Melatonin Treatment Inhibits the Growth of *Xanthomonas oryzae* pv. *oryzae*

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*Xanthomonas oryzae* pv. *oryzae* (Xoo) causes rice bacterial blight (BB), one of the most widespread and destructive diseases in rice-growing regions worldwide. Melatonin enhances pathogen resistance by inducing plant innate immunity, but the direct effect of melatonin on plant pathogenic bacteria is poorly understood. In this study, we investigated the direct effects of melatonin on Xoo. Exogenous melatonin at 200 µg/mL significantly inhibited the proliferation of Xoo and reduced the mRNA expression of five genes involved in cell division. This concentration of melatonin also inhibited the motility and biofilm formation of Xoo. Notably, melatonin was observed to alter the length of Xoo cells. To provide deeper insights into the mechanisms underlying this antibacterial activity, we examined global gene expression changes in Xoo strain PXO99 in response to the application of 200 µg/mL melatonin using RNA sequencing (RNA-Seq). A wide range of differentially expressed genes (DEGs) related to catalytic activity and metal-binding activity were downregulated in Xoo cells in response to the melatonin treatment. In addition, DEGs responsible for carbohydrate and amino acid metabolism were also downregulated. These results suggest that the inhibitory mechanism of melatonin on Xoo proliferation may involve the regulation of cell division in combination with a reduction in the concentration or activity of enzymes involved in metabolism.

**Keywords**: melatonin, *Xanthomonas oryzae* pv. *oryzae*, antibacterial action, growth, transcriptome

INTRODUCTION

Melatonin (N-acetyl-5-methoxytryptamine) is a highly evolutionarily conserved molecule that exists in the microbe (Manchester et al., 2015), insect (Vivien-Roels et al., 1984), animal (Menendez-Pelaez and Reiter, 1993), and plant kingdoms (Dubbels et al., 1995). In animals, melatonin was discovered in the bovine pineal gland in 1958 (Lerner et al., 1958). This indoleamine is a well-known animal neurohormone involved in numerous cellular and physiological functions, such as sleep (Garfinkel et al., 1995), circadian rhythms (Jung-Hynes et al., 2010), stem cell differentiation (Radio et al., 2006), and scavenging of reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Reiter et al., 2016). In plants, melatonin was simultaneously discovered by two research groups in 1995 (Dubbels et al., 1995; Hattori et al., 1995).
Since then, melatonin has been found in a variety of plant species (Kolar and Machackova, 2005). Plant melatonin is involved in many significant plant processes, including plant growth (Chen et al., 2009; Arnal and Hernández-Ruiz, 2017) and defence against both biotic (Vielma et al., 2014; Shi et al., 2016) and abiotic stresses (Byeon and Back, 2016; Zhang et al., 2017). In microbes, exogenous melatonin acts as a biocide against some fungi and bacteria (Wang et al., 2001; Hu et al., 2017). Melatonin shows antibacterial activity against Gram-positive and Gram-negative bacteria at a low concentration in vitro (Atroshi et al., 1998; Konar et al., 2000; Ozturk et al., 2000; Karakas et al., 2013). In vivo, the exogenous application of melatonin was shown to suppress PstDC3000 propagation in Arabidopsis leaves (Lee et al., 2014). Melatonin may prevent the uptake of free iron by bacteria (Limson et al., 1998; Tekbas et al., 2008), inhibit constitutive bacterial protein secretion (Bubis and Zisapel, 1995), and reduce intracellular substrates that are important for bacterial growth (Tekbas et al., 2008). However, the mechanisms underlying these inhibitory effects of melatonin on bacteria have been little studied.

Bacterial blight (BB) of rice caused by X. oryzae pv. oryzae (Xoo) is one of the most destructive diseases in most rice-growing countries, especially those in Asia (Mansfield et al., 2012). This disease leads to leaf blight during the growing season, hindering photosynthesis and diminishing the production and quality of crops (Mahmood et al., 2006). Despite attempts to control BB by broad-spectrum breeding with high-yield cultivars, this disease remains a major constraint on rice production (Suh et al., 2013). Earlier research demonstrated that N-acetylserylserotonin methyltransferase (ASMT), the last enzyme involved in the synthesis of melatonin, was induced during Xoo infection (Wei et al., 2016). However, there have been no reports on the relationship between melatonin and Xoo.

In this study, we used Xoo strain PXO99 to determine whether melatonin exhibits antibacterial activity against this pathogen. In addition, the relationships between melatonin and cell division and morphology were investigated. We also used RNA sequencing (RNA-Seq) to explore how melatonin, in its role as an antibiotic, inhibits the growth of PXO99. A genome-wide expression profiling analysis clearly demonstrated that many genes involved in metabolic and transcription processes were downregulated. Our results could help to gain a better understanding of the mechanisms by which melatonin inhibits the proliferation of Gram-negative bacteria.

