Transcriptome analysis of *Xanthomonas oryzae pv. oryzicola* exposed to H$_2$O$_2$ reveals horizontal gene transfer contributes to its oxidative stress response

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Abstract

*Xanthomonas oryzae pv. oryzicola* (*Xoc*), the causal agent of bacterial leaf streak, is one of the most severe seed-borne bacterial diseases of rice. However, the molecular mechanisms underlying *Xoc* in response to oxidative stress are still unknown. In this study, we performed a time-course RNA-seq analysis on the *Xoc* in response to H$_2$O$_2$, aiming to reveal its oxidative response network. Overall, our RNA sequence analysis of *Xoc* revealed a significant global gene expression profile when it was exposed to H$_2$O$_2$. There were 7,177 and 246 genes that were differentially regulated at the early, middle, and late stages after exposure, respectively. Three genes (*xoc_1643*, *xoc_1946*, *xoc_3249*) showing significantly different expression levels had proven relationships with oxidative stress response and pathogenesis. Moreover, a hypothetical protein (*XOC_2868*) showed significantly differential expression, and the *xoc_2868* mutants clearly displayed a greater H$_2$O$_2$ sensitivity and decreased pathogenicity than those of the wild-type. Gene localization and phylogeny analysis strongly suggests that this gene may have been horizontally transferred from a *Burkholderiaceae* ancestor. Our study not only provides a first glance of *Xoc*'s global response against oxidative stress, but also reveals the impact of horizontal gene transfer in the evolutionary history of *Xoc*.

Introduction

*Xanthomonas* consists of a large number of phytopathogenic bacteria that infect more than 400 host species, including a wide variety of economically important plants, such as rice, citrus, banana, cabbage, and bean [1, 2]. One of them, *Xoc*, is the causal agent of bacterial leaf streak (BLS) of rice (*Oryza sativa*), and is considered one of the most destructive seed-borne bacterial diseases of rice in many Asian countries and parts of Africa [3]. The interaction complexity between *Xoc* and rice results from the outcome of a longstanding and ongoing evolutionary battle, in which the bacteria attempts to invade and multiply, while the rice plant’s cells attempt to recognize and defend against this invasion. Of these plant responses, oxidative burst is one of the initial response against pathogens [4].
Oxidative burst is a process in which high concentrations of reactive oxygen species (ROS) are produced at the plasma membrane in the vicinity of a pathogen [4, 5]. This activity in plants may directly kill the pathogen, slow its growth by producing toxins, or even act as a signaling cascade leading to various defenses, including the hypersensitive response (HR) and cell wall modifications [6]. Since ROS activity is a common feature of plant defense systems and the mechanism involved in pathogen cell death, any pathogen that is able to resist this effect is likely privileged. These mechanisms require complex and well-orchestrated reactions involving both radical scavenging and enzymatic repair activities [7]. The cellular response to oxidative stress in the model organism, *Escherichia coli*, has been largely elucidated. When *E. coli* cells are treated with a low dose of H\textsubscript{2}O\textsubscript{2}, growth arrest occurs immediately and the expression of approximately 30 genes is maximally induced within 10–30 minutes of treatment [8]. Among them, the oxidized form of the transcriptional regulator, OxyR, induces many genes such as *katG* (encoding catalase G), *ahpCF* (encoding alkyl hydroperoxide reductase), and *trxC* (encoding reduced thioredoxin) to remove intracellular H\textsubscript{2}O\textsubscript{2}, maintain redox homeostasis and ultimately enable cells to resume growth [9, 10]. These studies demonstrate that the genes involved in oxidative-stress response are vital for bacteria survival. However, there is limited knowledge on oxidative response network of phytopathogens using time-course system analysis although it is important to elucidate the molecular mechanisms that plant pathogens use to resist oxidative stress.

RNA-seq is widely used to investigate differential gene expression in many bacteria [8]. Most of the oxidative stress research using this technology were carried out on human pathogens and environmental bacteria [11, 12]. The application of RNA-seq to *Xoc* on oxidative stress may provide new insights into molecular mechanisms on oxidative response network.

BLS256, the representative genome of *Xoc*, has been whole-genome sequenced [13]. Interestingly, more than 30% of the coding genes in this genome are hypothetical genes.

In this study, we identified differentially expressed genes in the *Xoc* strain BLS256 in response to a time-course H\textsubscript{2}O\textsubscript{2} treatment (early, middle, and late) using the Illumina RNA-Seq platform. Furthermore, we discovered one hypothetical protein, in addition to the genes previously confirmed to be triggered by oxidative stress, which showed high-fold differential expression compared with the control. This finding strongly suggests that this gene could have been horizontally transferred from other microbe organism.

