Center Zurich (FGCZ). Using the CLC Genomics Workbench v7.0 (QIAGEN CLC bio, Aarhus, Denmark) program, the obtained reads were trimmed to 70 bp and mapped to the *P. phymatum* STM815 genome, allowing up to two mismatches per read [32]. Afterwards the unique reads were analyzed statistically with the *DESeq* R-package (version 1.30.0) [33]. The top 200 significantly RpoN-regulated genes with a $\log_2(FC) \geq 1$ and ≤ -1 were considered and then ranked by ascending *p*-value. To get additional information, the top 200 differentially regulated genes were assigned to functional categories (EggNOG v3.0) [34]. Table S2 lists all *P. phymatum* STM815 genes and their expression profile in the wild type and the σ^{54} mutant. The RNA-Seq raw and processed data files of the *P. phymatum* STM815 wild-type and σ^{54} mutant strains are available with the GSE156048 accession number.

2.4. Phenotypic Analysis

Plates for the swimming motility test were prepared by using LB salt-free medium containing 1% tryptone (Difco), 0.5% yeast extract (Difco), and 0.2% agar. The bacterial cells were washed twice with 10 mM $MgSO_4$ and normalized to an OD_{600} of 0.5. The plates were inoculated with the bacterial

suspension using a 20 µL pipette tip. Subsequently, the plates were incubated at 30 °C inside a box containing wet paper towels. The diameter of the swimming zone was measured after 40 h incubation.

To perform competition experiments on plates, a target strain and an attacker strain were chosen, plated on salt-free LB without antibiotics and incubated overnight at 30 °C. The strains were washed twice with 10 mM $MgSO_4$ and the OD_{600} was adjusted (target strain OD_{600} = 0.2, attacker strain OD_{600} = 2.0). The strains were mixed in a 1:1 volume ratio. Twenty µl aliquots of the mixture were put on cellulose nitrate membrane filters (GE Healthcare Life Sciences, Marlborough, MA, USA) on ABG plates, and incubated at 30 °C. After 24 h, the cells on the filters were resuspended in one ml 10 mM $MgSO_4$. The colony forming units (CFU) were determined on salt-free LB plates, both with and without antibiotics, to recover the attacker and target strains, respectively.

2.5. Statistical Analysis

For category distribution, the percentages of upregulated and downregulated genes in each category were calculated as described previously [25]. One-way ANOVA (performed with Prism 7.0), in which the mean of each strain was compared to the mean of the wild type, was used to analyze the motility data. The one unpaired *t*-test corrected for multiple comparisons using the Holm–Sidak method (Prism 7.0) was employed to statistically analyze the results of the competition on plate. The induction of the promoter fusions was tested with two-way ANOVA corrected for multiple comparisons using the Sidak test (Prism 7.0).

3. Results

3.1. The P. phymatum STM815 σ^{54} Regulon in Free-Living Nitrogen-Limiting Conditions

RNA-Seq was employed to determine the *P. phymatum* STM815 σ^{54} regulon under nitrogen-limited free-living conditions. For each strain, three independent biological replicates were prepared, processed, and analyzed as described previously [25]. The unique read counts obtained per sample ranged from 6.9 to 13.2 million reads.

After a *DESeq* analysis comparing gene expression in the *P. phymatum* STM815 σ^{54} mutant versus wild type, the top 200 differentially regulated genes (*DESeq* analysis *p*-value $< 2.2 \times 10^{-8}$, with $\log_2$ [FC] ≥ 1 and ≤ -1) were identified and classified in eggNOG functional categories [34] (Figure 1). Among these 200 top-regulated genes, 81 showed increased expression, while 119 were downregulated in the σ^{54} mutant (and were therefore positively regulated by σ^{54}). While the two categories "signal transduction mechanisms" and "cell motility" were over-represented among the downregulated genes (Table 1), one category ("cell wall, membrane, envelope biogenesis") was found to be over-represented in the upregulated genes.

In the category "cell motility", we found several genes involved in flagella biosynthesis (in the Bphy_2926-64 cluster) and a gene involved in chemotaxis (Bphy_5592). In the category "signal transduction mechanisms", several transcriptional regulatory genes (e.g., Bphy_3958, Bphy_5314, and Bphy_5669) and genes coding for histidine kinases (e.g., Bphy_0226, Bphy_3957, Bphy_3963, Bphy_4749, Bphy_5668, and Bphy_5975) were identified. Among these top differentially regulated genes, six might be part of a two-component regulatory system (TCRS): Bphy_3957-58, Bphy_3962-63, and Bphy_5668-69. Upstream of Bphy_5668-69, we found Bphy_5667, which codes for a citrate carrier and has an RpoN-binding sequence in the promoter region (Figure 2A). Moreover, downstream of Bphy_5668-69 we found a gene coding for a diguanylate phosphodiesterase potentially involved in c-di-GMP metabolism (Bphy_5670, Figure 2A). The histidine kinase Bphy_0226 (*dctB*) is located downstream of a gene coding for a C_4-dicarboxylate transporter (*dctA*, Bphy_0225), which was also on the list of top-regulated genes and contained an RpoN box in the promoter region (Figure 2B). In several bacteria, the DctA transporter has been shown to be involved in the assimilation of C_4 dicarboxylates, i.e., fumarate, malate, and succinate [4,35,36], which are important energy sources for symbiotic rhizobia [37]. Interestingly, most of the genes (13 of 20) belonging to one of the two T6SS