### MATERIALS AND METHODS

#### Bacterial Strain and Plants

Xoo strain PXO99 was streaked on nutrient agar (NA) medium (beef extract, 3 g/L; yeast extract, 1 g/L; polypeptone, 5 g/L; sucrose, 10 g/L; and agar, 15 g/L) and incubated at 28°C for 2 days. Rice seedlings of the Nipponbare (Oryza sativa spp. Nipponbare) cultivar were germinated and grown in a growth chamber under an alternating 12-h light/12-h dark cycle with a photon flux density of 200 μmol/m²·s¹. Rice leaves were inoculated with Xoo strain PXO99 (race P6) for pathogenicity tests using the leaf clipping method (Kauffman et al., 1973). Tobacco plants (Nicotiana benthamiana, Nb) were grown in a growth chamber under an alternating 12-h light/12-h dark cycle at 25°C with a photon flux density of 120 μmol/m²·s¹. Tobacco leaves were inoculated with PXO99 for hypersensitive reaction (HR) assays using the needleless syringe method (Xu et al., 2015). Statistical analyses were performed using SPSS Version 20.0. The variables were analyzed using Student’s t-tests and were tested for significance at the P < 0.05 (*), P < 0.01 (**), P < 0.001 (***) levels.

#### Measurement of the Effect of Melatonin on Bacterial Growth

Xoo strain P6 was incubated with shaking in nutrient broth (NB) medium (NA without agar) at 28°C until an OD$_{600}$ = 1.0 (early logarithmic phase) was reached. The cells were harvested and resuspended in an equal volume of sterilized ddH$_2$O. Next, 0.5 mL of the cell suspension was added to 50 mL of NB liquid medium containing different concentrations of melatonin (0, 200, 400, or 1000 μg/mL). Methanol (MeOH) solvent without melatonin (0 μg/mL) served as a control. All cultures were shaken (200 rpm) at 28°C in the dark, and the OD$_{600}$ was measured every 3 h until bacterial growth reached the stationary phase. Each experiment was performed three times, with three replicates per experiment.

#### Transmission Electron Microscope (TEM) Observations

The concentration of fresh bacteria in sterilized ddH$_2$O was adjusted to OD$_{600}$ = 1.0. Next, 0.5 mL of cell suspension was added into 50-mL fresh NB medium containing different concentrations of melatonin (0, 200, or 400 μg/mL). Methanol (MeOH) solvent without melatonin (0 μg/mL) served as a control. All cultures were grown at 28°C with shaking at 200 rpm for 12 h. Bacterial samples were placed on copper mesh grids with formvar membranes and negatively stained with phosphotungstic acid (2% v/v, pH = 6.7). The samples were then observed using a TEM (Hitachi H-7650) at 80 kV and photographed with a Gatan832 CCD camera (Gatan, Pleasanton, CA, United States).

#### Determination of Cell Motility and Biofilm Formation

Swimming motility and biofilm formation assays were performed as described previously (Tian et al., 2015). The concentration of fresh bacteria in sterilized ddH$_2$O was adjusted to OD$_{600}$ = 1.0. Next, a 5-μL aliquot of the bacterial suspension was spotted onto semi-solid NA (0.3% agar) containing different concentrations of melatonin (0, 10, 40, or 250 μg/mL). Methanol (MeOH) solvent without melatonin (0 μg/mL) served as a control. Cell motility was monitored after a 96 h incubation at 28°C in darkness. Each experiment was performed three times, with five replicates per experiment. For the biofilm formation assay, a 30-μL cell suspension was inoculated into 3 mL NB liquid medium.

1http://www.genome.jp/kegg/
containing different concentrations of melatonin (0, 10, 40, or 250 µg/mL). After inoculation, the cultures were incubated at 28°C for 5 days without shaking. After gently removing the cultures, the cells adhered to the culture tubes were stained with two volumes of 10% (w/v) crystal violet solution and incubated at 28°C without shaking for 1 h, followed by gentle washing with sterilized ddH₂O three times, and air drying for 1 h. The crystal violet in the stained cells was dissolved using destaining buffer [40% methanol (v/v), 10% glacial acetic acid (v/v), 50% ddH₂O (v/v)], and the absorbance at 595 nm (OD₅₉₅) was measured using a spectrophotometer (Eppendorf, Germany). Each experiment was performed three times, with six replicates each time.

Measurement of Endogenous Melatonin
To determine the melatonin content of Xoo cells, a direct sample extraction method was used. The concentration of fresh bacteria in sterilized ddH₂O was adjusted to OD₆₀₀ = 1.0. Next, 0.5 mL of cell suspension was added to 50 mL of fresh NB medium containing different concentrations of melatonin (0 or 200 µg/mL). Methanol (MeOH) solvent without melatonin (0 µg/mL) served as a control. All cultures were grown at 28°C with shaking at 200 rpm for 24 h. The two cultures were adjusted to the same concentration (OD₆₀₀ = 1.0) and washed with sterilized ddH₂O three times. Next, the cultures were centrifuged and the pellets were suspended in 10 mL of acetonitrile buffer. The bacterial cells were homogenized using a sonicator (Scientz, Ningbo). After centrifugation, the supernatants were subjected to LC−MS as described previously (Huang and Mazza, 2011).