**Materials and methods**

**Strains, plasmids and culture conditions**

The bacterial strains and plasmids used in this study are listed in S1 Table. *Escherichia coli* strains were cultivated at 37 °C in Luria-Bertani (LB) medium or on LB agar plates. Unless otherwise specified, *Xoc* strains were grown at 28 °C in nutrient broth (NB) (0.1% yeast extract, 0.3% beef extract, 0.5% polypeptone, and 1% sucrose), nutrient agar (NA) (NB with 15 g L\textsuperscript{-1} agar), NA without sucrose (NAN), NA with 10% sucrose (NAS), and NB without beef extract and sucrose (NY). In some experiments, strains were grown in MMX minimal medium [0.5% glucose, 0.2% (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}, 0.1% trisodium citrate dihydrate, 0.4% K\textsubscript{2}HPO\textsubscript{4}, 0.6% KH\textsubscript{2}PO\textsubscript{4}, 0.02% MgSO\textsubscript{4} \cdot 7H\textsubscript{2}O]. Kanamycin (Km) and Ampicillin (Amp) were added in concentration of 50μg mL\textsuperscript{-1} and 100μg mL\textsuperscript{-1}, respectively, as required.

**Oxidative stress treatments and total RNA preparation**

H\textsubscript{2}O\textsubscript{2} resistance assays were performed as described previously described [14] with some modifications. *Xoc* strains were cultured to the mid-log phase (OD 600 = 1.0 ~ 1.2) in NB medium. Cultures were treated with 0.1mM H\textsubscript{2}O\textsubscript{2} (Fluka) and constantly stirred at 330 rpm in a 28 °C
shaking incubator. At time of 0, 7, 15, and 45 min, aliquots were withdrawn and pelleted by centrifugation at 4 °C. Cell The cell pellets was were washed two times with cold PBS and the total RNA immediately extracted using the RNeasy Protect Bacteria Mini Kit (QIAGEN) protocol. Two biological replicates were performed in this experiment.

**mRNA purification and cDNA synthesis**

Ten micrograms from each total RNA sample were treated with the MICROBExpress Bacterial mRNA Enrichment kit and RiboMinus™ Transcriptome Isolation Kit (Bacteria) (Invitrogen). Bacterial mRNAs were chemically fragmented to the size range of 200–250 bp using 1 X fragmentation solution for 2.5 min at 94°C. cDNA was generated according to instructions given in the SuperScript Double-Stranded cDNA Synthesis Kit (Invitrogen). Each mRNA sample was mixed with 100 pmol of random hexamers, incubated at 65 °C for 5 min, and chilled on ice for 2 min. 4 μL of First-Strand Reaction Buffer (Invitrogen), 2 μL of 0.1 M DTT, 1 μL of 10 mM RNase-free dNTPmix, 1 μL of SuperScript III reverse transcriptase (Invitrogen) were then added to each sample and incubated at 50 °C for 1 h. To generate the second strand, the following Invitrogen reagents were then added: 51.5 μL of RNase-free water, 20 μL of second-strand reaction buffer, 2.5 μL of 10 mM RNase-free dNTP mix, 50 U *E. coli* DNA Polymerase, and 5 U *E. coli* RNase H, and incubated at 16 °C for 2.5 h.

**Library construction and Illumina sequencing**

The Illumina Paired End Sample Prep kit was used for RNA-Seq library creation according to the manufacturer’s instructions. Fragmented cDNA was end-repaired, ligated to Illumina adaptors, and amplified by 18 cycles of PCR. Paired-end 100-bp reads were generated by high-throughput sequencing with the Illumina Hiseq2000 Genome Analyzer instrument.

**RNA-Seq data analysis**

After removing the low quality reads and adaptors, pair-end reads were mapped to the reference genome BLS256 using Bowtie1.1.1 with default parameters [15]. If reads mapped to more than one location, then only the reading having the highest alignment score was kept. Reads mapping to rRNA and tRNA regions were removed from further analysis. Four time-points samples were prepared in this study: 0 min, 7 min, 15 min and 45 min, defined as WT, early, middle and late stage, respectively. All samples were compared with the 0 min samples to detect the differential expressed genes (DEGs). After obtaining the number of reads for every sample, the edgeR with TMM normalization method [16] was used to determine the DEGs [17]. FDR value < 0.05 was selected as the cutoff for further analysis. Cluster 3.0 and TreeView were used to represent the cluster of DEGs over time-series samples [18].