clusters present in *P. phymatum* STM815 (Bphy_5978-97, T6SS-b, Figure 2C) grouped in the category "function unknown" were downregulated in the σ^{54} mutant grown under nitrogen starvation. By contrast, the expression of the second T6SS cluster (Bphy_6107-6129, T6SS-3) was not dependent on σ^{54}. T6SSs are contact-dependent secretion systems that have been shown to be important for the transport of effectors to other prokaryotic or eukaryotic cells [38]. Interestingly, several genes located in a cluster coding for components of a type IV secretion system (T4SS, Bphy_7524-37) also showed significant downregulation in the σ^{54} mutant. Similar T4SSs are usually involved in conjugation [39].

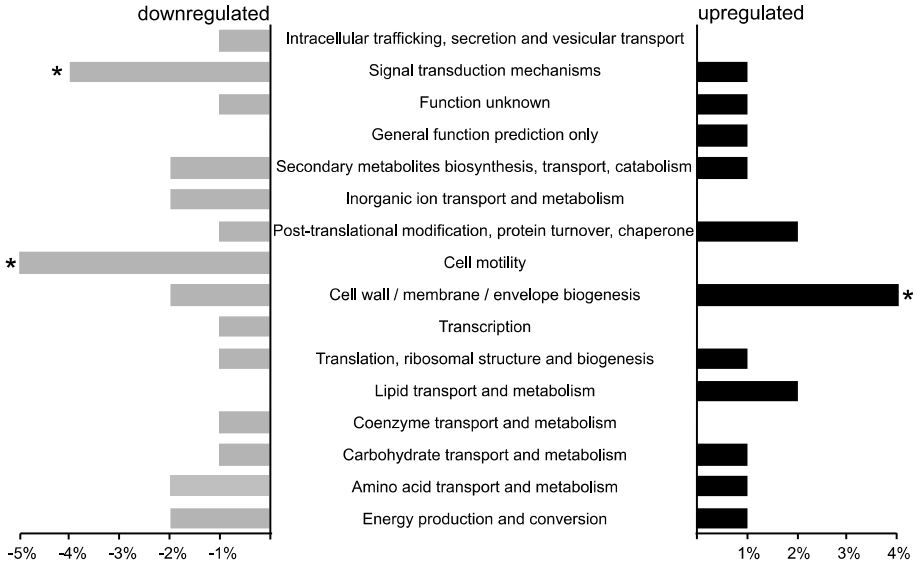

Figure 1. Functional categories of the top 200 differentially expressed genes of the *P. phymatum* STM815 wild type versus σ^{54} mutant (gray, downregulated genes, black upregulated genes) under nitrogen starvation according to eggNOG classification [34]. The asterisks (*) indicate statistical significance for over-represented genes in a particular category (p-value < 0.02).

In the category "carbohydrate transport and metabolism", we found several genes whose expression was downregulated in the σ^{54} mutant: Bphy_2145, coding for a trehalose-6-phosphate synthase (*otsA*), as well as two genes involved in glycogen metabolism (Bphy_5335 and Bphy_5336, *glgX* and *glgA*, Figure 2D). All three genes display an RpoN-binding sequence in their promoter region (Table 1). In the operon Bphy_3959-61, which encodes a potential nitrate/sulfonate/bicarbonate transporter, we found the most highly downregulated genes ($\log_2$(FC) = −7.1, −7.4, −6.0, for Bphy_3959 to Bphy_3961, respectively). Additionally, the expression of three genes in a cluster potentially coding for a polysaccharide (Bphy_3730-1 and Bphy_3733) was downregulated in the σ^{54} mutant compared to the wild type.

As previously mentioned, among the 81 genes upregulated in the mutant, the category "cell wall/membrane/envelope biogenesis" was over-represented, with several genes coding for porins (Bphy_0154, Bphy_1082 and Bphy_2684), as well as a cluster coding for the polysaccharide cepacian (Bphy_1056-77) [40]. Out of the 200 genes significantly regulated by RpoN, ten were located on the symbiotic plasmid including the T4SS and an aminotransferase class-III (Bphy_7651), which were all positively regulated by σ^{54}.

By applying a less stringent p-value cut-off than that selected for the top 200 differentially regulated genes (*DESeq* analysis p-value $\leq 2 \times 10^{-5}$), additional genes associated with nitrogen metabolism were found to be positively regulated by σ^{54}, such as the TCRS *ntrBC* (Bphy_1479-80), *urtB* (Bphy_2252) coding for a urea ABC transporter permease and the *glnB1-amtB* operon, coding for the nitrogen regulatory protein P-II and the ammonium transporter (Bphy_0256-57).

Table 1. List of 119 genes positively controlled by σ^{54}. Over-represented categories are marked with and asterisk (Fischer test, p-value < 0.02). Genes harboring a putative RpoN-box in their promoter region are shown in bold.

