Sustainable natural bioresources in crop protection: antimicrobial hydroxycoumarins induce membrane depolarization-associated changes in the transcriptome of *Ralstonia solanacearum*

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Running title: Antimicrobial hydroxycoumarins induce transcriptome change of *Ralstonia solanacearum*

Abstract:

BACKGROUND: *Ralstonia solanacearum* is one of the most devastating pathogen affect crop production worldwide. Our previous studies showed that hydroxycoumarin (umbelliferone, esculetin, daphnetin), natural plant secondary metabolites, significantly enhance antibacterial activity against *R. solanacearum* and imply as sustainable natural bioresources on controlling of plant bacterial wilt. However, the antibacterial mechanism of hydroxycoumarins against plant pathogen still remains poorly understood.

RESULTS: Here we characterized the effect of three hydroxycoumarins on the transcriptome of *R. solanacearum*. All three hydroxycoumarins were able to kill *R. solanacearum*, but their antibacterial activity impacted differently the bacterial transcriptome, indicating that their modes of action might be different. Treatment of *R. solanacearum* cultures with hydroxycoumarins resulted in a large number of the differentially expressed genes (DEGs), involved in basic cellular functions and metabolic process, such as downregulation of genes involved in fatty acid synthesis, lipopolysaccharides biosynthesis, RNA modification, ribosomal submits, and oxidative phosphorylation and electrontansport, as well as upregulation of genes involved in transcriptional regulators, drug efflux, and oxidative stress responses. Future studies based on *in vitro* experiments are proposed to investigate lipopolysaccharides biosynthesis pathway leading to *R. solanacearum* cell death caused by hydroxycoumarins. Deletion of *lpxB* substantially inhibited the growth of *R. solanacearum*, and reduced virulence of pathogen on tobacco plants.

CONCLUSION: Our transcriptomic analyses show that xxx. These findings provide evidence that hydroxycoumarins inhibit *R. solanacearum* growth through

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multi-target effects. Hydroxycoumarins could serve as sustainable natural bioresources against plant bacterial wilt through membrane destruction targeting the lipopolysaccharides biosynthesis pathway.

Keywords: transcriptome analysis, *R. solanacearum*; hydroxycoumarins, antibacterial activity, membrane

1 INTRODUCTION

Ralstonia solanacearum (*R. solanacearum*) represents one of the most devastating plant bacterial pathogens among the top ten plant pathogens, infecting more than 250 plant species and causing bacterial wilt worldwide. [1, 2] The limitation of control methods may aggravate the harm of bacterial wilt in agriculture. [3] In addition, the increasing host range of *R. solanacearum*, its complex pathogenicity and the wide range of hosts makes it a threat to agriculture. [4-6] Currently, the primary method for controlling bacterial wilt is by using chemical pesticides[7]; however, the extensive application of synthetic pesticide resulted in resistance in pathogen populations, and safety environmental concerns. [3] Thus, development of the potential control methods from natural bioresources for bacterial wilt is highly demanded. In continuation of discovering new natural products bactericidal agents, we found that coumarins inhibit bacteria growth and suppress the virulence-associated factors of *R. solanacearum*. [8, 9] Furthermore, coumarins could preserve the host endogenous microbiome and exert little selective pressure, avoiding the rapid appearance of resistance. [10]

Coumarins are natural secondary metabolites composed of fused benzene and α-pyrone rings produced via the phenylpropanoid pathway and accumulate in response to infection by bacteria, fungi, virus and oomycetes. [11-13] The extent and timing of coumarin accumulation has often been associated with the level of disease resistance. For instance, young leaves of *Nicotiana attenuata* show higher resistance against *Alternaria alternata* than mature leaves, which is correlated with stronger induction of scopoletin. [14] *Nicotiana tabacum* cv. Petit Havana resistance to *Botrytis cinerea* is due to the accumulation of scopoletin and PR proteins. [15] Similarly,

Scopoletin accumulated in the resistant tomato line 902 upon tomato *yellow leaf curl virus* (TYLCV) infection. [16] Besides their role in aboveground plant tissues, the coumarins scopolin, coniferin and syringing have shown to be rapidly processed in the *Arabidopsis* roots upon infection by the oomycete pathogen *Pythium sylyaticum*, giving rise to cell wall-fortifying lignin and antimicrobial scopoletin. [17] Moreover, in the wild tobacco *N. attenuata*, the content of the phytoalexin scopoletin in roots was enhanced after infection by fungus *A. alternata*. [18] The accumulation of specific coumarins in roots plays a role in defense against soil-borne pathogens. For instance, umbelliferone suppresses the expression of T3SS regulatory and effector genes and alters the virulence of *R. solanacearum* on tobacco. [19] However, the ecological relevance and the underlying biological mechanisms of coumarins against pathogens remain largely unknown. [13]

Several studies have proven that diverse coumarins showed antimicrobial activities against plant and animal pathogens. Recently discovered coumarins from plant sources, exerted antibacterial activity against *R. solanacearum* and the hydroxylation on C-6, C-7 and C-8 enhanced this activity. [20] Similarly, the phenolic coumarin scopoletin showed strong antibacterial against *Escherichia coli* by reducing biofilm formation. [21] Recent studies evidenced that coumarins induced strong non-receptor mediated membrane lysis as their primary microbicide strategy. [8, 22] Exposure to coumarin and umbelliferone clearly reduce fimbriae production and biofilm formation of *Escherichia coli*. [21] Indeed, scopoletin and daphnetin were proved as promising inhibitors of the bacterial cell division protein FtsZ, and its hydroxyl, diethyl, or dimethyl amino substituents in the 7th carbon enhanced this inhibitory activity, halting the first step of bacterial cell division. [23] Moreover,

coumarins inhibit proliferation of *Mycobacteria* by targeting the assembly of MtbFtsZ. [24] Hydroxycoumarins also displayed antibacterial activity through inhibiting isoleucyl-transfer RNA (tRNA) synthetase gene expression. [25] Besides their mentioned role damaging the cell membrane, coumarins might efficiently traverse them and bind to the DNA or RNA ligase to reduce the biosynthesis of these molecules. These actions can then control the expression of genes encoding transcriptional regulators and other downstream genes. However, the antibacterial mechanism of coumarins against plant pathogen still remains poorly understood.

Recently, transcriptional analysis was proven a useful means to reveal antibacterial mechanism of certain compounds against pathogen. [26-28] For instance, Genome-wide gene expression profiling enables to investigate the antimicrobial mechanism of peptides against *Streptococcus pneumonia*. [26] Transcriptome analysis of *Escherichia coli* exposed to lysates of lettuce leaves revealed the upregulation of numerous genes associated with attachment and virulence, oxidative stress, antimicrobial resistance to detoxification of noxious compounds, as well as DNA repair. [29] Hydroxycoumarins were proven to destabilize the cell membrane and inhibit biofilm formation. [20] Transcriptome analysis of *R. solanacearum* provides a way to understand the antibacterial mechanism of hydroxycoumarins.

In this work, we aimed to investigate the effect of three hydroxycoumarins (umbelliferone, esculetin and daphnetin) on the gene expression of *R. solanacearum* using RNA sequencing (RNA-seq) approach. To better understand their mechanism of action and how their hydroxylation at the C-6, C-7 or C-8 position significantly enhanced the antibacterial activity against *R. solanacearum*.

2 MATERIALS AND METHODS

120 **2.1 Strain and compounds**

121 The bacterial wilt pathogen *R. solanacearum* CQPS-1 (phylotype I, race 1, biovar
122 3) used in this study (accession number NZ_CP016914.1), was originally isolated from
123 an infected tobacco plant in Chongqing, China by Laboratory of Natural Products
124 Pesticides. [30] The strain was preserved in nutrient broth supplemented with 25%
125 glycerol stocked and stored at -80 °C and grown in rich B medium or minimal medium
126 (M63) incubated at 28 °C.

127 Umbelliferone (7-hydroxycoumarin, ES), esculetin (6,7-dihydroxycoumarins, ES),
128 and daphnetin (7,8-dihydroxycoumarins, DA) were purchased from Shanghai Yuanye
129 Bio-Technology Co., Ltd. (Shanghai, China), and the purity of compounds (> 98%) was
130 validated by using High Performance Liquid Chromatography and Mass Spectrometry.