**Quantitative real-time PCR**

Bacterial total RNA was extracted following RNeasy Protect Bacteria Mini Kit (QIAGEN) instructions and used in generating the first strand complementary DNA (cDNA) per Takara PrimeScript RT reagent Kit with gDNA Eraser’s (Takara) protocol. After detecting RNA quantity and quality by Nanodrop ND1000 spectrophotometer V 3.5.2 (NanoDrop Technologies, Wilmington, DE, USA), 1 μg of the total RNA was incubated at 42 °C for 20 mins to eliminate the gDNA by gDNA Eraser before obtaining cDNA. The reverse transcription reaction was accomplished by incubated at 37 °C for 15 min and then 85 °C for 5 s in the presence of random RT primers. The cDNA was then used directly as the template for qRT-PCR using a SYBR Green master mix (Applied Biosystems) on an ABI Prism 7000 sequence detection.
system (Applied Biosystems). Normalized expression levels of the target gene transcripts were calculated relative to the rRNA using the ΔΔCT method, where CT is the threshold cycle [19]. Three biological replicates were carried out in this experiment.

**Construction of defective deletion mutant and complementation**

To investigate the role of interested genes in Xoc, In-frame deletion mutations were constructed in BLS256 using homologous recombination. Briefly, two fragments flanking the left and right of corresponding genes were amplified from the wide-type genomic DNA with primer pairs listed in S2 Table. The amplified fragments were cloned into pMD18-T vectors (TaKaRa), confirmed by sequence analysis, and then digested and subcloned into vector pKMS1 [20] at BamHI and PstI (or SalI) sites. The resultant recombinant plasmids were introduced into BLS256 by electroporation, and transformants were plated on NAN plates supplemented with kanamycin. Colonies resulting from a single homologous crossover (integration of deletion construct at either the left or right border of target gene) were then transferred to NBN broth, grown for 12 h at 28˚C, and then plated on NAS plates for sucrose-positive deletion mutant selection. Sucrose resistant colonies were visible within 3 to 4 days and then transferred to NA plates and NA plus kanamycin plates. Since kanamycin-sensitive colonies could be mutants containing a second homologous crossover, these were further examined by PCR amplification with the primer pairs.

In order to complement the deletion mutants, the full-length of corresponding genes including promoter regions were amplified using primer pairs listed in S2 Table. The amplified DNA fragments were cloned into pUFR034 vectors [20] at BamHI and PstI (or SalI) sites to create the recombinant plasmids. The recombinant plasmids were transferred into corresponding mutants by electroporation, and transformants were screened on NA plates with kanamycin.

**H₂O₂ resistance assay**

The assay plates were prepared by adding H₂O₂ to the sterilized NB medium in concentrations of 0, 0.1 and 0.25 mM, respectively. Strains were cultured to the mid-log phase (OD 600 = 1.0) in NB medium, and three-fold and nine-fold dilutions were made. 5-μL aliquots of the initial culture and diluted cultures for each strain were spotted onto NB agar plates (in triplicate) and cultured for 36 h at 28 ˚C [21].

**Pathogenicity assays**

Bacteria were prepared based on previous reported method [21]. Briefly, *Xanthomonas* cells were grown in NB broth at 28˚C and 200 rpm for 16 h, when cells approached the exponential phase of growth. Bacterial cells were then harvested by centrifugation, washed twice, and resuspended in sterile water to an optical density at 600 nm (OD600) of 0.3 (approximately 1×10⁸ CFU/mL). Bacteria were inoculated into leaves of adult rice plants (*Oryza sativa* cv. IR24, susceptible to Xoc, 2 months old) using leaf piercing for lesion length measurement for evaluating water-soaked symptoms. All plants were maintained in a greenhouse as described previously [13]. Five leaves were inoculated for each independent experiment, and each treatment was repeated at least three times.

**Phylogenetic analysis**

Interested gene was first compared against non-redundant database for homologs searching with E<0.0001 as cutoff. Next, 40% alignment similarity and 80% coverage parameter was
used to define the real homologue genes. MAFFT was used to generate the multiple sequences alignment [22]. Maximum Likelihood phylogeny was finally evaluated and built by PhyML [23] using a JTT model and a gamma distribution with eight rate categories. We performed 1,000 bootstraps to gain branch support values.

Results

Global overview of the Xoc transcriptome in response to H$_2$O$_2$

In this study, 100-bp paired-end deep sequencing was performed on all the tested samples. In general, more than 40M reads were generated from each single sample. After adaptor removal, quality control, and removal of reads, mapped to ribosomal RNA, 7M to 10M confident reads remained. The sequencing depth in this experiment was more than 150 X, which is sufficient for further statistical analysis. In general, 7, 177, and 246 genes were differentially regulated in the early, middle, and late stages, respectively (Fig 1, S3 Table). The overall number of differentially expressed genes (DEGs) was similar to the number found in recent research related to human pathogens and environmental bacteria [24, 25], with the exception of DEGs in the early state (7 min), which were almost 10 times smaller than in our study. This number, indicates the oxidative response difference that may occur between plant pathogen versus other bacteria.

All of the RNA-seq data has been deposited in BioProject database (https://www.ncbi.nlm.nih.gov/bioproject) and the accession number is PRJNA350867.