RNA Sequencing and Data Analysis
RNA was extracted from strain PXO99 treated with MeOH (M0) and 200 µg/mL melatonin (M200) and used for RNA sequencing. After a 21 h incubation, bacterial cultures at OD₆₀₀ = 1.0 (early logarithmic phase) in broth were harvested. Total RNA was extracted from the mock and melatonin-treated samples using TRIzol reagent (Invitrogen, Carlsbad, CA, United States) according to the procedure recommended by the manufacturer. The following steps were then completed by a commercial company (GenePioneer Biotechnologies Corporation, Nanjing, China). Three micrograms of RNA per sample was used for library construction. For direct comparisons, two libraries (M0 and M200) were prepared in the same manner and sequenced on an Illumina HiSeq Xten platform. We selected genes with a log₂FC > 2 and p < 0.01 for further analysis. Differentially expressed genes (DEGs) between the melatonin-treated and mock samples were analyzed by Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment.
RNA Extraction and Quantitative Real-Time PCR Analysis

Specific primers for quantitative real-time PCR (qRT-PCR) were designed with Primer 5 (version 5) using the corresponding gene sequences from the NCBI database (Supplementary Table S1). Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, United States) according to the procedure recommended by the manufacturer, treated with DNase I (Takara, Japan) to eliminate genomic DNA, and then converted into cDNA using a PrimeScript™ RT Reagent Kit (Takara, Japan). Next, qRT-PCR was performed using diluted cDNA and SYBR Green PCR Master Mix (Takara, Japan) on a Quant Studio 6 Real Time PCR system (Thermo Fisher Scientific, United States). The expression data, given as quantification cycle (Cq), were collected and statistically processed using the $2^{-\Delta\Delta Ct}$ method. RecA was used as an internal control, and each experiment was conducted three times with three replicates.

RESULTS AND DISCUSSION

Melatonin Inhibits Xoo Growth

Melatonin has been previously observed to play multiple roles in a wide variety of significant processes in plants, animals, and humans (Dollins et al., 1994; Guerrero and Reiter, 2002; Shi et al., 2015; Fu et al., 2017). However, the impact of melatonin on agriculturally relevant bacteria has not been explored. To this end, we assessed the bacterial growth rates of Xoo treated with methanol (mock control) and various concentrations of melatonin (Figure 1A). The OD$_{600}$ value of PXO99 at 24 h pre-treated with melatonin (200 µg/mL) was approximately 1.0, only half that of the control group (Figure 1B). Thus, 200 µg/mL of melatonin effectively reduced the growth of PXO99. When the concentration was elevated, the bacterial density showed
a greater decrease. No PXO99 cells survived 24 h in broth containing 1000 µg/mL melatonin or 50 µg/mL kanamycin. Melatonin represses the growth of human pathogenic bacteria at certain concentrations (Hu et al., 2017), including that of Streptococcus agalactiae at 2 µg/mL (Atroshi et al., 1998) and Saccharomyces cerevisiae at 1000 µg/mL (Konar et al., 2000). Our growth inhibition results showed that melatonin inhibited PXO99 growth in a concentration-dependent manner, and the inhibitory effect may be dose dependent. In plants, we also observed that 200 µg/mL melatonin suppressed the HR induced by PXO99 on tobacco leaves (Supplementary Figure S1). Overexpression of a melatonin-induced gene (OsMAPK12-1) resulted in plants with enhanced disease resistance against PXO99 (Xiao et al., 2017). Melatonin-induced plant resistance is mediated by MAPK signaling (Lee and Back, 2017) Thus, whether the suppression of HR caused by melatonin-induced plant innate immunity or melatonin inhibiting the proliferation of PXO99 caused the observed plant disease resistance still requires further investigation.

**Melatonin Reduces Xoo Swimming Motility but Increases Biofilm Formation**

Swimming motility is necessary for biofilm formation and is crucial for bacterial attachment (O'Toole and Kolter, 1998; de Kerchove and Elimelech, 2008). However, the impact of melatonin on bacterial swimming motility has rarely been reported. To study the influence of melatonin on bacterial motility, the swimming motility diameter of Xoo was measured in the presence and absence of melatonin. In initial experiments, we observed that a higher concentration of melatonin disrupted the swimming ability of Xoo. Thus, melatonin was used at no more than 200 µg/mL in subsequent tests. As shown in Figure 2A, the colony diameters in plates with 10 µg/mL melatonin were decreased by more than 30% compared with that observed in the mock control. When the melatonin concentration was increased, the swimming motility diameter was further decreased. The colony diameters in plates with 200 µg/mL melatonin were reduced by more than half compared with that observed in the mock control. Thus, melatonin affected the motility of Xoo in a concentration-dependent manner. These results suggest that the inhibition of bacterial motility by melatonin may occur through increasing cell death, although further investigation of this possibility is necessary.