131 **2.2 Total RNA extraction**

132 *R. solanacearum* was overnight inoculated in rich B medium, then the bacterial
133 suspension (10^8 to 10^9 CFU per mL) was centrifugated at 5000 rpm for 10 min, the
134 bacteria were collected and diluted in M63 medium adjusted to with OD_{600} 0.2 and
135 incubated on a shaker at 180 rpm and 28 °C for 4-5 h. Then bacterial cells were
136 treated with hydroxycoumarins at the concentration with half of minimum inhibitory
137 concentration (MIC) for 1 h (UM 128 mg L^{-1}, ES 96 mg L^{-1}, and DA 32 mg L^{-1}). The 0.1%
138 DMSO treatment was used as the control (CK). [20] The 1 h treatment duration was
139 chosen as it was found that treatment for 2 h or more caused lower yield and poor
140 quality of the RNA obtained, probably due to bacterial lysis by hydroxycoumarins and
141 release of RNA before extraction. The samples were harvested by centrifugation at
142 5000 rpm for 10 min at 4 °C, the supernatants were removed, and the treated
143 bacterial cells were collected and frozen in liquid nitrogen if RNA isolation was not

conducted immediately. RNA was extracted by using TRNzol reagent according to the manufacturer's instructions (Tiangen Biotech Co. Ltd, Beijing, China) and then treated with RNase-free DNase I (Tiangen Biotech Co. Ltd, Beijing, China) to remove genomic DNA contaminations. RNA degradation and contamination were checked on 1% agarose gels and RNA concentration and purity were monitored using the Nanovue UV-Vs spectrophotometer (GE Healthcare Bio-Science, Uppsala, Sweden). All experiments were performed three times, which constituted biological replicas.

2.3 RNA-Seq library construction

RNA concentrations were assessed using Qubit® RNA Assay Kit in Qubit® 2.0 Flurometer (Life Technologies, CA, United States) and Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, CA, United States). The quality standard of RNA samples including minimum RNA integrity number (RIN) of 7, absorbance values A260/280 in the range 1.8-2.0 and A260/230 over 1.8. Libraries construction and RNA-Seq were performed by Shenzhen Hengchuan (Shenzhen, China). RNA-Seq libraries were generated using NEBNext® Ultra ™ RNA library Prep Kit for Illumina (NEB, United States) following manufacturer's recommendations. After synthesis first strand cDNA and second strand cDNA, the samples were sequenced on the Agilent Bioanalyzer 2100 system. The length of the reads was around 150 bp. Quality control of the RNA-Seq raw data was performed using FastQC. [31]

2.4 Mapping and differential gene expression analysis

The reference genome of *R. solanacearum* CQPS-1 was downloaded from GenBank (NZ_CP016914.1). [30] The raw data were filtered by discarding low-quality sequences and removing adaptor sequences. Read mapping against the reference

genome was performed by using HITAT2. [32]

To determinant the expression level for each gene, we measured numbers of reads uniquely mapped to the specific gene and total number of uniquely mapped reads in the sample using the feature Counts tool.

Differentially expressed genes (DEGs) upon hydroxycoumarin treatments were obtained using the DESeq2. [33] To extract genes with differentially expression changes, the cutoff of q-value < 0.05 and $|\log_2$ Fold change$| > 2$ was applied. [34] Moreover, we performed gene set enrichment analysis (GSEA) on the basis of q-values resulted from differential expression analysis with the OmicsBox 1.2.4 (https://www.biobam.com/omicsbox/).

2.5 Validation of the RNA-Seq using qRT-PCR

To validate the results of RNA-Seq, ten differentially expressed genes (DEGs) (seven down-regulated and three up-regulated) were examined using quantitative real-time PCR (qRT-PCR). Independent RNA samples were collected as described for RNA-Sequencing and first-strand cDNA was synthesized using the iScript gDNA clear cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. Primers were synthesized by BGI Technologies (Shenzhen, Guangzhou, China) (Table S1) and qRT-PCR analysis was carried out in 96-well plates in a 20 µL reaction system with C1000 Thermal Cycler (Bio-Rad, Hercules, CA, USA). Three technical replicate reactions were used for each sample. Normalized gene expression was calculated by Bio-Rad CFX and SerC was used as the reference gene to normalize gene expression. [35] All assays were carried out three times using biological replicas.

2.6 Construction of *lpxB* deletion mutant of *R. solanacearum*

In this study, the *lpxB* deletion mutant was generated by pK18mobsacB- based homolog recombination as previously described. [36] The primer pairs lpxB_A1B (GCGGATCCTGCATGCGACCATGCT) with lpxB_B1C (ATCTTCTGAACTTGCGTCATTCAGTCG GCCCACGCCGTCT) and lpxB_A2C (AGACGGCGTGGGCCGACTGAATGACGCAAGTTCAGAA GAT) with lpxB_B2H (ATAAGCTTGCCCAATCGCCCACTTCC) were used for constructing plasmid *pK18-lpxB-d*. After validating the sequence, the *pK18-lpxB-d* was horizontal transferred from *E. coli* S17-1 into *R. solanacearum* strain CQPS-1. The *lpxB* deletion mutant were confirmed by cloning PCR.

2.7 Bacterial biofilm formation, swimming activity and virulence assay

Biofilm formation of *R. solanacearum* and *lpxB* mutant were performed in 96-well polystyrene microtiter plates. [37] Briefly, bacteria suspension mix with B medium were inoculated in plates at 30 °C for 24 h. Then biofilms were stained with crystal violet, dissolved in 95% ethanol and quantified by absorbance at 530 nm (OD_{530}).

Swimming motility were assessed on M63 minimal medium supplemented with 20 mM L-Glutamate. [38] The bacteria suspension was stab-inoculated into agar with sterilized tips. The diameter of swimming halo was measured after 36 h, 48 h and 60 h cultivation at 30 °C.

The drenching assay was used to evaluate virulence of *R. solanacearum* and *lpxB* mutant as described previously. [9] Tobacco plants (Yunyan 87) were used to virulence assay with soil-soaking, which mimics the natural invasion through the roots. Each assay was repeated independently three times with 16 plants. Wilt symptoms of plants were scared daily using disease index (scale of 1-4) and the mean values of all

experiments were averaged with SD. The data were analyzed with the SPSS 17.0 statistical software program using student's t test under the significance level of 0.05 (P-value=0.05)

3 RESULTS

3.1 *R. solanacearum* transcriptome signatures influenced by hydroxycoumarins

We profiled *R. solanacearum* transcriptomes under three hydroxycoumarin treatments in M63 medium. As showed in Figure 1B, all three treatments significantly affect the transcriptome of *R. solanacearum* compared to the control treatment. Furthermore, the effect of daphnetin (DA) and esculetin (ES) on bacteria were similar, but different to the umbelliferone (UM) treatment.

In total, differential expression of 344 genes in *R. solanacearum* supplemented with UM treatment (representing 6.46% of the predicted protein-coding sequences in the CQPS-1 genome) could be observed, including 163 genes up-regulated and 181 genes down-regulated. Esculetin affected a higher number of genes than UM, causing up- and down-regulation of 326 and 372 genes. Furthermore, DA, which exhibits the strongest antibacterial activity against *R. solanacearum* showed the biggest number of differential expressed genes, including up-regulation of 420 genes and down-regulation of 502 genes (Fig. 2A, Fig. S1). There were 191 common genes involving in three hydroxycoumarins treated bacteria, including 90 upregulated genes and 94 downregulated genes (Fig. 2, Fig. S2).

To validate the RNA-Seq results, a total of ten genes were selected from DGEs for qRT-PCR analysis. The results indicated gene-expression data obtained by this RNA-Seq strategy strongly correlated with RT-PCR measurements (Fig.1(C)).

3.2 Treatment by any of the hydroxycoumarins alters expression of 191 *R. solanacearum* genes

We investigated the functional categories enriched by con differential expression genes treated with three hydroxycoumarins. In cellular component category, intracellular, intracellular organelle, intracellular non-membrane-bounded organelle, non-membrane-bounded organelle, organelle, and protein-containing complex were enriched. Then, structural molecule activity, structural constituent of ribosome, binding, transporter activity were enriched in molecular function category. In biological process, cellular metabolic process, primary metabolic process, peptide process, protein metabolic process, transport, transmembrane transport were enriched (Table 1).