General gene expression kinetics among the time-course samples

Previous research indicates that bacteria require dynamic regulatory networks at different time-points when they are exposed to environmental stress [24, 26]. To capture this variable activity, gene expression among the time-course samples was generated and results are shown in Fig 1. In general, clusters 1 and 2 had a decreased induction whereas clusters 4 and 5 had an increased induction through time (Fig 1). By contrast, the clusters 3 and 6 consisted of those genes whose expression peaked at the middle time stage.

Gene Ontolog (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis were carried out based on the STRING V10 database [27], with a $p < 0.01$ after a Bonferroni correction [28] set as the cutoff. The GO enrichment analysis revealed the categories of transport (GO:0006810), cell outer membrane (GO:0009279), and acyl-CoA dehydrogenase activity (GO:0003995) as significant regulation based on this cutoff for clusters 1 and 2 (Table 1). The regulation of cellular process (GO:0050794), chemotaxis (GO:0006935), single organism signaling (GO:0044700), response to external stimulus (GO:0009605), molecular transducer activity (GO:0060089), and signal transducer activity (GO:004871) were significant for clusters 4 and 5 (Table 1). Membrane protein (GO:0016020), outer membrane protein (GO:0019867), and cellular component (GO:0005575) were significant for clusters 3 and 6 (Table 1). The KEGG enrichment results suggested that only valine, leucine, and isoleucine degradation was significant for clusters 1 and 2 (Table 1). Interestingly, we found many TonB-dependent receptors (TBDRs) that were differentially expressed in all the three time stages, indicating their importance in oxidative stress (S3 Table).

Gene clusters involved in the DEGs

Since those genes forming a bacterial gene cluster are always involved in a common pathway or function [29], the DEGs located in the gene clusters we identified may play a fundamental role in oxidative stress. Based on our criterion—that at least three tandem genes must be
differentially expressed to constitute a gene cluster—we identified all the possible clusters and listed them in S4 Table. Not surprisingly, many reported oxidative stress-associated gene clusters were found involved, such as those for the alkyl hydroperoxide reductase, suf operon and pst operon [30, 31]. Furthermore, we also found a large gene cluster encoding ribosomal proteins that were up-regulated in our study (S3 Table). In addition, we found an F1F0 ATPase complex cluster that was up-regulated under oxidative stress.

**Fig 1.** Time-course transcriptome study of Xoc (Xanthomonas oryzae pv. oryzicola) resistance to an oxidative stress response. Hundreds of genes are differentially expressed at different time points (early, middle, and late) during the H2O2 treatment when compared with the 0-min sample. These differentially expressed genes can be classified into different clusters based on their expression patterns. The line from -8.0 to 8.0 represents the Log2 fold change value.

| Functional group            | Clusters | p value*   |
|-----------------------------|----------|------------|
| GO category                 |          | 1 and 2    | 4 and 5 | 3 and 6 |
| Transport (GO:0006810)      | +        | 6.50E-02   |         |         |
| Cell outer membrane (GO:0009279) | +        | 3.13E-02   |         |         |
| Acyl-CoA dehydrogenase activity (GO:0003995) | +        | 1.61E-03   |         |         |
| Regulation of cellular process (GO:0050794) | +        | 1.97E-03   |         |         |
| Chemotaxis (GO:0006935)     | +        | 1.60E-02   |         |         |
| Single organism signaling (GO:0044700) | +        | 5.32E-02   |         |         |
| Response to external stimulus (GO:0009605) | +        | 9.49E-02   |         |         |
| Molecular transducer activity (GO:0060089) | +        | 5.94E-03   |         |         |
| Signal transducer activity (GO:0004871) | +        | 7.08E-02   |         |         |
| Membrane (GO:0016020)       | +        | 1.09E-03   |         |         |
| Outer membrane (GO:0019867) | +        | 5.20E-02   |         |         |
| Cellular component (GO:0005575) | +        | 7.46E-02   |         |         |
| KEGG category               |          |            |         |         |
| Valine, leucine and isoleucine degradation | +        | 4.06E-02   |         |         |

*Bonferroni test

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Quantitative real time PCR experiments confirms the gene expression profiles

The RNA-seq results were validated with a qRT-PCR analysis of four selected genes (xoc_1643, xoc_1946, xoc_2868, and xoc_3249) that showed different levels of expression at 7, 15, and 45 minutes (S3 Table). The mRNA abundance of these transcripts at these three time-points after the H$_2$O$_2$ treatment followed a profile similar to that of the microarray dataset (S3 Table), thus validating the quality of our assay. The Pearson correlation test of the microarray against the qRT-PCR measurements yielded a correlation coefficient of $R^2 = 0.81$ (n = 4), suggesting that RNA-seq dataset correlated positively and tightly with the qRT-PCR quantification (Fig 2).