Locus ID [1]	Description [1]	Gene Name	Log_2FC (σ^{54} mt vs. wt) [2]
	Amino Acid Transport and Metabolism		
Bphy_0112	extracellular ligand-binding receptor		−1.1
Bphy_0588	extracellular ligand-binding receptor		−1.2
Bphy_0589	inner-membrane translocator		−1.8
Bphy_0590	inner-membrane translocator		−1.5
Bphy_0591	ABC transporter-like protein		−1.4
Bphy_2572	polar amino acid ABC transporter inner membrane subunit		−1.2
Bphy_2573	extracellular solute-binding protein		−1.3
Bphy_3017	extracellular ligand-binding receptor		−1.2
Bphy_3043	extracellular ligand-binding receptor		−1.4
Bphy_3046	inner-membrane translocator		−1.6
Bphy_3047	extracellular ligand-binding receptor		−1.2
Bphy_3780	cationic amino acid ABC transporter		−1.4
Bphy_5470	5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase		−1.9
Bphy_5613	methionine gamma-lyase		−2.1
	Carbohydrate Transport and Metabolism		
Bphy_1237	periplasmic binding protein/LacI transcriptional regulator		−1.2
Bphy_2145	trehalose-6-phosphate synthase	*otsA*	−2.4
Bphy_2615	monosaccharide-transporting ATPase		−1.1
Bphy_5335	glycogen debranching enzyme	*glgX*	−2.3
Bphy_5336	glycogen synthase	*glgA*	−2.6
Bphy_5372	extracellular solute-binding protein		−1.3
	Cell Motility		
Bphy_2938	flagellar motor switch protein FliM	*fliM*	−3.2
Bphy_2939	flagellar motor switch protein FliN	*fliN*	−3.6
Bphy_2956	flagellar rod assembly protein/muramidase FlgJ	*flgJ*	−2.9
Bphy_2962	flagellar biosynthesis regulator FlhF	*flhF*	−4.1
Bphy_5592	methyl-accepting chemotaxis sensory transducer		−2.0
	Cell Wall/Membrane/Envelope Biogenesis		
Bphy_0254	Porin		−1.1
Bphy_1082	Porin		−1.6
Bphy_2167	OmpC family outer membrane porin		−1.2
Bphy_2684	Porin		−2.2
Bphy_3730	glycosyl transferase family protein		−1.7
Bphy_3733	exopolysaccharide tyrosine-protein kinase		−2.0
Bphy_5991	aspartate racemase		−1.5
	Coenzyme Transport and Metabolism		
Bphy_3573	putative aminotransferase		−1.4
Bphy_7651	aminotransferase class-III		−1.3
	Energy Production and Conversion		
Bphy_0010	aldehyde dehydrogenase		−2.3
Bphy_0225	C4-dicarboxylate transporter	*dctA*	−4.7
Bphy_1368	isocitrate lyase		−1.4
Bphy_3971	cytochrome o ubiquinol oxidase subunit IV		−1.4
Bphy_4774	oxidoreductase alpha (molybdopterin) subunit		−1.1
Bphy_5667	citrate carrier protein		−7.1
Bphy_5990	sodium:dicarboxylate symporter		−1.7
Bphy_5992	sodium:dicarboxylate symporter		−1.2

Table 1. *Cont.*

Locus ID [1]	Description [1]	Gene Name	Log_2FC (σ^{54} mt vs. wt) [2]
	Function Unknown		
Bphy_0467	hypothetical protein		−1.1
Bphy_0675	hypothetical protein		−1.4
Bphy_0848	CsbD family protein		−1.3
Bphy_0980	Ku protein		−1.4
Bphy_1016	hypothetical protein		−1.3
Bphy_1217	hypothetical protein		−1.0
Bphy_2791	hypothetical protein		−1.2
Bphy_2880	hypothetical protein		−1.8
Bphy_3671	hypothetical protein		−1.7
Bphy_3731	hypothetical protein		−1.7
Bphy_3807	hypothetical protein		−2.5
Bphy_3844	hypothetical protein		−3.6
Bphy_3917	hypothetical protein		−1.4
Bphy_4035	hypothetical protein		−1.5
Bphy_4046	MbtH domain-containing protein		−2.3
Bphy_4787	hypothetical protein		−3.1
Bphy_5272	hypothetical protein		−1.6
Bphy_5337	putative lipoprotein		−3.7
Bphy_5431	hypothetical protein		−4.9
Bphy_5573	hypothetical protein		−1.5
Bphy_5699	hypothetical protein		−2.9
Bphy_5724	hypothetical protein		−2.5
Bphy_5976	hypothetical protein		−1.3
Bphy_5977	hypothetical protein		−2.3
Bphy_5978	type VI secretion protein		−2.1
Bphy_5979	EvpB family type VI secretion protein		−2.1
Bphy_5980	hypothetical protein		−2.2
Bphy_5981	type VI secretion system lysozyme-related protein		−2.0
Bphy_5982	type VI secretion protein		−1.9
Bphy_5983	type VI secretion protein		−1.5
Bphy_5985	type VI secretion system Vgr family protein		−1.4
Bphy_5996	IcmF-like protein		−1.0
Bphy_5998	hypothetical protein		−1.1
Bphy_7681	hypothetical protein		−1.3
Bphy_7682	hypothetical protein		−1.5
	General Function Prediction Only		
Bphy_5298	PfpI family intracellular peptidase		−2.7
Bphy_5303	transport-associated		−5.2
Bphy_5721	amidohydrolase 3		−2.1
	Inorganic Ion Transport and Metabolism		
Bphy_3175	NMT1/THI5-like domain-containing protein		−1.8
Bphy_3959	nitrate/sulfonate/bicarbonate ABC transporter periplasmic ligand-binding protein		−7.2
Bphy_3960	ABC transporter related		−7.4
Bphy_3961	binding-protein-dependent transport systems inner membrane component		−6.0
Bphy_4041	periplasmic binding protein		−1.8
	Intracellular Trafficking, Secretion and Vesicular Transport		
Bphy_7526	P-type DNA transfer ATPase VirB11		−1.3
Bphy_7530	VirB8 family protein		−1.5
	Lipid transport and metabolism		
Bphy_0466	GDSL family lipase		−1.9
	Post-translational Modification, Protein Turnover and Chaperones		
Bphy_2992	TPR repeat-containing protein		−1.3
Bphy_5984	type VI secretion ATPase		−1.4