Hydroxycoumarin treatments up-regulated expression of different family transcriptional regulators (*Rsc0149*, *Rsc1997*, *Rsc1851*, *Rsp0447*, *Rsp0443*, *Rsp1668* and *Rsp0816*), genes coding for drug efflux lipoprotein and transmembrane proteins (*Rsc0009*, *Rsc2499*, *Rsc1852*, *Rsp0819*, *Rsp0818*, *Rsc1294* and *Rsp0817*), several genes involved in putative signal peptide proteins (*Rsc0153*, *Rsc3092*, *Rsc2725*, and *Rsp0992*), and genes encoding stress-related proteins (*coxM* and *Rsp0993*) (Table S2).

Otherwise, hydroxycoumarins significantly suppressed expression of genes involved in intracellular organelle, such as fatty acid synthesis genes (*accC1*, *accB1*, *Rsp0035*, *Rsp0782*, and *Rsp0783*) and lipopolysaccharides biosynthesis genes (*Rsc0686* and *Rsc0685*). Genes coding for peptide proteins (*Rsc3300*, *Rsc3285*, *Rsp1461*, *Rsp1269*, *Rsp0811*, and *Rsp0699*), RNA modification related genes (*Rsc1419* and *Rsp0782*), translation (*tsf*, *infA*, and *Rsp0039*), subunits of 50S and 30S ribosomal proteins (*rplS*, *rplJ*, *rplL*, *rpoC*, *rplE*, *rpsH*, *rpsF*, *rpsR*, *rpsE*, *rpmD*, *rplO*, *rpmJ*, *rpsM*,

rpsK, *rplQ*, *prmA*, *rpmG*, *rplT*, *rplI*, and *rpsR*), and oxidative phosphorylation and electron transport (*cyoB1*, *cyoC1*, and *cyoD1*) were down-regulated by hydroxycoumarins (Table S2).

3.3 DA and ES treatment cause similar change on *R. solanacearum* transcriptome

We investigated the functional categories enriched by 395 differential expression genes treated with DA and ES treatment. As showed in Table 1, In cellular component category, membrane and plasma membrane were enriched. In molecular function, rRNA binding, heterocyclic compounds binding, organic cyclic compound binding and nucleic acid binding were enriched. There were more biological process categories were enriched, such as cellular biosynthetic process, metabolic process, translation and gene expression.

Based on enriched GO terms and the antibacterial effect of DA and ES treatment against *R. solanacearum*, we choose the differential expression genes enriched in GO terms and involved in bacterial basic processed. Lipopolysaccharides and fatty acid play a key role in cell membrane components in *R. solanacearum*. As showed in Table 2, the gene expression of lipopolysaccharides biosynthesis clusters (LpxA, LpxB, LpxD and FabZ) and lipid A biosynthesis lauroyl acyltransferase (Rsc0135 and Rsc0136) were significantly inhibited by DA and ES treatments. Mostly of fatty acid synthesis pathway genes (*fabB*, *fabD*, *fabG*, *fabH*, *fabI*) and fatty acid synthesis regulated associated genes (*Rsc0265*, *Rsp0652*, *Rsp0648*, *acpF*, *Rsc0434*, and *Rsc2546*) were down-regulated by DA and ES treatments. Meanwhile, genes coding for modification of RNA (*cysS*, *yibK*, *hrpA*), elongation factor Tuf showed decreased transcriptional expression. Certain genes involved in transcription, transcriptional regulation and membrane transport were down-regulated. The expression of genes coding for the

two component response regulator transcription regulators Rsc3160, cold shock-like transcription regulator CspC, and response transcription regulators (Rsc2430 and Rsc1584), LPS export ABC transporter permease (LptG and LptF), D-xylose ABC transporter substrate-binding proteins (XylF, XylG and XylH) and protein translocase subunit (SecF and SecD) were significantly reduced by DA and ES treatments. Furthermore, DA and ES affect energy production in *R. solanacearum* by suppression gene expression of oxidative phosphorylation and electron transport (*ctaG*, *xyoA1*, *atpB*, *atpE*, *atpF*, *atpA*, *atpD*, *atpC*, *nuoH*, *nuoJ*, *nuoK*, *nuoL*, *nuoM* and *nuoN*).

Furthermore, we analysis the differential genes involved in generally upregulated function by DA and ES treatments. 6 genes coding of fatty acid degradation pathway (*pcaB*, *pcaC*, *pcaD*, *pcaF*, *pcaJ*, *Rsc0161*) were upregulated. Several genes involved in transcriptional regulation and membrane transport were significantly induced by DA and ES treatments, such as *RSc2361*, *RSc2114*, *RSc1857*, *RSc1185*, *RSc0993*, *RSp0415*, *fur2*, *emrB*, *exbD1*, *exbB1*. Furthermore, the expression of two genes involved in stress related proteins (MsrA and RpoN2) were increased (Table 2).

3.4 Hydroxycoumarins specifically alter the expression of *R. solanacearum* gene sets

ES and DA treatment resulted in similar expression changes, and UM treatment showed different expression pattern (Fig. 3). In order to investigate the specific gene regulation in hydroxycoumarin treatments, we used the Venn diagrams of differential expression genes to identify specific genes of each hydroxycoumarin treatment (UM, ES and DA).

UM treatment specifically resulted in 33 genes down-regulation and 44 genes

up-regulation (Table S3), such as flagellar-associated genes *flgC*, *flgF*, *flgH*, *fliJ* and *fliK* were down-regulated about 4-fold; also type III secretion system transcription regulator gene *prhG* was down-regulated by UM treatment. Among the up-regulated genes, there were several genes coding for myo-inositol catabolim pathway (*Rsc1247*, *Rsc1246*, *iolH* and *Rsc1242*) were up-regulated about 4-fold.

There were 44 genes down-regulated and 48 genes up-regulated in ES treatment. Among the down regulation genes, 8 open reading frames coding for hypothetical proteins, seven genes coding for transcriptional regulator (*Rsc1016*, *livH2*, *Rsc2437*, *Rsc0002*, *Rsp0985*, *and Rsp0983*). In addition, there were 48 specifically up-regulated genes by ES treatment, including 10 genes involved in hypothetical proteins, 4 genes involved in transcriptional regulator (*Rsc2325*, *Rsc0029*, *Rsp0821* and *BC350_RS25620*), and several genes involved in virulence-associated genes (*fliO, hrcC* and *hrpK*). Two genes related with tryptophan synthesis pathway *(trpE* and *trpB*) were up-regulated in ES treatment (Table S4).

Due to the strongest antibacterial activity against *R. solanacearum*, DA treatment resulted in 126 specifically genes up-regulation and 150 genes down-regulation. As showed in Table S5, the significant enrich gene ontology (GO) terms of specifically genes were focused on biological process, such as nucleotide biosynthetic process, ion transmembrane transport and phosphorus metabolic process. In the molecular function, RNA binding, nucleotide binding, nucleoside phosphate binding and cation transmembrane were enriched. Among the up-regulated genes, certain genes were involved in basic cellular functions, such as transcriptional regulators (*Rsc1201*, *Rsc0635*, *Rsc0302*, *Rsc2505*, *Rsc2498*, *Rsc2466*, *BC350_RS12595*, *Rsc2018*, *Rsc1960*, *Rsc1511*, *BC350_RS16690*, *Rsp0440*, *Rsp1512*,

Rsp1616, *Rsp1667*, *Rsp1178* and *BC350_RS23395*), molecular chaperone *DnaK* and two DNA damage-inducible mutagenesis protein (*Rsp0799* and *imuA*) involved in DNA damage. Gene coding for basic biological process such as *prpC*, *prpB*, *paaE*, *paaB*, *paaA*, *otsB*, *paaC*, *paaD*, *trxB*, *xdhB* and *xdhC* were enriched. The expression of 150 specifically genes were suppressed by DA treatment, including several genes encoding extracellular polysaccharide (*epsA*, *epsP*, *epsB*, *epsC*, *wecC*, *epsF*, *Rsp1013*, *Rsp1012*, *Rsp1011*, *Rsp1010* and *xpsR*), type III secretion system (*prhA*, *hrpG* and *prhJ*), type VI secretion system (*Rsp0746*, *Rsp0745*, *Rsp0629* and *tssH*), ATP synthase subunit (*atpH* and *atpG*), IS3 and IS4 family transposase (*BC350_RS12015* and *BC350_RS15970*) (Table S5).