A hypothetical gene plays some role in the oxidative stress response in Xoc

Sensing, detoxification, and adaptation to oxidative stress play a critical role during successful pathogen infection and pathogenesis by Xanthomonas [30]. The XOC_1643 (outer membrane channel protein), XOC_3249 (membrane protein YnfA) and XOC_2868 (hypothetical protein) and mutants clearly displayed a greater sensitivity to H$_2$O$_2$ than did the BLS256 wild-type and complemented strains (Fig 3). Not surprisingly, these three mutants showed decreased pathogenicity when compared with the wild-type (Fig 4). However, the XOC_1946 (TonB-dependent receptor) mutants showed a greater resistance to H$_2$O$_2$ but a decreased pathogenicity when compared with the wild-type (Fig 4). TonB-dependent receptor was proven to be involved in the transport of plant-derived molecules such as sucrose and maltodextrins in previous studies and yet delayed the disease symptom development in plants to some extent [31, 32]. Our studies show similar results with earlier researches, suggests that XOC_1946 is

Fig 2. Real-time quantitative PCR analysis. Transcript levels of the four candidates under oxidative stress at 7, 15, and 45 min after H$_2$O$_2$ treatment. Values given are the means of five replicate measurements from a representative experiment. The experiment was repeated five times, and similar results were obtained. Columns with the same letters are not significantly different from each other by t-test (i.e., $P \geq 0.05$). Error bars represent the standard deviations.

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Fig 3. Gene mutations changed the resistance to H$_2$O$_2$ in *Xanthomonas oryzae* pv. *oryzicola* (*Xoc*). *Xoc* strains, including the wild-type strain BL5256, the gene deletion mutants Δ1643, Δ1946, Δ2868, and Δ3249, and their complemented strains Δ1643 (1643), Δ1946 (1946), Δ2868 (2868), and Δ3249 (3249), were grown on nutrient broth agar (NA) plates with 0 mM H$_2$O$_2$, 0.1 mM H$_2$O$_2$, or 0.25 mM H$_2$O$_2$. Three replicates for each treatment were used, and the experiment was repeated three times. “1x”: original cultures; “3x”: three-fold dilutions of cultures; “9x”: nine-fold dilutions of cultures.

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involved in the transport of $\text{H}_2\text{O}_2$. As genes \textit{xoc\_1643}, \textit{xoc\_1946} and \textit{xoc\_3249} have proven relationships with an oxidative stress response, this result confirmed the accuracy of our RNA-seq result in this study. Interestingly, \textit{XOC\_2868}, a hypothetical protein, was also involved in the stress response. Sequence analysis revealed that this gene has a MntR domain, thus indicating its potential role in superoxide resistance regulation [33].
xoc_2868 is a horizontally transferred gene

A comprehensive BLAST sequence analysis revealed that this MntR-like gene exists widely in Xoc but not in other Xanthomonas species (Fig 5). Interestingly, homologs of this gene were found to present in many Burkholderiaceae family bacteria, such as Burkholderia, Ralstonia, and Cupriavidus (Fig 5). The phylogenetic analysis further suggested that this gene in Xoc might have originated from a transfer from a Burkholderiaceae ancestor over the course of evolutionary history, an inference with high bootstrapping support (Fig 5). Notably, many genes located adjacent to xoc_2868 are transposase (i.e., from Xoc_2859 to Xoc_2865). Ochman et al. suggested that HGT is mediated by bacteriophage integrases or mobile element transposases, while Keeling et al. suggested that a phylogenetic tree is the gold standard by which to identify HGT [34, 35]. The results presented here strongly suggest that xoc_2868 is a horizontally transferred gene.
Discussion

In this study, we demonstrated, for the first time, that TonB-dependent receptors (TBDRs) is involved in resistance to $H_2O_2$ and virulence in $Xoc$. Indeed, the trigger of these TBDRs is reportedly involved in many different kinds of stress responses such as oxidative stress, iron stress and zinc stress [36]. Researchers also found that TBDRs are required for full virulence of $Xanthomonas campestris$ pv. $campestris$ to Arabidopsis [30]. We may infer that TBDRs may play some role in the oxidative response in $Xoc$. Hence, the function of one TBDRs gene $Xoc_1946$ was confirmed by $H_2O_2$ resistance assays and pathogenicity assays. Our results show that $Xoc_1946$ did play a role in oxidative response and pathogenicity. However, the role of the TBDRs in these bacterial species remains to be investigated.

There are many oxidative-associated gene clusters were found involved in DEGs on our study. Alkyl hydroperoxide reductase have been reported responsible for alkyl peroxide metabolism in $Xanthomonas$. It is the best characterized microbial enzyme involved in organ peroxide metabolism[30]. $suf$ operon, which consists of a list of cysteine desulfurase encoding genes, is involved in the assembly of $[Fe-S]$ clusters under oxidative stress, it is also known as being necessary for virulence of the plant pathogen $Erwinia chrysanthemi$[31]. Another operon $pst$, which has been found differentially expressed in this study, was also been considered to improve heat, oxygen and starvation stress resistance in $Lactococcus lactis$[37]. Our results indicate that the role of these clusters in bacterial resistance to oxidative-stress might not be diverse in different bacterial species.