Biofilm formation plays a crucial role in plant pathogen infections (Parsek and Singh, 2003). Melatonin was reported to inhibit the biofilm formation of Candida parapsilosis and S. aureus ATCC29213 at 2.9 and 340 µg/mL, respectively (Yang et al., 2014; Romic et al., 2016). Biofilm-associated pathogens can form light-colored rings on the wall of a culture tube at the interface between air and broth. To evaluate the effect of melatonin on the attachment of Xoo, the biofilm formation of PXO99 in response to melatonin challenge was analyzed. As shown in Figure 2B, the presence of 10 µg/mL melatonin slightly increased the biofilm formation of PXO99. When the melatonin concentration was increased, the CV absorbance at OD₅₉₅ showed a greater increase. The observed OD₅₉₅ value from tubes containing 40 µg/mL melatonin was threefold higher than that of the mock control. However, the opposite effect was observed when melatonin was present at higher concentrations. The OD₅₉₅ value in the tubes containing melatonin at 200 µg/mL was 40% lower than that of the control. Thus, the effects of melatonin on PXO99 biofilm formation did not resemble the observed effects on swimming motility or growth inhibition. When melatonin was present at a high concentration, no swimming motility, or biofilm formation was observed. Our results suggest that melatonin may induce biofilm formation in Xoo at low concentrations but inhibit its formation at high concentrations. Interestingly, we observed that both the bacterial abundance and lesion length in rice leaves infected with PXO99 treated with melatonin (200 µg/mL) was similar to that of the control group (Supplementary Figure S2). Moreover, the HR in tobacco leaves induced by PXO99 treated with melatonin (200 µg/mL) was similar to that of the control group (Supplementary Figure S3). Thus, the results indicated that melatonin may not affect Xoo pathogenicity.

**Xoo Becomes Highly Enriched With Melatonin**

Melatonin has been observed to easily pass through cell walls (Tekbas et al., 2008). In this study, we evaluated the content of endogenous melatonin in PXO99 cell treated with melatonin by LC-MS. The endogenous melatonin was 14.43 ng in POX99 cells that were harvested from 30 mL broth cultures after incubating for 24 h. In contrast, the 156.13 ng of endogenous melatonin was detected in PXO99 cells that were incubated with exogenous melatonin and harvested from 30 mL broth cultures after incubating for 24 h (Figure 3A). The results showed that melatonin can easily pass through the cell wall and become enriched in Xoo cells. The endogenous melatonin detected in the treatment group was approximately 100 times that in the control group (Figure 3B). This disruption in normal endogenous melatonin levels in Xoo may inhibit the proliferation of this bacterium. Because melatonin was detected in PXO99, we...
### TABLE 1 | Differentially expressed genes in Xoo treated with melatonin.