3.5 Enrichment analysis of GO pathway

Based on the Gene Ontology (GO) - cellular component (CC), molecular function (MF) and biological process (BP), we observed that among the DEGs in *R. solanacearum* responded to hydroxycoumarins, the GO terms of DA treatment was more than other two hydroxycoumarins (ES and UM). In cellular component, ribosome, membrane, plasma membrane, and cell periphery were enriched. Structural constituent of ribosome, heterocyclic compound binding, organic cyclic compound binding, nucleotide binding, transporter activity, transmembrane transporter activity and nucleic acid binding were enriched in molecular function category. Primary metabolic process, protein metabolic process, transport, peptide biosynthetic process, translation, cation transport, cation transmembrane transport and lipid metabolic process were enriched in biological process category (Table 1).

3.6 DA and ES inhibit bacterial growth and virulence of *R. solanacearum* by altering *lpxB* expression

DA and ES treatment significantly reduced expression of genes coding for lipopolysaccharides synthase pathway (Table 2). Especially, expression of *lpxB* involved in lipid-A-disaccharide synthase was most down-regulated with 9.58-fold and 10.83-fold. LpxB involved in one of key step for lipid A biosynthesis and is important for bacterial cell membrane (Fig. 5(A)). So, we generated a *lpxB* in-frame-deleted mutant (Δ*lpxB*) to confirm its effect on bacterial growth and virulence.

Compared with WT (CQPS-1), *lpxB* mutant exhibited slower bacterial growth in liquid medium ($P < 0.05$) (Fig. 5(C)). Biofilm formation and swimming motility are important for virulence of *R. solanacearum* in host plants. The *lpxB* mutant forms red and small colonies with less mucoid, indicating that EPS production was suppressed by *lpxB* deletion (Fig. 5(B)). The biofilm formation and swimming motility of *lpxB* deletion were significantly inhibited (Fig 6(A), (B)). The *lpxB* mutant significantly altered the disease progress of bacterial wilt ($P < 0.05$). The results suggest that *lpxB* is required for EPS production, biofilm formation, swimming motility and virulence of *R. solanacearum*.

4 DISCUSSION

Coumarins are produced via the phenylpropanoid pathway and accumulated in plant tissues, respond to infection of a diversity of pathogens and play dual roles in plant defense due to the antimicrobial activity and plant defense signaling. [12, 13] Further, coumarins play role in the interaction of plant and soil-borne pathogens. As a landmark discovery, advances in the study of interaction between plant pathogen and host have provided evidence that metabolites could inhibit pathogen growth and alter the transcriptome of *R. solanacearum*. [39, 40] Plant-derived antibacterial

compounds were originally proposed to change plasma membrane permeability, leading to membrane rupture and rapid lysis of microbial cells. Recently, it has been proposed that coumarin induces strong non-receptor mediated membrane lytic mechanism as the primary microbicide strategy. [8] In this study, potent antibacterial properties of three hydroxycoumarins were demonstrated, indicating the potential use in plant protection. Hydroxycoumarins (UM, ES, DA) were proved strong antibacterial activity against *R. solanacearum*. [20] At present, the mode of action of hydroxycoumarins is not known, although in our previous work PI stain results indicated that the compounds changed the permeability of the bacterial membranes. [9] To further investigate the molecular mechanism of actions of hydroxycoumarins against *R. solanacearum*, we perform RNA-Seq to study the transcriptomic response of *R .solanacearum* treated with three hydroxycoumarins. Our results revealed that the expression of genes involved in fatty acid synthesis, lipopolysaccharides biosynthesis, RNA modification, ribosomal submits was extensively down regulated by hydroxycoumarins treatment (Fig. 1, Table 2).

In addition, the bactericidal action of hydroxyl-substituents on C-6, C-7 and C-8 in three hydroxycoumarins might be differentially accessible in various species. Coumarins, naturally plant derived secondary metabolites composed of fused benzene and a-pyrone rings. Umbelliferone (7-hydroxycoumarin), esculetin (6, 7-dihydroxycoumarins) and daphnetin (7, 8-dihydroxycoumarins) have different number of hydroxyl-substituents in different position. Further, our previous study indicated that three hydroxycoumarins have different antibacterial activity, the MICs of UM, ES and DA were 256 mg L^{-1}, 192 mg L^{-1} and 64 mg L^{-1}, respectively. [20] Based on the previous results, we hypothesize that these compounds might destroy cell

membranes or affect specific action targets. In this study, the antibacterial mechanisms affected upon *in vitro* exposure to the hydroxycoumarins were studied by transcriptome analysis of *R. solanacearum*. We infer similar or different modes of action of the tested compounds from the changes in the expression of different genes at the tested time. This does not preclude the possibility that similar genes are expressed with a different timing, which would not imply a similar mode of action, as has been widely demonstrated in plant immunity (https://pubmed.ncbi.nlm.nih.gov/20585331/ ; https://pubmed.ncbi.nlm.nih.gov/29794063/). The effect of ES and DA on the bacterium were similar, but different to the UM treatment (Fig. 1A, Fig. 3), indicating that the number of hydroxyl-substituent was more important than the hydroxyl-position in antibacterial activity. DA, which exhibits the strongest antibacterial activity against *R. solanacearum* showed the biggest number of differential expressed genes. There were 191 common genes involving in three hydroxycoumarins treated bacterial, including 90 upregulated genes and 94 downregulated genes (Fig. 2, Fig. S2). These fiunding indicates that the core potent target protein might play an important role in the antibacterial activity of Hycs.

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The outer membrane (OM) of Gram-negative bacteria is essential for sustaining cell morphology and poses a significant barrier to unwanted molecules from entering the cell and thus accumulating to toxic levels inside of the pathogen. [41, 42] The membrane of bacteria usually contains three major macromolecules, including lipopolysaccharides, outer membrane proteins and lipoproteins. [41] Since the OM serves as a protective barrier, disruption or interference with the biosynthesis of the OM presents an attractive strategy for antibacterial drug discovery. It's been proved

that polymyxin involves binding to the lipid A component of lipopolysaccharide portion of OM, indicated strong antibacterial activity. [43] In this study, the expression of genes involved in the biosynthesis of lipopolysaccharides (*lpxA*, *lpxB*, *lpxD*, *fabZ, Rsc0135* and *Rsc0136*) were significantly suppressed supplemented with ES and DA treatment (Table 2).

Fatty acids are essential components of membranes and are important sources of metabolic energy in bacteria. There, fatty acid biosynthesis and degradation pathways could be switched on and off according to the availability of fatty acids to maintain membrane lipid homeostasis. [44] The indispensable fatty acid synthase pathway is a special attractive target for antibacterial agents. Platensimycin, platencin and phomallenic were demonstrated to inhibit the condensation step in in the bacteria fatty acid biosynthesis pathway. [45] Recent studies showed that antibacterial peptides NCR335 reduced the expression of fatty acid biosynthesis genes. [46] In the current study, we found that Hycs might altered the expression of genes involved in basic cellular function. DA and ES treatment suppressed the expression of several genes involved in fatty acid synthesis pathway (*fabB*, *fabD*, *fabG*, *fabH* and *fabI*). Further, a variety of genes coding fatty acid degradation pathway (*pcaB*, *pcaF*, *gabD*, *pcaJ* and *Rsc0161*) were induced. These results indicated that Hycs might destroyed membrane lipid homeostasis by suppressing gene expression of lipopolysaccharide synthesis pathway in *R. solanacearum*, and imbalance availability of fatty acid by suppressing gene expression of fatty acid biosynthesis pathway and inducing gene expression of fatty acid pathway degradation pathway.