Table 1. *Cont.*

Locus ID [1]	Description [1]	Gene Name	Log_2FC (σ^{54} mt vs. wt) [2]
	Secondary Metabolites Biosynthesis, Transport and Catabolism		
Bphy_0174	hypothetical protein		−1.1
Bphy_4036	lysine/ornithine N-monooxygenase		−1.9
Bphy_4038	amino acid adenylation domain-containing protein		−2.2
Bphy_5720	isochorismatase hydrolase		−2.4
	Signal Transduction Mechanisms		
Bphy_0226	integral membrane sensor signal transduction histidine kinase		−2.4
Bphy_3957	histidine kinase		−1.8
Bphy_3958	two component transcriptional regulator		−2.3
Bphy_3963	heavy metal sensor signal transduction histidine kinase		−1.2
Bphy_4749	PAS/PAC sensor hybrid histidine kinase		−1.2
Bphy_5314	sigma-^{54} dependent transcriptional regulator		−1.6
Bphy_5338	response regulator receiver protein		−3.5
Bphy_5668	integral membrane sensor signal transduction histidine kinase		−5.8
Bphy_5669	two component, sigma^{54} specific, Fis family transcriptional regulator		−4.9
Bphy_5670	diguanylate phosphodiesterase		−3.5
Bphy_5974	non-specific serine/threonine protein kinase		−2.6
Bphy_5975	histidine kinase		−1.7
Bphy_5989	extracellular solute-binding protein		−1.4
Bphy_6398	CheB methylestease		−2.6
	Transcription		
Bphy_2946	anti-sigma28 factor FlgM	*flgM*	−3.4
Bphy_3962	two component heavy metal response transcriptional regulator		−1.5
Bphy_4638	transcriptional activator FlhC	*flhC*	−2.3
Bphy_5304	RNA polymerase factor sigma-^{54}		−2.9
Bphy_5333	response regulator receiver protein		−3.7
Bphy_5662	MarR family transcriptional regulator		−1.3
Bphy_7189	Fis family GAF modulated sigma^{54} specific transcriptional regulator		−1.1
	Translation, Ribosomal Structure and Biogenesis		
Bphy_0314	50S ribosomal protein L25/general stress protein Ctc		−1.0
	Not Assigned to a Functional Category		
Bphy_1514	putative lipoprotein		−3.3
Bphy_1768	PRC-barrel domain-containing protein		−1.6
Bphy_5614	phosphoesterase PA-phosphatase related		−3.3

[1] Locus identifier and description were extracted from the GenBank files (NC_010622.1, NC_010623.1, NC_010625.1, NC_010627.1); [2] Log_2 of the fold change (FC) in expression of σ^{54} mutant (σ^{54} mt) versus the wild type (wt) in free-living conditions under nitrogen limitation; ABC, ATP-binding cassette; ATP, Adenosine tri-phosphate.

3.2. Phenotypic Characterization of the σ^{54} Mutant

3.2.1. Role of σ^{54} for Assimilation of C_4 Dicarboxylates

As mentioned above, one of the genes significantly downregulated under nitrogen-limited conditions in our transcriptomic analysis was *dctA* (Bphy_0225), which codes for a C_4 dicarboxylate transporter. *DctA* is located upstream of a gene cluster encoding the sensor kinase DctB and the response regulator DctD, which belongs to the σ^{54}-interacting protein family. While *dctB* expression was downregulated in absence of σ^{54}, *dctD* expression did not change, suggesting that *dctD* is in a separate transcriptional unit (Figure 2B). In the promoter region of *dctA*, an RpoN-binding box was identified.