| No. | Gene ID     | FC* | Annotation                                      |
|-----|-------------|-----|------------------------------------------------|
|     |             |     | **Transcription and translation**               |
| 1   | PXO_RS00800 | −2.83 | Transposase                                    |
| 2   | PXO_RS24475 | +1.60 | Transposase                                    |
| 3   | PXO_RS24500 | +1.97 | Transposase                                    |
| 4   | PXO_RS27155 | −2.48 | Isrso17-ISXo8 transposase protein              |
| 5   | PXO_RS08305 | −1.56 | AcrB family transcriptional regulator         |
| 6   | PXO_RS05275 | −2.34 | LysR transcriptional regulator                |
| 7   | PXO_RS17440 | −1.84 | TetR family transcriptional regulator         |
| 8   | PXO_RS09765 | −2.27 | PhoU family transcriptional regulator         |
| 9   | PXO_RS08695 | −1.98 | GntR family transcriptional regulator         |
| 10  | PXO_RS19870 | −2.38 | PhoB family transcriptional regulator         |
| 11  | PXO_RS20385 | −1.91 | AraC family transcriptional regulator         |
| 12  | PXO_RS13760 | −1.88 | MarR family transcriptional regulator         |
| 13  | PXO_RS23815 | −3.61 | Alkaline phosphatase D                        |
| 14  | PXO_RS23810 | −4.78 | Alkaline phosphatase                          |
| 15  | PXO_RS18050 | −2.20 | DNA-binding protein                           |
| 16  | PXO_RS08310 | −1.56 | DNA-binding protein                           |
| 17  | PXO_RS03000 | −1.60 | Disulphide-isomerase                          |
| 18  | PXO_RS20265 | −2.92 | Chlamydia polymorphic membrane family protein |
| 19  | PXO_RS07715 | −1.80 | MFS transporter                               |
|     |             |     | **Carbon and protein metabolism**              |
| 20  | PXO_RS22385 | −2.39 | Acetyl-CoA acetyltransferase                  |
| 21  | PXO_RS12655 | −2.11 | Acetyl-CoA acetyltransferase                  |
| 22  | PXO_RS11725 | −2.01 | Acetyl-CoA acetyltransferase                  |
| 23  | PXO_RS22370 | −2.39 | 3-Oxoadipate:succinyl-CoA transferase, partial|
| 24  | PXO_RS01885 | 2.03  | 3-Methylcrotonyl-CoA carboxylase subunit alpha |
| 25  | PXO_RS11720 | −2.07 | 3-Hydroxyacyl-CoA dehydrogenase               |
| 26  | PXO_RS12650 | −2.07 | 3-Hydroxyacyl-CoA dehydrogenase               |
| 27  | PXO_RS20635 | −1.90 | 2-Methylisocitrate lyase                      |
| 28  | PXO_RS20105 | −2.20 | Malate dehydrogenase                          |
| 29  | PXO_RS02070 | −2.19 | NADH-dependent FMN reductase                  |
| 30  | PXO_RS27075 | +1.86 | Fumaroylacetate hydrolase domain protein       |
| 31  | PXO_RS08315 | −2.97 | Bilfunctional aconitate hydratase 2/2-methylisocitrate dehydratase |
| 32  | PXO_RS21380 | −2.23 | Chorismate mutase                             |
| 33  | PXO_RS20590 | −4.82 | Glycerophosphodiester phosphodiesterase       |
| 34  | PXO_RS00340 | −2.69 | Aldolase                                      |
| 35  | PXO_RS00350 | −2.69 | Aldolase                                      |
| 36  | PXO_RS23355 | −1.69 | Celulase                                      |
| 37  | PXO_RS05615 | −3.88 | Xylanase                                      |
| 38  | PXO_RS01665 | −2.32 | Xylose isomerase                              |
| 39  | PXO_RS01605 | −4.72 | Beta-1,4-xylanase                             |
| 40  | PXO_RS19450 | +2.54 | Glycosidases                                  |
| 41  | PXO_RS19890 | −2.48 | Glycosyl transferase                          |
| 42  | PXO_RS21085 | −1.75 | Mannose-1-phosphate guanyltransferase         |
| 43  | PXO_RS23035 | +1.61 | Fucose permease                               |
| 44  | PXO_RS19800 | −2.48 | UDP-2,3-diacylgalactosamine hydrolase         |
| 45  | PXO_RS18550 | −1.50 | Ubiquinol oxidase subunit II                  |
| 46  | PXO_RS22130 | −2.61 | Lipase                                        |
| 47  | PXO_RS15625 | −2.94 | Peptidase S53                                 |
| 48  | PXO_RS06470 | −2.30 | Peptidase C1                                  |
| 49  | MSTRG.1600  | +1.94 | Pentapeptide repeats family protein           |
| 50  | PXO_RS16310 | −3.64 | Oar protein                                   |