The global transcriptional response of *R. solanacearum* to Hycs indicated that exposure to these chemical is stressful to the pathogen. Compared with DMSO

454 treatment, Hycs downregulated these genes involved in basic cellular functions, such
455 as transporter activity, oxidative phosphorylation and ribosomal. Compared with the
456 limited data available on the effect of antibacterial agents, salicylic acid
457 demonstrated similar result in down-regulation of the transcription-translation
458 machinery in *R. solanacearum*. [39] Furthermore, The F_0F_1 ATP synthase genes involved
459 in oxidative phosphorylation were down-regulated by DA and ES treatment. Similar
460 with diarylquinolines target subunit c of mycobacterial ATP synthase. [47] Hycs
461 inhibited the expression of ribosomal subunits, like the nodule-specidic cysteine-rich
462 reptides down-regulated the expression of ribosomal subunits and shown antifungal
463 activity against *Sinorhizobium meliloti*. [46] Meanwhile, antimicrobial peptide MAF-1A
464 reduced the ribosomal subunits transcription level in *Candida albicans*. [27]Oxidative
465 stress is caused by exposure to reactive oxygen intermediates, which can damage cell
466 membranes proteins, and nucleic acids. [48] Recent studies proven that oxidative stress
467 could suggested as a key antibacterial mechanism of nanoparticles (NPs), such as
468 fullerene and graphene oxide. [49] The produced ROS mediated by oxidative stress can
469 irreversibly damage bacteria (e. g., their membrane, DNA), resulting in bacteria death.
470 [50] Salicylic acid was demonstrated to cause oxidative stress in *R. solanacearum*,
471 upregulated expression of oxidative stress genes. [39] In this study, Hycs treatment
472 induced oxidative stress genes in *R. solanacearum* (*coxM* and *Rsp0993)*. In order to
473 adapt to antibacterial agent stress, bacteria encode drug efflux pump protein to
474 exudate the toxic chemicals. In present study, we found that Hycs treatment
475 up-regulated expression of drug efflux pump genes (*Rsc0009*, *Rsc2499*, *Rsc1852*,
476 *Rsp0819*, *Rsp0818*, *Rsc1294* and *Rsp0817*). Similarly, *R. solanacearum* also
477 upregulates drug efflux pump genes in response to a high concentration of 500 μM

478 SA. [39] Following exposure to UM, *R. solanacearum* cells displayed reduced expression
479 of virulence genes encoding type III secretion components (PrhG) and
480 flagellar-associated genes. These results were similar with our previously study,
481 which proven that UM could suppressed expression of T3SS regulators through the
482 HrpG-HrpB and PrhG-HrpB pathways. [19] This is consistent with multiple effect of
483 plant-derived compounds on virulence genes in plant pathogen. For example,
484 Oleanolic acid induces the type III secretion system of *R. solanacearum*. [51] Salicylic
485 acid derivative compound inhibited the expression of type III secretion components.
486 [52] Interesting, extracellular polysaccharide biosynthesis enzymes were specially
487 suppressed by DA treatment. Hycs indicated inhibition effect on virulence genes,
488 might suggest these compounds not only could use as antibacterial agents, but also
489 might be virulence inhibitor under low concentration.

490 In conclusion, it was demonstrated that plant-derived metabolites
491 hydroxycoumarins ((umbelliferone, esculetin and daphnetin) significantly alter the
492 transcriptome level of *R. solanacearum*. The transcription change pattern of DA was
493 similar with ES treatment, different to the pattern exposure to UM. Compared with
494 the hydroxyl substituent site, the number of hydroxylation substituent of
495 hydroxycoumarins shown more important role in changing the gene expression in *R.*
496 *solanacearum*. Transcriptome analysis of cells treated with Hycs revealed
497 characteristic genes expression change, mainly included fatty acid pathway,
498 lipopolysaccharides biosynthesis pathway and ATP synthase pathway, accompanied
499 with the stress caused by the disruption of bacteria cell membrane, which can cause
500 the death of bacterial cells. This study provided important insights into the
501 bactericidal actions of Hycs against *R. solanacearum*.

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SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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Figure 1. *R. solanacearum* transcriptome signatures influenced by hydroxycoumarins. (A) Chemical structure of hydroxycoumarins (umbelliferone, UM; esculetin, ES; daphnetin, DA). (B) Principal component analysis (PCA) analysis of transcriptome level supplemented with hydroxycoumarins. (C) Comparison of ten genes expression levels between RNA-Seq and qRT-PCR. Choose genes involved in F_0F_1 ATP synthase subunits (*atpB*, *atpD*, *atpD* and *atpG*), lipopolysaccharides biosynthesis (*lpxB* and *Rsc0135*), fatty acid biosynthesis (*accC1*), and fatty acid degradation (*Rsc0064* and *Rsc0161*) and out membrane drug efflux lipoprotein *Rsp0817*. The gene-expression data obtained by RNA-Seq strategy strongly correlated with RT-PCR measurements.

Figure 2. Differentially expressed genes supplemented with three hydroxycoumarins (UM, ES and DA). (A) Differentially up and down-regulated genes number under hydroxycoumarins. (B) The volcano plots of up-regulated genes in *R. solanacearum* regulated by hydroxycoumarins. (C) The volcano plots of down-regulated genes in *R. solanacearum* regulated by hydroxycoumarins.

Figure 3. Heatmaps of read counts of differentially expressed genes (q-value ≤ 0.05**,** $|\log_2$ Fold change$|$ > 2)**.** The color going white to red represents the number of reads from low to high, respectively. The grouping of samples is indicated in the tips of the vertical clustering tree with red (CK), green (UM), purple (DA) and blue colors (ES). Each group is classified into eight clusters.

Figure 4. Hydroxycoumarins significantly reduce expression of genes coding for lipopolysaccharides biosynthesis. (A) Biosynthesis pathway of lipopolysaccharides in *R. solanacearum*, there were nine enzymes involved in the pathway. (B) *R. solanacearum* wild-type (CQPS-1) and *lpxB* mutant grow on solid medium after

665 inoculated 48 h. (C) The growth curve of wild-type (CQPS-1) and *lpxB* mutant in rich B
666 liquid medium. Bacterial density was measured at OD_{600} every two hour during 24
667 hours inoculation in liquid medium. * indicates statistically significant differences
668 between *lpxB* mutant and WT (CQPS-1) with student's *t* test analysis ($P<0.05$).

669 **Figure 5. Effect of LpxB on biofilm formation, swimming motility and virulence of *R.***
670 ***solanacearum* on tobacco.** (A) Biofilm formation of *R. solanacearum* in polystyrene
671 microtiter plates. Bacterial suspension were inoculated in rich medium and kept at
672 30 °C for 24 h without shaking. Biofilm formation was measured by OD530 after
673 stained with crystal violet. (C) Swimming motility of *R. solanacearum* in minimal
674 medium. The diameter of swimming halo was measured after 36 h, 48 h and 60 h
675 cultivation at 30 °C.

676 **Table 1. The significant enrich Gene ontology (GO) terms of differentially expressed**
677 **genes (DEGs) of *R. solanacearum* under hydroxycoumarins treatment.**

678 **Table 2. Selected differentially expression genes in *R. solanacearum* regulated by**
679 **DA and ES treatments based on GO term enrichment.**

680

681 **Figure S1. The volcano plots of differentially expression genes in *R. solanacearum***
682 **regulated by hydroxycoumarins.** (A) Differentially expressed genes of *R.*
683 *solanacearum* treated with umbelliferone (UM). (B) Esculetin treatment (ES). (C)
684 Daphnetin treatment (DA). Significantly differentially expressed genes were marked
685 with red dots (including upregulated and downregulated), and others indicated with
686 blue dots. The cutoff of q-value < 0.05 and $|\log_2$ Fold change$| > 2$ was identified as
687 significantly differentially expressed gene. The abscissa indicated fold change, and
688 the ordinate represents adjusted P value (q-value).

Figure S2. The Venn diagram of significantly differentially expressed genes supplemented with three hydroxycoumarins (UM, ES and DA). The number in each circle represents the number of differentially expressed genes that were expressed in UM, ES and DA treatment. And the overlapping part of circles indicated that the gene was co-expressed in two treatments or three treatments.

694 Table 1 The significant enrich Gene ontology (GO) terms of differentially expressed genes (DEGs) of *R.*
695 *solanacearum* under hydroxycoumarins treatment.