Prior research has demonstrated that $H_2O_2$ causes a slower rate of ribosomal run-off, while the expression of several ribosomal proteins associated with the translation of the stress response-associated genes was increased [38]. Our findings show that there is a gene cluster encoding ribosomal proteins were up-regulated, this result indicate that this gene cluster contributes to the translation of oxidative stress associated genes in our $Xoc$ strain.

It is well-known that the oxidative stress generated in the plant response to pathogens will decrease the intracellular pH [38]. Although direct evidence is lacking for $Xanthomonas$ species, the mutants of these genes from several other bacteria showed clear growth defects under low pH, thus indicating the $F1F0$ ATPase complex cluster which was found up-regulated in our study is important for maintain the $\Delta pH$ [39].

It is now widely appreciated that a time-course transcriptome analysis can help us to better understand how organisms react to stress conditions over time [40]. Here, we set three time points corresponding to the early, middle, and late response stages. Very few genes were significantly differentially expressed in the early stage (S3 Table). Interestingly, however, we did find that one gene encoding SoxR, a redox-sensitive transcriptional activator, was the highest up-regulated gene in this time stage. In $E. coli$, soxR and soxS were shown to control the superoxide response regulon of $E. coli$ [41]. Since $Xoc$ lacks the homolog of soxS, and soxR is the only transcriptional regulator, we may infer that this gene triggers the $Xoc$ oxidative stress response.

Conclusions

In this research, gene expressions of $Xoc$ strain BLS256 in response to a time-series $H_2O_2$ treatment have been presented by RNA-Seq analysis. In general, 7, 177, and 246 genes were differentially regulated in the early, middle, and late stages, respectively. The soxR gene was highly up-regulated in the early stage, indicating that this gene triggers the $Xoc$ oxidative stress response. In addition, the sensitivity to $H_2O_2$ and pathogenicity of four DEGs’ mutants were investigated, and the results prove strong relationships between these DEGs and oxidative stress response. Interestingly, the results about a hypothetical protein XOC_2868 presented here strongly suggest that it is encoded by a horizontally transferred gene.
Supporting information

S1 Table. Strains and plasmids used in this study.
(DOCX)

S2 Table. Primers used in this study.
(DOCX)

S3 Table. Differential expressed genes in the early, middle and late stage.
(XLSX)

S4 Table. Gene clusters that were differential expressed in the early (red color), middle (yellow color) and late (blue color) stages.
(XLSX)

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References

1. Liang X, Duan Y, Yu X, Wang J, Zhou M. Photochemical degradation of bismuthiazol: structural characterisation of the photoproducts and their inhibitory activities against Xanthomonas oryzae pv. oryzae. Pest Manag Sci. 2016; 72; 997–1003. https://doi.org/10.1002/ps.4080 PMID: 26174501

2. Ryan RP, Vorhölder FJ, Potnis N, Jones JB, Van Sluys MA, Bogdanove AJ, et al. Pathogenomics of Xanthomonas: understanding bacterium-plant interactions. Nat Rev Microbiol. 2011; 9; 344–355. https://doi.org/10.1038/nrmicro2558 PMID: 21478901

3. Niño-Liu DO, Ronald PC, Bogdanove AJ. Xanthomonas oryzae pathovars: model pathogens of a model crop. Mol Plant Pathol. 2006; 7; 303–324. https://doi.org/10.1111/j.1364-3703.2006.00344.x PMID: 20507449

4. Wojtaszek P. Oxidative burst: an early plant response to pathogen infection. Biochem J. 1997; 322; 681–692. https://doi.org/10.1042/bj3220681 PMID: 9148737

5. Doke N, Miura Y, Sanchez LM, Park HJ, Noritake T, Yoshioka H, et al. The oxidative burst protects plants against pathogen attack: mechanism and role as an emergency signal for plant bio-defence—a review. Gene 1996; 179; 45–51. https://doi.org/10.1016/s0378-1119(96)00423-4 PMID: 8955628

6. Torres MA, Jones JDG, Dangl JL. Pathogen-induced, NADPH oxidase–derived reactive oxygen intermediates suppress spread of cell death in Arabidopsis thaliana. Nat Genet. 2005; 37; 1130–1134. https://doi.org/10.1038/ng1639 PMID: 16170317

7. Liochev SI. Reactive oxygen species and the free radical theory of aging. Free Radic Biol Med. 2013; 60; 1–4. https://doi.org/10.1016/j.freeradbiomed.2013.02.011 PMID: 23943764