(Continued)
### TABLE 1 | Continued

| No. | Gene ID         | FC\(^a\) | Annotation                                            |
|-----|----------------|----------|-------------------------------------------------------|
| 51  | PXO_RS19895     | −2.48    | Phosphoesterase                                       |
| 52  | PXO_RS05010     | −1.93    | Phosphoanhydride phosphohydrolase                     |
| 53  | PXO_RS09785     | −2.66    | Phosphate-binding protein                              |
| 54  | PXO_RS09790     | −2.35    | Phosphate-binding protein                              |
| 55  | PXO_RS09775     | −1.88    | Phosphate transporter permease subunit PtsA           |
| 56  | PXO_RS09780     | −1.88    | Phosphate ABC transporter permease                    |
| 57  | PXO_RS09770     | −2.44    | Phosphate ABC transporter ATP-binding protein         |
| 58  | MSTRG.1547      | −2.42    | Putative ABC transporter phosphate-binding protein    |
| 59  | PXO_RS17230     | −2.63    | Sulfitreductase                                       |
| 60  | PXO_RS17465     | −1.51    | Serine/threonine protein kinase                       |
| 61  | MSTRG.60        | −1.83    | Serine kinase                                         |
| 62  | MSTRG.2902      | −1.51    | Cytochrome D ubiquinol oxidase subunit II, partial    |
| 63  | PXO_RS18555     | −1.50    | Cytochrome bd-type quinol oxidase, subunit 1          |
|     |                 |          |                                                       |
| 64  | PXO_RS13030     | +1.50    | Flagellar biosynthesis                                |
| 65  | PXO_RS12100     | +1.68    | Flagellar biosynthesis                                |
| 66  | PXO_RS00345     | −2.69    | Flagellar biosynthesis protein PiP                    |
| 67  | PXO_RS12815     | +1.63    | Flagellar basal body rod protein FlgB                 |
| 68  | PXO_RS11885     | +1.76    | Flagellar basal body rod protein FlgB                 |
|     |                 |          |                                                       |
| 69  | PXO_RS19875     | −2.08    | Two-component system sensor protein                   |
| 70  | PXO_RS08560     | −2.18    | Type VI secretion protein                             |
| 71  | PXO_RS08540     | −2.18    | Type VI secretion protein                             |
| 72  | PXO_RS00385     | −2.69    | Type III secretion system protein                     |
| 73  | PXO_RS23030     | −2.97    | Type III secretion system effector protein            |
| 74  | PXO_RS02310     | −2.84    | Type III secretion system effector protein            |
| 75  | PXO_RS03830     | −2.24    | Type III secretion system effector protein            |
| 76  | PXO_RS00355     | −2.69    | Type III secretion protein                            |
| 77  | PXO_RS08565     | −2.18    | Tal3b, TAL effector AvrBs3/PthA family                |
| 78  | PXO_RS08545     | −2.12    | Tal3a, TAL effector AvrBs3/PthA family                |
| 79  | PXO_RS00740     | −4.91    | Tat pathway signal protein                            |
| 80  | MSTRG.3198      | −1.97    | TonB-dependent receptor                               |
| 81  | PXO_RS19075     | −4.14    | TonB-dependent receptor                               |
| 82  | PXO_RS20595     | −3.71    | TonB-dependent receptor                               |
| 83  | PXO_RS20360     | −1.91    | TonB-dependent receptor                               |
| 84  | MSTRG.2688      | −2.54    | TonB-dependent receptor, partial                       |
| 85  | PXO_RS00735     | −3.43    | TonB-dependent receptor, partial                       |
| 86  | PXO_RS17235     | −2.63    | TonB-dependent receptor                               |
| 87  | PXO_RS00360     | −2.69    | Hypersensitivity response secretion protein hrcV      |
| 88  | PXO_RS00320     | −3.97    | HrpE                                                  |
| 89  | PXO_RS00370     | −3.09    | HPr kinase                                            |
| 90  | PXO_RS00330     | −2.69    | HPr kinase                                            |
| 91  | PXO_RS00325     | −2.66    | HPr kinase                                            |
| 92  | PXO_RS00375     | −2.63    | HPr kinase                                            |
| 93  | PXO_RS00315     | −3.13    | protein HpaB                                          |
| 94  | PXO_RS00335     | −2.69    | Protein HpaA                                          |
| 95  | PXO_RS06005     | −2.01    | Putative sulfotransferase required for AvrXa21 activity ST (raxST) |
| 96  | PXO_RS25330     | −1.75    | Xanthomonadin biosynthesis protein                    |
| 97  | PXO_RS21615     | −2.04    | Adhesin                                               |
| 98  | PXO_RS22720     | +1.51    | Ankyrin                                               |
| 99  | PXO_RS22730     | +1.58    | Hemolysin D                                           |

(Continued)
TABLE 1 | Continued

| No. | Gene ID       | FC \(^a\) | Annotation                                      |
|-----|---------------|-----------|------------------------------------------------|
| 100 | PXO_RS05270   | −2.49     | Protocatechuate degradation protein             |
| 101 | PXO_RS05265   | −2.24     | Protocatechuate 3,4-dioxygenase subunit beta     |
| 102 | PXO_RS22360   | −1.98     | Protocatechuate 3,4-dioxygenase subunit beta     |
| 103 | PXO_RS22355   | −1.75     | Protocatechuate 3,4-dioxygenase subunit alpha    |
| 104 | PXO_RS13750   | −2.23     | Multidrug transporter                            |
| 105 | PXO_RS08690   | −1.65     | Multidrug transporter                            |
| 106 | PXO_RS13755   | −1.88     | Multidrug RND transporter                        |
| 107 | emrB          | −1.68     | Multidrug resistance protein B                   |
| 108 | PXO_RS27000   | −2.18     | Multidrug resistance efflux pump                |
| 109 | PXO_RS09795   | −2.78     | Porin                                           |
| 110 | PXO_RS25045   | −3.49     | Hypothetical protein                            |
| 111 | PXO_RS24060   | −2.67     | Hypothetical protein                            |
| 112 | PXO_RS26160   | +1.56     | Hypothetical protein                            |
| 113 | PXO_RS25965   | +1.52     | Hypothetical protein                            |
| 114 | PXO_RS00415   | −4.29     | Hypothetical protein                            |
| 115 | PXO_RS19070   | −4.12     | Hypothetical protein                            |
| 116 | PXO_RS00605   | −3.49     | Hypothetical protein                            |
| 117 | PXO_RS00425   | −3.28     | Hypothetical protein                            |
| 118 | PXO_RS00580   | −3.09     | Hypothetical protein                            |
| 119 | PXO_RS22960   | −3.07     | Hypothetical protein                            |
| 120 | PXO_RS20695   | −3.03     | Hypothetical protein                            |
| 121 | PXO_RS01615   | −2.32     | Hypothetical protein                            |
| 122 | PXO_RS20585   | −2.10     | Hypothetical protein                            |
| 123 | PXO_RS00380   | −2.10     | Hypothetical protein                            |
| 124 | PXO_RS01795   | −1.95     | Hypothetical protein                            |
| 125 | PXO_RS03845   | −1.84     | Hypothetical protein                            |
| 126 | PXO_RS09760   | −1.73     | Hypothetical protein                            |
| 127 | PXO_RS03505   | −1.64     | Hypothetical protein                            |
| 128 | PXO_RS20275   | −1.50     | Hypothetical protein                            |
| 129 | PXO_RS22735   | +1.62     | Hypothetical protein                            |
| 130 | PXO_RS17460   | −2.95     | Hypothetical protein                            |
| 131 | PXO_RS17455   | −2.95     | Hypothetical protein                            |
| 132 | PXO_RS06000   | −2.85     | Hypothetical protein                            |
| 133 | PXO_RS21520   | −2.36     | Hypothetical protein                            |
| 134 | PXO_RS17445   | −2.33     | Hypothetical protein                            |
| 135 | PXO_RS08495   | −2.32     | Hypothetical protein                            |
| 136 | PXO_RS20175   | −1.60     | Hypothetical protein                            |
| 137 | PXO_RS25040   | −2.83     | Hypothetical protein                            |
| 138 | PXO_RS25015   | −2.62     | Hypothetical protein                            |