GO Category	GO ID	GO Name	FDR q-value		
			DA vs CK	ES vs CK	UM vs CK
Cellular component	GO:0005622	intracellular	1.68×10^{-7}	7.32×10^{-3}	1.61×10^{-2}
	GO:0043229	intracellular organelle	2.50×10^{-4}	7.66×10^{-3}	1.16×10^{-2}
	GO:0043232	intracellular non-membrane-bounded organelle	4.39×10^{-4}	6.92×10^{-3}	1.56×10^{-2}
	GO:0005840	ribosome	1.22×10^{-3}	9.73×10^{-3}	7.26×10^{-3}
	GO:0043228	non-membrane-bounded organelle	1.24×10^{-3}	1.74×10^{-2}	1.37×10^{-2}
	GO:0043226	organelle	1.63×10^{-3}	1.62×10^{-2}	1.60×10^{-2}
	GO:0110165	cellular anatomical entity	1.87×10^{-3}	3.08×10^{-2}	1.78×10^{-2}
	GO:0032991	protein-containing complex	3.38×10^{-2}	3.63×10^{-2}	1.44×10^{-2}
	GO:0016020	membrane	1.78×10^{-3}	2.45×10^{-4}	NA
	GO:0005886	plasma membrane	1.83×10^{-3}	4.55×10^{-2}	NA
	GO:0005737	cytoplasm	2.81×10^{-2}	NA	NA
	GO:0071944	cell periphery	NA	NA	1.63×10^{-2}
Molecular function	GO:0005198	structural molecule activity	2.71×10^{-4}	1.24×10^{-2}	1.09×10^{-2}
	GO:0003735	structural constituent of ribosome	2.32×10^{-4}	1.46×10^{-2}	1.65×10^{-2}
	GO:0005488	binding	2.68×10^{-3}	4.18×10^{-2}	0.011
	GO:0005215	transporter activity	2.91×10^{-2}	4.56×10^{-2}	1.94×10^{-2}
	GO:0019843	rRNA binding	2.29×10^{-3}	1.86×10^{-2}	NA
	GO:1901363	heterocyclic compound binding	6.55×10^{-3}	4.62×10^{-2}	NA
	GO:0097159	organic cyclic compound binding	8.54×10^{-3}	4.41×10^{-2}	NA
	GO:0003676	nucleic acid binding	3.63×10^{-2}	3.76×10^{-2}	NA
	GO:0022857	transmembrane transporter activity	3.38×10^{-2}	NA	2.81×10^{-2}
	GO:0000166	nucleotide binding	2.43×10^{-2}	NA	NA
	GO:0003723	RNA binding	7.14×10^{-3}	NA	NA
	GO:1901265	nucleoside phosphate binding	3.01×10^{-2}	NA	NA
	GO:0008324	cation transmembrane transporter activity	4.05×10^{-2}	NA	NA
Biological process	GO:0044237	cellular metabolic process	3.14×10^{-11}	1.67×10^{-2}	4.03×10^{-2}
	GO:1901564	organonitrogen compound metabolic process	1.32×10^{-13}	3.13×10^{-2}	3.27×10^{-2}
	GO:0044238	primary metabolic process	2.45×10^{-4}	1.42×10^{-2}	2.07×10^{-2}
	GO:0019538	protein metabolic process	3.25×10^{-4}	1.14×10^{-2}	7.13×10^{-3}
	GO:0051234	establishment of localization	6.11×10^{-4}	3.19×10^{-2}	3.13×10^{-2}
	GO:0006810	transport	5.75×10^{-4}	3.11×10^{-2}	1.14×10^{-2}
	GO:0044267	cellular protein metabolic process	6.06×10^{-4}	9.22×10^{-3}	1.99×10^{-2}
	GO:0043043	peptide biosynthetic process	7.96×10^{-4}	1.79×10^{-2}	1.54×10^{-2}
	GO:0006518	peptide metabolic process	7.60×10^{-4}	1.77×10^{-2}	1.64×10^{-2}
	GO:0055085	transmembrane transport	1.76×10^{-3}	1.70×10^{-2}	2.07×10^{-2}
	GO:0043603	cellular amide metabolic process	2.37×10^{-3}	2.84×10^{-2}	1.87×10^{-2}
	GO:0044249	cellular biosynthetic process	2.21×10^{-14}	4.66×10^{-2}	NA
	GO:0008152	metabolic process	1.76×10^{-12}	3.10×10^{-2}	NA
	GO:0071704	organic substance metabolic process	2.08×10^{-8}	1.09×10^{-2}	NA
	GO:0009058	biosynthetic process	1.90×10^{-4}	3.67×10^{-2}	NA

GO:1901576	organic substance biosynthetic process	2.95×10^{-4}	4.53×10^{-2}	NA
GO:0044260	cellular macromolecule metabolic process	2.07×10^{-3}	8.97×10^{-3}	NA
GO:0034645	cellular macromolecule biosynthetic process	2.31×10^{-3}	1.57×10^{-2}	NA
GO:0006412	translation	2.63×10^{-3}	1.70×10^{-2}	NA
GO:0009059	macromolecule biosynthetic process	2.79×10^{-3}	2.10×10^{-2}	NA
GO:0043170	macromolecule metabolic process	3.33×10^{-3}	8.21×10^{-3}	NA
GO:0006807	nitrogen compound metabolic process	3.94×10^{-3}	7.76×10^{-3}	NA
GO:0043604	amide biosynthetic process	6.80×10^{-3}	2.68×10^{-2}	NA
GO:0044271	cellular nitrogen compound biosynthetic process	1.30×10^{-2}	3.34×10^{-2}	NA
GO:0010467	gene expression	2.95×10^{-2}	8.65×10^{-3}	NA
GO:0051179	localization	5.76×10^{-4}	NA	1.02×10^{-2}
GO:0044255	cellular lipid metabolic process	2.01×10^{-2}	NA	1.20×10^{-2}
GO:0006629	lipid metabolic process	2.26×10^{-2}	NA	8.47×10^{-3}
GO:0009987	cellular process	2.14×10^{-4}	NA	NA
GO:1901566	organonitrogen compound biosynthetic process	5.44×10^{-4}	NA	NA
GO:0019637	organophosphate metabolic process	3.61×10^{-3}	NA	NA
GO:0006812	cation transport	5.78×10^{-3}	NA	NA
GO:1901293	nucleoside phosphate biosynthetic process	5.89×10^{-3}	NA	NA
GO:0009165	nucleotide biosynthetic process	6.44×10^{-3}	NA	NA
GO:0090407	organophosphate biosynthetic process	7.12×10^{-3}	NA	NA
GO:0098655	cation transmembrane transport	9.48×10^{-3}	NA	NA
GO:1901135	carbohydrate derivative metabolic process	1.38×10^{-2}	NA	NA
GO:0098662	inorganic cation transmembrane transport	1.36×10^{-2}	NA	NA
GO:0034220	ion transmembrane transport	1.60×10^{-2}	NA	NA
GO:0009117	nucleotide metabolic process	2.04×10^{-2}	NA	NA
GO:0006753	nucleoside phosphate metabolic process	2.45×10^{-2}	NA	NA
GO:1901137	carbohydrate derivative biosynthetic process	2.47×10^{-2}	NA	NA
GO:0006796	phosphate-containing compound metabolic process	2.50×10^{-2}	NA	NA
GO:0098660	inorganic ion transmembrane transport	2.98×10^{-2}	NA	NA
GO:0015672	monovalent inorganic cation transport	3.02×10^{-2}	NA	NA
GO:1902600	proton transmembrane transport	3.42×10^{-2}	NA	NA
GO:0006793	phosphorus metabolic process	3.38×10^{-2}	NA	NA

(DA indicates daphnetin treatment, ES indicates esculetin treatment and UM means umbelliferone treatment)

697 Table 2. Selected differentially expression genes in *R. solanacearum* regulated by DA and ES treatments according
698 to GO term enrichment.