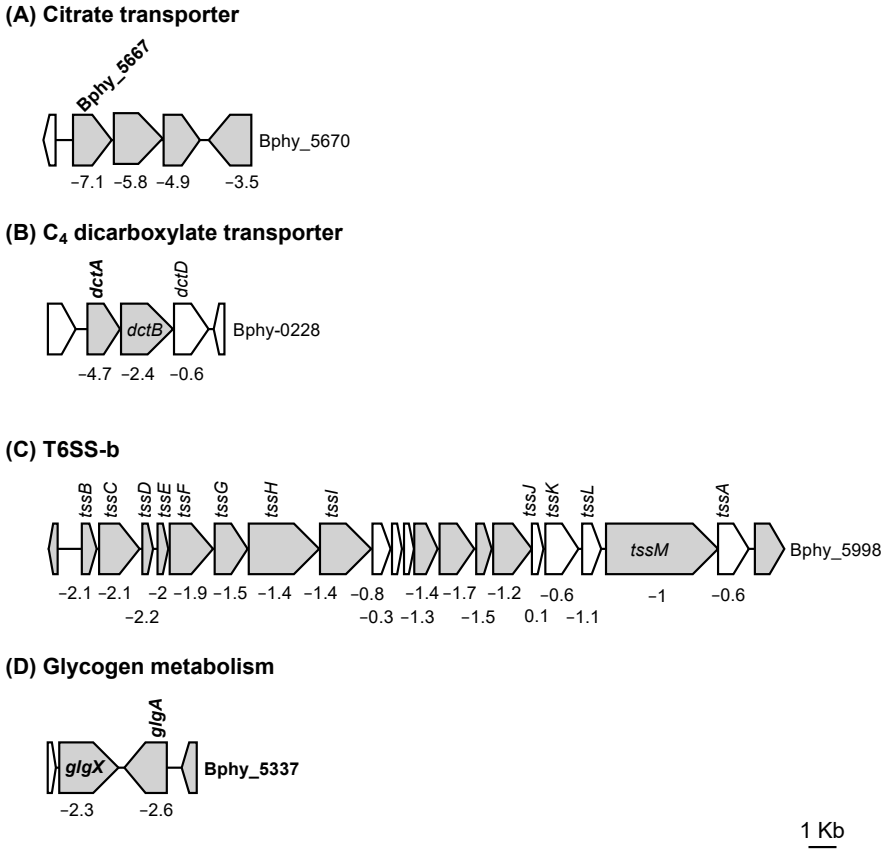

Figure 2. Four *P. phymatum* STM815 gene clusters showing significant downregulation in a σ^{54} mutant compared to the wild type: (**A**) Citrate carrier protein and associated two-component regulatory system (TCRS), (**B**) C_4 dicarboxylate transporter T6SS-b, (**C**) T6SS-b, and (**D**) glycogen metabolism. Genes containing an RpoN box in their promoter region are indicated in bold. The top 200 regulated genes are colored gray. The $\log_2$ of the fold change has been indicated below the genes in the cluster of interest.

A similar genomic constellation to *dctA-dctB-dctD* is found for dicarboxylate transport systems in several bacteria, however, *dctB* and *dctD* are usually transcribed divergently compared to *dctA* [4]. To verify that expression of the DctA transporter gene was regulated by σ^{54}, we examined the ability of the wild-type, the σ^{54} mutant, and the complemented σ^{54} strains to grow in minimal medium in the presence of C_4-carbon sources. The growth of these *P. phymatum* STM815 strains was tested in defined buffered AB minimal medium supplemented with fumaric acid, malic acid, and succinic acid. We found that the σ^{54} mutant strain was impaired in the utilization of all tested C_4 organic acids and that this defect could be restored in the σ^{54} complemented strain (Table 2). The results confirmed the validity of our transcriptomic analysis and suggested that σ^{54} is involved in the assimilation of C_4 compounds in *P. phymatum* STM815.

Table 2. Utilization of different C_4 sources by *P. phymatum* STM815 wild-type (wt), σ^{54} mutant (σ^{54} mt), and σ^{54} complemented (σ^{54} comp) strains [1].

Carbon Source	Utilization of Carbon		
	wt	**σ^{54} mt**	**σ^{54} comp**
Fumaric acid	+	−	+
Malic acid	+	−	+
Succinic acid	+	−	+

[1] Growth was assessed in three independent replicates by measuring the optical density at 600 nm after incubation in AB minimal medium supplemented with 15 mM of different carbon sources for 30 h at 30 °C and 220 rpm. The "−" sign corresponds to $OD_{600} \leq 0.08$.

3.2.2. σ^{54} Positively Controls Swimming Motility

In the list of top genes positively regulated by σ^{54} at the transcript level (Table 1), the category "cell motility" was over-represented. The expression of Bphy_2938-39 (*fliMN*), Bphy_2956 (*flgJ*), and Bphy_2962 (*flhF*), all of which encode flagellar biosynthesis proteins, was significantly downregulated in a σ^{54} mutant (Table 1). Since flagella are used for motility, the swimming ability of *P. phymatum* STM815 wild type, the σ^{54} mutant, and the complemented strain was tested on salt-free LB plates, and the diameter of the swimming cells was measured after 40 h of incubation at 30 °C. In agreement with our RNA-Seq data, the σ^{54} mutant was 40% less motile, and the complemented strain showed increased motility relative to the wild-type strain (Figure 3).