\(^a\) The symbol “+” indicates upregulated genes in Xoo cells treated with melatonin, while the symbol “−” indicates downregulated genes in Xoo cells treated with melatonin.

can assume that Xoo has the ability to synthesize melatonin and may have a biosynthetic pathway that is similar to that present in plants or animals. However, the function of melatonin in Xoo needs further study.

**Melatonin Inhibits Xoo Cell Division**

Bacterial cell division occurs by the formation of a Z-ring at the site of division (Lutkenhaus and Addinall, 1997). The dynamics of the Z-ring are regulated by the cell division-related genes ZapE and FtsZ, and the role of ZapA is to recruit ZapB to the inner face of the Z-ring (Galli and Gerdes, 2010; Marteyn et al., 2014). To investigate whether melatonin inhibits bacterial proliferation by disrupting or inhibiting cell division, the mRNA expression of nine cell division-related genes in PXO99 challenged with melatonin (200 g/mL) was analyzed by qRT-PCR. The mRNA expression of many internal genes has been reported to be affected by melatonin treatment (Sheshadri et al., 2018). In preliminary experiments, we tested the stability of two PXO99 internal candidate reference genes and observed that RecA was the more stable of the two in PXO99 cells treated with melatonin.
As shown in Figure 4, four cell division-related genes (FetQ, ZapE, FetL, and FetE) were upregulated, and five (ZipA, FetB, ZapA, FetD, and FetZ) were downregulated in Xoo cells treated with melatonin compared to the control cells. Our results indicate that the melatonin treatment resulted in a decrease in Xoo cell division. Because bacterial proliferation depends on the ability of cells to divide (Pardee, 1989), the reduction in cell division could result in an inhibition of Xoo growth.

**Melatonin Alters Xoo Morphology**
A previous study showed that *P. infestans* cells treated with melatonin exhibited reduced lipid droplet production and inhibited the proliferation of *P. infestans* (Zhang et al., 2017). In this study, we investigated the effect of melatonin on the cellular morphology of PXO99 by making TEM observations. As shown in Figure 5, both bacterial size and shape were easily distinguished by TEM using a negative...
staining method. The width and length of individual PXO99 cells ranges from 0.6 to 1.0 µm and from 1.0 to 2.7 µm, respectively, and our observations agreed with these specifications (Figure 5A). By contrast, the width of PXO99 cells treated with melatonin was slightly shorter than in the control, and the length of PXO99 treated with 200 µg/mL of melatonin exhibited a significant reduction (20%) compared to the control (Figure 5B). These data indicate that the reduction in the cell length of PXO99 treated with melatonin may result from the inhibition of Xoo proliferation.