8. Manlu Z, Xiongfeng D. Maintenance of translational elongation rate underlies the survival of Escherichia coli during oxidative stress. Nucleic Acids Res. 2019; https://doi.org/10.1093/nar/gkz467 PMID: 31131413

9. Imlay JA. The molecular mechanisms and physiological consequences of oxidative stress: lessons from a model bacterium. Nat Rev Microbiol. 2013; 11;443–454. https://doi.org/10.1038/nrmicro3032 PMID: 23712352
10. Imlay JA. Transcription factors that defend bacteria against reactive oxygen species. Annu Rev Microbiol. 2015; 69:93–108. https://doi.org/10.1146/annurev-micro-091014-094322 PMID: 26070785

11. Perkins TT, Kingsley RA, Fookes MC, Gardner PP, James KD, Yu L, et al. A strand-specific RNA-Seq analysis of the transcriptome of the typhoid Bacillus salmonellae typhi. PLoS Genet. 2009; 5; e1000569. https://doi.org/10.1371/journal.pgen.1000569 PMID: 19609351

12. Shimizu R, Chou K, Orita I, Suzuki Y, Nakamura S, Fukui T. Detection of phase-dependent transcriptomic changes and Rubisco-mediated CO2 fixation into poly (3-hydroxybutyrate) under heterotrophic condition in Raistonia eutropha H16 based on RNA-seq and gene deletion analyses. BMC Microbiol. 2013; 13; 169. https://doi.org/10.1186/1471-2180-13-169 PMID: 23879744

13. Bogdanove AJ, Koebnik R, Lu H, Furutani A, Angiuoli SV, Patil PB et al. Two new complete genome sequences offer insight into host and tissue specificity of plant pathogenic Xanthomonas spp. J Bacteriol. 2011; 193; 5450–5464. https://doi.org/10.1128/JB.05262-11 PMID: 21784931

14. Upadhyra R, Campbell LT, Donlin MJ, Aurora R, Lodge JK. Global transcriptome profile of Cryptococcus neoformans during exposure to hydrogen peroxide induced oxidative stress. PLoS ONE 2013; 8; e55110. https://doi.org/10.1371/journal.pone.0055110 PMID: 23383070

15. Langmead B, Trapnell C, Pop M, Salzberg SL. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome Biol. 2009; 10; R25. https://doi.org/10.1186/gb-2009-10-3-r25 PMID: 19261174

16. Maza E. In papyro comparision of TMM (edgeR), RLE (DESeq2), and MRN normalizaion methods for a simple Two-Conditions-Without-Replicates RNA-Seq experimental design. Front Genet. 2016; 16; 164.

17. Robinson MD, McCarthy DJ, Smyth GK. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics 2010; 26; 139–140. https://doi.org/10.1093/bioinformatics/btp161 PMID: 19910368

18. de Hoon MJL, Imoto S, Nolan J, Miyano S. Open source clustering software. Bioinformatics 2004; 20; 1453–1454. https://doi.org/10.1093/bioinformatics/bth078 PMID: 14871861

19. Jozefczuk J, James A. Chapter Six—Quantitative real-time PCR-based analysis of gene expression. Methods Enzymol. 2011; 500;99–109. https://doi.org/10.1016/B978-0-12-385118-5.00006-2

20. Guo W, Cai LL, Zou H-S, Ma WX, Liu XL, Zou LF, et al. Ketoglutara te transport protein KgtP is secreted through the type III secretion system and contributes to virulence in Xanthomonas oryzae pv. oryzae. Appl Environ Microbiol. 2012; 78; 5672–5681. https://doi.org/10.1128/AEM.07997-11 PMID: 22685129

21. Qian G, Liu C, Wu G, Yin F, Zhao Y, Zhou Y, et al. AsnB, regulated by diffusible signal factor and global regulator Clp, is involved in ASPartate metabolism, resistance to oxidative stress and virulence in Xanthomonas oryzae pv. oryzae. Mol Plant Pathol. 2013; 14; 145–157. https://doi.org/10.1011/j.1364-3703.2012.00838.x PMID: 23157387

22. Katoh K, Standley DM. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Mol Biol Evol. 2013; 30; 772–780. https://doi.org/10.1093/molbev/mst101 PMID: 23326960

23. Guindon S, Dufayard J-F, Lefort V, Anisimova M, Hordijk W, Gascuel O. New algorithm s and methods for estimating maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. Syst Biol. 2010; 59; 307–321. https://doi.org/10.1093/sysbio/sys010 PMID: 20526388

24. Berghoff BA, Konzer A, Mank NN, Looso M, Rischo T, Forster Nu, et al. Integrative "omics"-approach discovers dynamic and regulatory features of bacterial stress responses. PLoS Genet. 2013; 9; e1003576. https://doi.org/10.1371/journal.pgen.1003576 PMID: 23818867