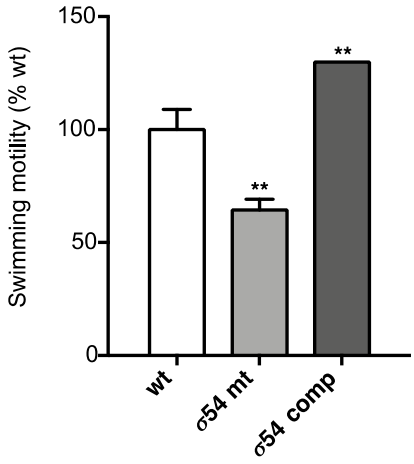

Figure 3. Swimming motility of *P. phymatum* STM815 wild-type (wt), σ^{54} mutant strain (σ^{54} mt) and σ^{54} mutant complemented strain (σ^{54} comp) on salt-free Luria-Bertani LB plates. The plates were incubated at 30 °C and the diameter measured after 40 h. The experiment was performed in triplicates, and the obtained results were analyzed using one-way ANOVA (**, p-value ≤ 0.01). The standard deviation is shown.

3.2.3. The Presence of σ^{54} Influences Interbacterial Competition

Our RNA-Seq data showed that the expression of one *P. phymatum* STM815 cluster coding for a T6SS (Bphy_5978-97, T6SS-b cluster) was dependent on σ^{54} under nitrogen-limiting growth conditions (Figure 2C). Both *P. phymatum* STM815 T6SS systems were previously shown to be important for competition with other related *Paraburkholderia* strains such as *P. diazotrophica* [22]. In order to validate the RNA-Seq data, the promoters of Bphy_5978 (T6SS-b cluster) or Bphy_6115 (T6SS-3 cluster) were fused with *gfp*, and expression was measured in the *P. phymatum* STM815 wild-type and the σ^{54} mutant background. In accordance with the transcriptome data, only the expression of the T6SS-b cluster (p5978), and not of the T6SS-3 cluster (p6115), was decreased in a *P. phymatum* STM815 σ^{54} mutant compared to the wild-type strain (Figure 4).

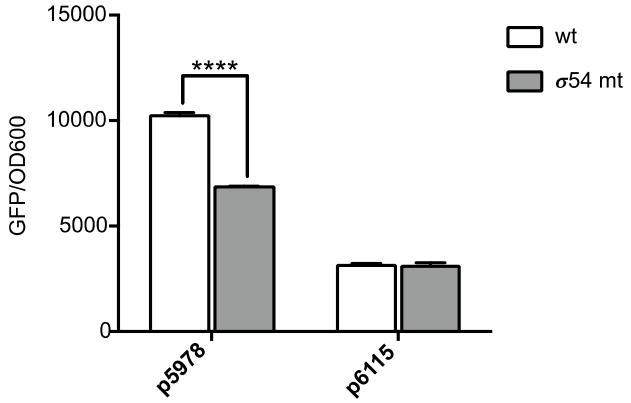

Figure 4. Expression of the T6SS-b cluster (p5978) and the T6SS-3 cluster (p6115) in *P. phymatum* STM815 wild-type (wt) and σ^{54} mutant (σ^{54} mt) strains. The histograms show *gfp* activity from the promoter (measured at a wavelength of 488 nm) normalized by the OD_{600}. Three independent biological replicates were performed. The statistical analysis was performed using two-way ANOVA corrected with Sidak's multiple comparisons test. ****, $p < 0.0001$.

We next tested the ability of the *P. phymatum* STM815 wild-type, the σ^{54} mutant, and the complemented strain to compete against *P. diazotrophica* for 24 h on ABG minimal medium (Figure 5). The σ^{54} mutant turned out to be slightly, but significantly, less competitive as compared to the *P. phymatum* STM815 wild type, indicating a role for this alternative sigma factor in controlling interbacterial competition. In line with this observation, a σ^{54} complemented strain, which expressed *rpoN* from the the *lac* promoter on the pBBR1MCS plasmid, out-competed *P. diazotrophica* more effectively than the wild-type strain.

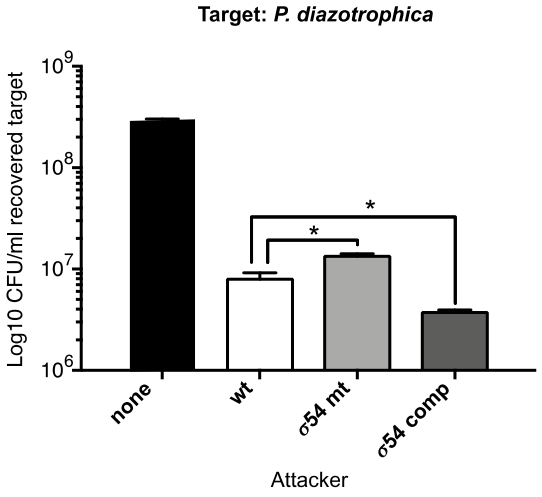

Figure 5. Competition assay with *P. phymatum* STM815 wild-type (wt), σ^{54} mutant (σ^{54} mt), and σ^{54} complemented (σ^{54} comp) strains using *P. diazotrophica* as target. The assay was performed on ABG plates for 24 h at 30 °C. The histogram shows the mean colony forming units (CFU) per ml of the recovered *P. diazotrophica* strain (target). Three independent biological replicates were carried out, and error bars indicate the standard deviation. The results were analyzed using an unpaired *t*-test corrected for multiple comparisons using the Holm-Sidak test. *, $p < 0.01$.