RNA-Seq Transcriptome Analysis of Melatonin-Treated Xoo

To further investigate the mechanism of the effects of melatonin on Xoo proliferation, total RNA from PXO99 cells that were treated or untreated with melatonin was collected and analyzed by RNA-Seq. An analysis of the gene expression changes obtained from the RNA-Seq assay showed that 138 genes had alterations in mRNA transcript levels in response to a melatonin challenge at 21 h post-treatment (Table 1), corresponding to 2.73% of the Xoo genome. Fourteen genes were upregulated, and 124 genes were downregulated, and these DEGs were characterized.
FIGURE 8 | Classification of differential genes in the metabolism in PXO99 under melatonin treatment.
both by using the GO database, which provides annotation information regarding cellular components, molecular functions and biological processes, and by using the KEGG database. Of the 14 upregulated genes, four were enriched in flagellar components, four were enriched in transporter activity, and three were involved in metabolic processes. Flagella are used for motility in PXO99. Whether the four flagellar genes regulated by melatonin are involved in swimming motility or biofilm formation requires further study. Transporters are well known to play a crucial role in substantial exchanges between cells and the outside environment, and the upregulated genes involved in transporter activity and metabolic processes may help PXO99 survive. Among the observed downregulated genes, a notable overrepresentation of genes associated with membrane and cellular components was observed in the cellular component category (Figure 6A). Moreover, genes encoding catalytic activity-related proteins were overrepresented in the molecular function category (Figure 6B). Consistently, a notable overrepresentation of genes associated with metabolic processes in the biological processes category was observed (Figure 6C). In the metabolic processes, 41 genes were dominant in the biological processes category (Figure 6C). Consistently, 56 and 27 genes involved in catalytic activity and metal-binding activity, respectively, were dominant in the category of molecular functions (Figure 6B). To verify the reliability of the transcriptomes, 18 randomly selected genes were analyzed using qRT-PCR. The results were consistent with the sequencing data (Figure 7). Genes related to oxidative phosphorylation, citrate cycle, protein secretion, and two component systems were downregulated in PXO99 treated with melatonin.

**Melatonin Regulates Xoo Metabolism**

Metabolism is an important characteristic of bacteria, and melatonin was reported to significantly reduce the expression mRNA of genes associated with metabolism in microbes (Zhang et al., 2017). In this study, we observed that genes involved in carbohydrate and amino acid metabolism were enriched (Figure 8). The best carbon and nitrogen sources for Xoo growth are sucrose and glutamate (Singh, 2016). Interestingly, we observed that many genes involved in sucrose and glutamate metabolism were downregulated in PXO99 when challenged with melatonin.

Bacterial pathogens are known to require iron for replication and infection (Schaible and Kaufmann, 2004; White and Yang, 2009; Skaar, 2010). Xoo requires ferrous sulfate for optimal proliferation and modulates copper redistribution in rice during infection (Yuan et al., 2010). According to the RNA-Seq results, the mRNA expression of the transporter TonB, which is responsible for iron absorption (Yue et al., 2003), was downregulated in PXO99 challenged with melatonin (Table 1). Other DEGs related to metal binding were also downregulated (Figure 6B). The mRNA levels of genes that encode metal-ion binding and cation binding proteins were previously observed to be downregulated in rice leaves treated with melatonin, similar to our results (Liang et al., 2015). The content of endogenous melatonin in melatonin-treated PXO99 was approximately 100 times that of the control group (Figure 3). Interestingly, 18 genes involved in xenobiotic metabolism were downregulated (Figure 8). Melatonin has a strong ability to bind copper and iron (III) (Limson et al., 1998). Thus, we speculate that melatonin can cause a free iron deficiency in bacterial cells and inhibit growth through the metal-binding activity of melatonin or by reducing the concentration and activity of metal-binding enzymes.

Phosphate is most commonly used in energy metabolic processes and serves as a buffering agent in cells (Lardy and Wellman, 1952). In this study, the mRNA expression of the transcription factor PhoU, which function in environmental phosphate (Pi) sensing and transportation (Muda et al., 1992), was reduced in PXO99 cells treated with melatonin (Table 1). In addition, DEGs encoding proteins located on the cell membrane related to phosphate transporter and phosphate binding proteins involved in energy metabolism were both downregulated (Figure 8). In humans, melatonin inhibits cancer cell growth by preventing the cell membrane from assimilating linoleic fatty acid (Blask et al., 1999). The results of this study indicated that the inhibitory mechanism of melatonin on bacterial growth may be related to reducing phosphate levels, although a detailed characterization of these mechanisms will require further investigation.

**CONCLUSION**

In this study, we investigated the potential effects of melatonin on *X. oryzae pv. oryzae*. Our data showed that melatonin can cross the cell wall and become enriched in *Xoo* cells, inhibiting the cell division and proliferation of this bacterium. Importantly, melatonin altered the cell structure and reduced the motility and attachment ability of *Xoo* cells. The results of the transcriptome analysis suggest that the inhibitory effects of melatonin on *Xoo* proliferation may occur through (i) decreasing cell division and (ii) reducing the concentration and activity of enzymes involved in metabolism. This work provides new insights into the inhibitory effect of melatonin on bacterial growth and gene expression.

**AUTHOR CONTRIBUTIONS**

FL, ZF, and XC designed the study. XC and CS performed the experiments. XC, CS, and YZ analyzed the data. XC, CS, and PL drafted the manuscript. FL, IP, JQ, and ZF reviewed and edited the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.02280/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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