locus_tag	GMI1000	gene	Description	log2 Fold change (DA-vs-CK)	log2 Fold change (ES vs CK)	log2 Fold change (UM vs CK)
Lipopolysaccharides biosynthesis						
BC350_RS06480	*RSc0136*	-	lipid A biosynthesis lauroyl acyltransferase	-2.15	-2.37	#N/A
BC350_RS06485	*RSc0135*	-	lipid A biosynthesis lauroyl acyltransferase	-3.16	-2.80	#N/A
BC350_RS17350	*RSc1370*	*lplT*	putative lysophospholipid transporter	-2.82	-2.68	#N/A
BC350_RS17110	*RSc1417*	*lpxB*	lipid-A-disaccharide synthase	-3.26	-3.44	#N/A
BC350_RS17115	*RSc1416*	*lpxA*	UDP-N-acetylglucosamine acyltransferase	-2.49	-2.60	#N/A
BC350_RS17125	*RSc1414*	*lpxD*	UDP-3-O-(3-hydroxymyristoyl)glucosamine N-acyltransferase	-2.42	-2.50	#N/A
BC350_RS17120	*RSc1415*	*fabZ*	3R-hydroxyacyl-ACP dehydratase FabZ	-2.75	-2.69	#N/A
BC350_RS03430	*RSc0684*	*rfbA*	glucose-1-phosphate thymidylyltransferase protein	-2.97	-2.00	#N/A
Genes encoding proteins involved in fatty acid synthesis and metabolism and membrane modification						
BC350_RS05760	*RSc0265*	-	acyl-CoA carboxylase subunit alpha	-2.48	-2.37	#N/A
BC350_RS25670	*RSp0652*	-	acyl-CoA dehydrogenase oxidoreductase protein	-2.11	-2.22	#N/A
BC350_RS25690	*RSp0648*	-	enoyl-CoA hydratase	-2.48	-3.91	#N/A
BC350_RS00385	*RSc1172*	*fabI*	enoyl-[acyl-carrier-protein] reductase FabI	-3.76	-2.96	#N/A
BC350_RS01040	*RSc1052*	*fabG*	3-ketoacyl-(acyl-carrier-protein) reductase	-2.22	-2.04	#N/A
BC350_RS01045	*RSc1051*	*fabD*	acyl-carrier-protein S-malonyltransferase	-2.35	-2.51	#N/A
BC350_RS01050	*RSc1050*	*fabH*	3-oxoacyl-(acyl carrier protein) synthase III	-2.85	-3.40	#N/A
BC350_RS22070	*RSp0357*	*fabB*	3-oxoacyl-[acyl-carrier-protein] synthase I	-2.30	-2.08	#N/A
BC350_RS01035	*RSc1053*	*acpF*	acyl carrier protein	-3.24	-3.76	#N/A
BC350_RS04755	*RSc0434*	-	acyl carrier protein	-3.97	-2.81	#N/A
BC350_RS11530	*RSc2546*	-	putative glycerol-3-phosphate acyltransferase PlsY	-3.15	-2.39	#N/A
BC350_RS13210	*RSc2253*	*pcaJ*	3-oxoadipate CoA transferase subunit B	2.03	2.84	#N/A
BC350_RS13215	*RSc2252*	*pcaF*	beta-ketoadipyl CoA thiolase	2.15	2.15	#N/A
BC350_RS13220	*RSc2251*	*pcaB*	3-carboxy-cis,cis-muconate cycloisomerase	2.65	2.49	#N/A
BC350_RS13225	*RSc2250*	*pcaD*	B-ketoadipate enol-lactone hydrolase transmembrane protein	2.10	2.11	#N/A
BC350_RS13230	*RSc2249*	*pcaC*	4-carboxymuconolactone decarboxylase	2.37	2.61	#N/A
BC350_RS06355	*RSc0161*	-	transmembrane aldehyde dehydrogenase oxidoreductase protein	3.04	2.88	#N/A
Genes encoding proteins involved in conformational modification of RNA						
BC350_RS00415	*RSc1167*	*cysS*	cysteine--tRNA synthetase	-3.15	-2.88	#N/A
BC350_RS05290	*RSc0358*	*yibK*	putative tRNA/rRNA methyltransferase protein	-2.58	-2.51	#N/A
BC350_RS17950	*RSc1251*	*hrpA*	ATP-dependent RNA helicase protein	-2.12	-2.31	#N/A
Genes encoding proteins involved in translation						
BC350_RS08680	*RSc3041*	*tuf*	elongation factor Tu	-2.73	-2.01	#N/A
BC350_RS13735	*RSc2152*	*greB*	transcription elongation factor GreB	2.84	2.38	#N/A
Genes encoding proteins involved in transcriptional regulation						
BC350_RS08275	*RSc3160*	-	two component sensor histidine kinase transcription regulator protein	-2.76	-2.26	#N/A
BC350_RS08305	*RSc3156*	*cspC*	cold shock-like transcription regulator protein	-2.85	-2.09	#N/A
BC350_RS12010	*RSc2430*	-	putative transcription regulator protein	-2.31	-3.68	#N/A
BC350_RS15545	*RSc1584*	-	putative transcription regulator protein	-2.35	-2.09	#N/A
BC350_RS12345	*RSc2361*	-	RNA polymerase sigma-E factor sigma-24 homolog transcription regulator protein	2.51	2.13	#N/A
BC350_RS13910	*RSc2114*	-	transcription regulator protein	2.92	2.81	#N/A
BC350_RS16745	*RSc1857*	-	putative transcription regulator	2.34	2.79	#N/A

			protein/PLP-dependent aminotransferase family protein			
BC350_RS00285	*RSc1185*	-	transcription regulator protein	2.80	2.44	#N/A
BC350_RS01335	*RSc0993*	-	putative transcriptional regulatory DNA-binding transcription regulator protein	2.82	2.40	#N/A
BC350_RS18850	*RSp0415*	-	extracytoplasmic function sigma factor transcription regulator protein	6.48	6.20	#N/A
BC350_RS21525	*RSp0247*	*fur2*	ferric uptake transcriptional (FUR)-like transcription regulator protein	4.47	4.72	#N/A
BC350_RS24180	*RSp0962*	-	putative transcription regulator protein	2.39	2.97	#N/A
BC350_RS24195	*RSp0959*	-	anaerobic nitric oxide reductase transcription regulator	2.57	2.23	#N/A
Genes encoding proteins involved in transcription						
BC350_RS24675	RSp0849	prhI	RNA polymerase sigma factor	-2.06	2.14	#N/A
BC350_RS00275	RSc1187	-	transcription termination factor Rho	-3.09	-2.86	#N/A
BC350_RS18350	RSp0553	-	metal/formaldehyde-sensitive transcriptional repressor	-2.71	-2.65	#N/A
Genes encoding proteins involved in membrane transport						
BC350_RS17375	*RSc1365*	-	putative multidrug resistance-like efflux transmembrane protein	-2.11	-2.57	#N/A
BC350_RS01230	*RSc1015*	-	transmembrane ABC transporter protein	-2.33	-3.12	#N/A
BC350_RS01425	*RSc0975*	-	ABC transporter ATP-binding protein	-2.79	-2.33	#N/A
BC350_RS04505	*RSc0484*	*gltL*	putative glutamate/aspartate transport ATP- binding ABC transporter protein	-3.07	-2.58	#N/A
BC350_RS04515	*RSc0482*	*gltJ*	glutamate/aspartate transmembrane ABC transporter protein	-2.47	-2.47	#N/A
BC350_RS04520	*RSc0481*	-	amino-acid-binding periplasmic (PBP) ABC transporter protein	-2.23	-2.04	#N/A
BC350_RS07625	*RSc3344*	-	ABC transporter ATP-binding protein	-2.47	-2.60	#N/A
BC350_RS07630	*RSc3343*	-	ABC transporter ATP-binding protein	-2.54	-2.45	#N/A
BC350_RS07635	*RSc3342*	-	putative substrate-binding periplasmic (PBP) ABC transporter protein	-2.96	-3.10	#N/A
BC350_RS07640	*RSc3341*	-	transmembrane ABC transporter protein	-3.10	-2.87	#N/A
BC350_RS07645	*RSc3340*	-	transmembrane ABC transporter protein	-2.30	-2.22	#N/A
BC350_RS07700	*RSc3329*	-	amino-acid-binding periplasmic (PBP) ABC transporter protein	-2.95	-2.42	#N/A
BC350_RS11350	*RSc2631*	-	transmembrane ABC transporter protein	-2.35	-2.32	#N/A
BC350_RS11955	*RSc2441*	-	putative amino acid-binding periplasmic ABC transporter proteinn	-4.01	-4.27	#N/A
BC350_RS12085	*RSc2417*	-	LPS export ABC transporter permease LptG	-2.90	-3.01	#N/A
BC350_RS12090	*RSc2416*	-	LPS export ABC transporter permease LptF	-3.14	-2.75	#N/A
BC350_RS15255	*RSc1529*	*pstS1*	phosphate ABC transporter substrate-binding protein PstS	-2.15	-2.59	#N/A
BC350_RS20000	*RSp1575*	-	amino-acid-binding periplasmic (PBP) ABC transporter protein	-2.98	-2.29	#N/A
BC350_RS20005	*RSp1576*	-	amino-acid transmembrane ABC transporter protein	-2.62	-2.59	#N/A
BC350_RS20255	*RSp1633*	*xylF*	D-xylose ABC transporter substrate-binding protein	-4.35	-3.95	#N/A
BC350_RS20260	*RSp1634*	*xylG*	xylose ABC transporter ATP-binding protein	-3.71	-3.66	#N/A
BC350_RS20265	*RSp1635*	*xylH*	xylose transmembrane ABC transporter protein	-3.41	-3.73	#N/A
BC350_RS20540	*RSp0016*	-	amino-acid ATP-binding ABC transporter protein	-2.66	-2.74	#N/A
BC350_RS20545	*RSp0017*	-	amino-acid ATP-binding ABC transporter protein	-2.36	-2.18	#N/A
BC350_RS21755	*RSp0292*	*cyaB*	cyclolysin-type secretion composite ATP-binding transmembrane ABC transporter protein	-3.52	-3.54	#N/A
BC350_RS04295	*RSc0522*	-	putative acyltransferase transmembrane protein	-3.03	-2.12	#N/A
BC350_RS08340	*RSc3150*	-	putative transmembrane protein	-2.63	-3.10	#N/A
BC350_RS09510	*RSc2976*	*mrcA*	penicillin-binding 1 transmembrane protein	-2.54	-2.32	#N/A
BC350_RS10520	*RSc2784*	-	putative thioredoxin-related transmembrane protein	-2.29	-2.02	#N/A
BC350_RS11620	*RSc2528*	*exbB2*	biopolymer transport EXBB-like	-3.72	-3.16	#N/A