4. Discussion

The alternative sigma factor σ^{54} was originally discovered in *Salmonella* as the sigma factor required for the synthesis of glutamine synthetase [41]. Subsequently, σ^{54} was identified in a wide

range of Gram-negative and Gram-positive bacteria, and found to play a general role in the control of nitrogen metabolism and to affect various other cellular functions such as motility, dicarboxylate transport, degradation of xenobiotics, and virulence in plant and human pathogens [42–48].

In the rhizobia, including *P. phymatum* STM815, σ^{54} is a master regulator during symbiosis since it controls the expression of the genes encoding nitrogenase, i.e., the enzyme that converts N_2 into a form that can be readily assimilated by the plant [49,50].

P. phymatum STM815 is a beta-rhizobium that previously belonged to the genus *Burkholderia*, which comprises versatile bacteria able to adapt and colonize different environmental niches. Our group has shown that in the closely related opportunistic pathogen *Burkholderia cenocepacia* H111, σ^{54} is an important regulator of several phenotypic traits, such as the utilization of nitrogen sources, motility, EPS and biofilm formation, biosynthesis of poly-hydroxybutyrate (PHB), and virulence towards *Caenorhabditis elegans* [48]. To control biofilm formation in H111, σ^{54} interacts with the EBP BerB, which binds c-di-GMP and thereby regulates expression of the exopolysaccharide Bep [51].

We report here that σ^{54} also plays an important role in *P. phymatum* STM815 grown under free-living conditions, where it regulates motility and the expression of T6SS-b, and controls the uptake of tricarbocylic acid cycle TCA cycle intermediates such as succinate, fumarate, and malate (Figure 6). Depending on the presence or absence of oxygen in the environment, dicarboxylates are taken up by bacteria using transporters of different protein families. The dicarboxylate transporter DctA, which belongs to the dicarboxylate amino acid-cation symporter (DAACS) family, has been shown to facilitate dicarboxylate uptake under aerobic conditions in several bacteria, including *E. coli* and *Rhizobium leguminosarum* [4,52,53]. We demonstrate here that the *P. phymatum* STM815 σ^{54} mutant is unable to take up succinate, fumarate, and malate under aerobic, free-living conditions, and that the expression of *dctA* (Bphy_0225), which is located upstream of the two component regulatory system genes *dctBD*, is activated by σ^{54}. DctB is a sensor kinase located in the membrane, which is autophosphorylated in the presence of C_4 dicarboxylates and transfers a phosphate group to its cognate response regulator DctD. DctD is a σ^{54}-dependent EBP that activates transcription of target genes including *dctA* [35]. DctA has been shown to be essential for symbiotic nitrogen fixation in *Sinorhizobium meliloti* and *R. leguminosarum* [54–56]. We are currently evaluating whether *dctA* is the main dicarboxylate transporter in *P. phymatum* STM815 and its role during symbiosis. Interestingly, the expression of the *B. cenocepacia* H111 *dctA* ortholog (I35_RS14715) is not regulated by σ^{54}, suggesting that C_4 uptake may not be dependent on this alternative sigma factor in opportunistic pathogens of the genus *Burkholderia*.

Among the most highly downregulated *P. phymatum* STM815 genes (Table 1), we found Bphy_3959-61, which encodes a potential nitrate/sulfonate/bicarbonate ABC transporter. Out of the five genes annotated as citrate transporter in the *P. phymatum* STM815 genome (Bphy_0810, Bphy_3035, Bphy_4278, Bphy_5667, and Bphy_5880), only Bphy_5667 was significantly regulated by σ^{54} (Figure 2A), suggesting that this transporter plays a key role in importing citrate under free-living conditions.

Several genes potentially involved in glycogen and trehalose metabolism were found among those that were statistically most significantly regulated by σ^{54}. In fact, the expression of genes encoding a glycogen synthase (Bphy_5336, *glgA*) (Figure 2D), a glycogen debranching enzyme (Bphy_5335, *glgX*) which degrades glycogen, and a trehalose-6-phosphate synthase (Bphy_2145, *otsA*) was activated by σ^{54} and RpoN-binding motifs were found in the promoter regions of these genes (Table 2).

Glycogen is a soluble polysaccharide composed of glucose in an α-1,4-linked linear arrangement with α-1,6-branches that serves as a storage molecule in many organisms, including eukaryotes and prokaryotes. During starvation periods, glycogen provides a source of stored energy and carbon. The non-reducing disaccharide trehalose (composed of two molecules of α –D-glucose) also acts as an energy reserve compound as well as an osmoprotectant. In *Bradyrhizobium diazoefficiens*, trehalose is produced to allow survival during desiccation, oxidative stress, and during nodule senescence [57–62]. The precursor of trehalose, trehalose 6-phosphate, has been proposed to be a signal for plant growth

and stress tolerance in *Rhizobium etli* [63]. In plants, trehalose- 6-phosphate is an important signal metabolite that coordinates carbon and nitrogen metabolism [64–66]. The three genes (*glgA*, *glgX* and *otsA*) are regulated by σ^{54} not only in free-living conditions, but also during symbiotic growth in root nodules [26]. Additionally, the gene encoding a trehalose synthase (Bphy_7407) was regulated by σ^{54} during symbiosis, but not under free-living conditions. To the best of our knowledge, the regulation of glycogen and trehalose synthesis via σ^{54} has not been reported previously. Trehalose biosynthesis was previously shown to be regulated by sigma factor σ^{38} (RpoS) in *E. coli* [67] and by the extra-cytoplasmic function (ECF) sigma factor RpoE2 of *S. meliloti* [68]. Interestingly and in contrast to H111, the synthesis of the storage compound PHB is not controlled by σ^{54} in *P. phymatum* STM815.

To date, not much is known about the regulation of T6SS expression in rhizobia. In this study, we show that σ^{54} is involved in the regulation of one of the two *P. phymatum* STM815 T6SS gene clusters (Figures 2 and 4), and that a σ^{54} mutant strain is slightly less competitive with *P. diazotrophica* than wild type *P. phymatum* STM815 (Figure 5). RpoN regulation of T6SS-b is probably indirect, since we did not find a RpoN-binding sequence in the promoter region of the first gene in the operon. However, the presence of two dicarboxylate transporter genes in T6SS-b (Bphy_5990 and Bphy_5992) could be the reason for the indirect control of this cluster by σ^{54}. Our results also suggest that the two *P. phymatum* STM815 T6SSs are used under different conditions and are therefore subject to different regulatory mechanisms, with T6SS-3 expression being σ^{54}-independent. Bernard and colleagues [69] showed that σ^{54} controls expression of T6SSs in several environmental strains, including *Vibrio cholerae*, *Aeromonas hydrophila*, *Marinomonas* MWYL1 and the plant pathogenic bacteria *Pectobacterium atrosepticum*, and *Pseudomonas syringae* pv. tomato [69]. Interestingly, several T6SS clusters investigated to date contain an EBP encoding gene (*vasH*), which has been demonstrated to be required for maximal activation of T6SS expression [69,70]. No *vasH* homolog was apparent in the two T6SS clusters present in *P. phymatum* STM815. Additionally, chromatin immunoprecipitation coupled with next-generation sequencing (ChIP-Seq) and RNA-Seq experiments performed in *V. cholerae* identified two genes coding for two T6SS hallmark proteins, Hcp and VgrG3, as direct σ^{54} targets [71,72].

In summary, in the beta-rhizobial strain *P. phymatum* STM815 σ^{54} controls important phenotypic traits, such as motility, competition, and transport of C4-dicarboxylates, which all contribute to the high competitiveness of this strain in infecting the roots of various legumes. The identity of the different EBPs that work together with σ^{54} to control expression of these different traits will be an interesting area for future investigations.

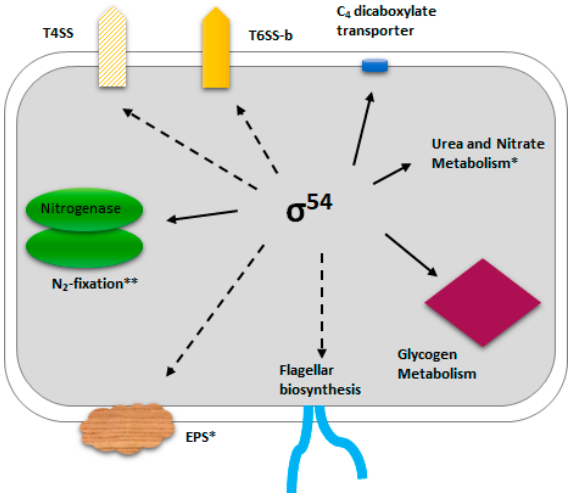

Figure 6. Schematic representation of the phenotypic traits regulated by σ^{54} in *P. phymatum* STM815. Direct σ^{54} targets (with an RpoN-binding motif in the promoter region) are indicated with a solid line. * Lardi et al. 2017; ** Lardi et al. 2018; T6SS, Type 6 Secretion System; T4SS, Type 4 Secretion System; EPS, Exopolysaccharide.

Supplementary Materials: The following are available online at http://www.mdpi.com/2504-3129/1/2/8/s1, Table S1: Bacterial strains and plasmids used in this study, Table S2: List of all *P. phymatum* STM815 genes, $\log_2$ of the fold changes in expression and the *p*-values in free-living conditions under nitrogen starvation by σ^{54} mutant versus wild type as assessed by *DESeq* analysis.

Author Contributions: M.L. and G.P. conceived and designed experiments; M.L., Y.L., S.H., and S.B.d.C. performed the experiments; M.L., Y.L., S.H., L.E., and G.P. analyzed the data; M.L. and G.P. wrote the paper. All authors have read and agreed to the published version of the manuscript.

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