			transmembrane protein			
BC350_RS11645	*RSc2523*	-	putative transmembrane protein	-2.39	-2.13	#N/A
BC350_RS15570	*RSc1588*	-	amino-acid transporter transmembrane protein	-2.73	-2.74	#N/A
BC350_RS16185	*RSc1757*	-	lysine-specific permease transmembrane protein	-2.25	-2.52	#N/A
BC350_RS16190	*RSc1758*	*lysP*	lysine-specific permease transmembrane protein	-2.59	-2.55	#N/A
BC350_RS19280	*RSp1423*	-	putative transmembrane protein	-2.78	-2.29	#N/A
BC350_RS20250	*RSp1632*	*oprB*	putative porin B precursor outer (glucose porin) transmembrane protein	-3.70	-3.38	#N/A
BC350_RS21080	*RSp0150*	-	general secretion pathway GSPG-like transmembrane protein	-2.20	-2.38	#N/A
BC350_RS21145	*RSp0169*	-	putative transmembrane protein	-3.05	-2.28	#N/A
BC350_RS21700	*RSp0282*	-	amino-acid permease transmembrane protein	-3.70	-3.17	#N/A
BC350_RS21750	*RSp0291*	-	hemolysin secretion-like transmembrane protein	-3.20	-3.62	#N/A
BC350_RS22515	*RSp1294*	-	serin-rich transmembrane protein	-3.49	-2.52	#N/A
BC350_RS24670	*RSp0850*	*prhR*	3-compartiment signal transduction system, component PRHR transmembrane protein	-2.78	2.19	#N/A
BC350_RS25805	*RSp0628*	*hoxN*	high affinity cobalt transporter transmembrane protein	-4.07	-2.57	#N/A
BC350_RS17745	*RSc1292*	*emrB*	multidrug resistance B (translocase) transmembrane protein	4.97	4.48	#N/A
BC350_RS10805	*RSc2726*	-	multidrug ABC transporter transmembrane protein	2.20	2.39	#N/A
BC350_RS06340	*RSc0164*	-	ABC transporter ATP-binding protein	3.62	3.92	#N/A
BC350_RS16435	*RSc1808*	-	ABC transporter ATP-binding protein	4.05	4.77	#N/A
BC350_RS16440	*RSc1809*	-	ABC transporter ATP-binding protein	4.72	5.10	#N/A
BC350_RS17290	*RSc1382*	-	transmembrane ABC transporter protein	4.27	4.56	#N/A
BC350_RS17295	*RSc1381*	-	transmembrane ABC transporter protein	4.34	4.90	#N/A
BC350_RS17300	*RSc1380*	-	substate-binding periplasmic (PBP) ABC transporter protein	4.46	5.07	#N/A
BC350_RS17305	*RSc1379*	-	ABC transporter ATP-binding protein	4.75	4.96	#N/A
BC350_RS23245	*RSp1145*	-	ABC transporter ATP-binding protein	2.54	2.08	#N/A
BC350_RS14640	*RSc1965*	*exbD1*	biopolymer transport transmembrane protein	2.81	2.49	#N/A
BC350_RS14645	*RSc1964*	*exbB1*	biopolymer transport transmembrane protein/MotA/TolQ/ExbB proton channel family protein	3.16	2.51	#N/A
BC350_RS00565	*RSc1138*	-	putative transmembrane protein	2.29	2.84	#N/A
BC350_RS07350	*RSc3400*	-	tranporter transmembrane protein	5.09	4.32	#N/A
BC350_RS12875	*RSc2324*	-	putative transport transmembrane protein	4.60	4.70	#N/A
BC350_RS12880	*RSc2323*	-	transport transmembrane protein	2.23	2.20	#N/A
BC350_RS12945	*RSc2310*	-	putative GSPG-related transmembrane protein	2.60	2.12	#N/A
BC350_RS14650	*RSc1963*	*tonB*	TONB transmembrane protein	4.03	4.63	#N/A
BC350_RS20335	*RSp1650*	-	putative transmembrane protein	2.53	2.11	#N/A
BC350_RS21465	*RSp0235*	-	putative maltooligosyl trehalose synthase transmembrane protein	4.40	3.09	#N/A
BC350_RS21540	*RSp0250*	-	putative transmembrane protein	3.68	2.69	#N/A
BC350_RS25600	*RSp0663*	-	transport transmembrane protein	2.71	2.96	#N/A
Genes encoding proteins involved in oxidative phosphorylation and electron transport						
BC350_RS05260	*RSc0365*	*ctaG*	cytochrome c oxidase assembly protein	-2.42	-2.39	#N/A
BC350_RS09820	*RSc2917*	*cyoA1*	transmembrane cytochrome O ubiquinol oxidase subunit II	-2.92	-2.23	#N/A
BC350_RS07730	*RSc3323*	*atpB*	F0F1 ATP synthase subunit A	-2.31	-2.53	#N/A
BC350_RS07735	*RSc3322*	*atpE*	F0F1 ATP synthase subunit C	-2.62	-2.01	#N/A
BC350_RS07740	*RSc3321*	*atpF*	F0F1 ATP synthase subunit B	-2.43	-2.25	#N/A
BC350_RS07750	*RSc3319*	*atpA*	ATP synthase subunit alpha	-2.94	-2.03	#N/A
BC350_RS07760	*RSc3317*	*atpD*	ATP synthase subunit beta	-2.96	-2.17	#N/A
BC350_RS07765	*RSc3316*	*atpC*	ATP synthase subunit epsilon	-2.80	-2.46	#N/A
BC350_RS14210	*RSc2055*	*nuoH*	NADH dehydrogenase subunit H	-2.09	-2.03	#N/A
BC350_RS14220	*RSc2053*	*nuoJ*	NADH dehydrogenase subunit J	-2.02	-2.19	#N/A

BC350_RS14225	*RSc2052*	*nuoK*	NADH dehydrogenase subunit K	-2.21	-2.10	#N/A
BC350_RS14230	*RSc2051*	*nuoL*	NADH dehydrogenase subunit L	-2.41	-2.61	#N/A
BC350_RS14235	*RSc2050*	*nuoM*	NADH dehydrogenase subunit M	-2.26	-2.20	#N/A
BC350_RS14240	*RSc2049*	*nuoN*	NADH dehydrogenase subunit N	-2.43	-2.21	#N/A
Genes encoding stress-related proteins						
BC350_RS03010	*RSc0764*	*msrA*	methionine sulfoxide reductase A	2.81	3.19	#N/A
BC350_RS20430	*RSp1671*	*rpoN2*	RNA polymerase factor sigma-54 factor	3.60	2.92	#N/A
Protein export						
BC350_RS10860	*RSc2716*	*secF*	protein translocase subunit SecF	-2.13	-2.26	#N/A
BC350_RS10865	*RSc2715*	*secD*	protein translocase subunit SecD	-2.17	-2.16	#N/A

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