25. Nobre LS, Saraiva LM. Effect of combined oxidative and nitrosative stresses on Staphylococcus aureus transcriptome. Appl Microbiol Biotechnol. 2013; 97; 2563–2573. https://doi.org/10.1007/s00253-013-4730-3 PMID: 23389340

26. Buescher JM, Liebermeister W, Jules M, Uhr M, Muntel J, Botella E, et al. Global network reorganization during dynamic adaptations of Bacillus subtilis metabolism. Science 2012; 335; 1099–1103. https://doi.org/10.1126/science.1206871 PMID: 22383848

27. Szklarczyk D, Franceschini A, Wyder S, Forslund K, Heller D, Huerta-Cepas J, et al. STRING v10: protein–protein interaction networks, integrated over the tree of life. Nucleic Acids Res 2014; 43; 447–452.

28. Rice WR. Analyzing tables of statistical tests. Evolution 1989; 43; 223–225. https://doi.org/10.1111/j.1558-5646.1989.tb04220.x PMID: 28568501

29. Ballouz S, Francis AR, Lan R, Tanaka MM. Conditions for the evolution of gene clusters in bacterial genomes. PLoS Comput Biol. 2010; 6; e1000672. https://doi.org/10.1371/journal.pcbi.1000672 PMID: 20168992

30. Mongkolus S, Whangsu K, Vattanaviboon P, Loprasert S, Fuangthong M. A Xanthomonas alkyl hydroperoxide reductase subunit C (ahpC) mutant showed an altered peroxide stress response and
complex regulation of the compensatory response of peroxide detoxification enzymes. J Bacteriol. 2000; 182; 6845–6849. https://doi.org/10.1128/jb.182.23.6845-6849.2000 PMID: 11073935
31. Nachin L, Loiseau L, Expert D, Barras F. SuF: an unorthodox cytoplasmic ABC/ATPase required for [Fe—S] biogenesis under oxidative stress. The EMBO J. 2003; 22; 427–437. https://doi.org/10.1093/emboj/cdg061 PMID: 12554644
32. Bianvillain S, Meyer D, Boulanger A, Lautier M, Guynet C, Denancé N, et al. Plant carbohydrate scavenging through TonB-dependent receptors: a feature shared by phytopathogenic and aquatic bacteria. PLoS ONE 2007; 2; e224. https://doi.org/10.1371/journal.pone.0000224 PMID: 17311090
33. Horsburgh MJ, Wharton SJ, Cox AG, Ingham E, Peacock S, Foster SJ. MntR modulates expression of the PerR regulon and superoxide resistance in Staphylococcus aureus through control of manganese uptake. Mol Microbiol. 2002; 44; 1269–1286. https://doi.org/10.1046/j.1365-2958.2002.02944.x PMID: 12028379
34. Ochman H, Lawrence JG, Groisman EA. Lateral gene transfer and the nature of bacterial innovation. Nature 2000; 405; 299–304. https://doi.org/10.1038/35012500 PMID: 10830951
35. Keeling PJ, Palmer JD. Horizontal gene transfer in eukaryotic evolution. Nat Rev Genet. 2008; 9; 605–618. https://doi.org/10.1038/nrg2386 PMID: 18591983
36. Kim HS, Park HJ, Heu S, Jung J. Molecular and functional characterization of a unique sucrose hydrolyase from Xanthomonas axonopodis pv. glycines. J Bacteriol. 2004; 186; 411–418. https://doi.org/10.1128/JB.186.2.411-418.2004 PMID: 14702310
37. Duwat P, Ehrlich SD, Gruss A. Effects of metabolic flux on stress response pathways in Lactococcus lactis. Mol Microbiol. 1999; 31; 845–858. https://doi.org/10.1046/j.1365-2958.1999.01222.x PMID: 10048028
38. Hu P, Brodie EL, Suzuki Y, McAdams HH, Andersen GL. Whole-genome transcriptional analysis of heavy metal stresses in Caulobacter crescentus. J Bacteriol. 2005; 187; 8437–8449. https://doi.org/10.1128/JB.187.24.8437-8449.2005 PMID: 16321948
39. van de Guchte M, Seror P, Cherval C, Smokvina T, Ehrlich SD, Maguin E. Stress responses in lactic acid bacteria. Antonie Van Leeuwenhoek 2002; 82; 187–216. PMID: 12369188
40. Jo K, Kwon H-B, Kim S. Time-series RNA-seq analysis package (TRAP) and its application to the analysis of rice, Oryza sativa L. ssp. Japonica, upon drought stress. Methods 2014; 67; 364–372. https://doi.org/10.1016/j.ymeth.2014.02.001 PMID: 24518221
41. Wu J, Weiss B. Two divergently transcribed genes, soxR and soxS, control a superoxide response regulon of Escherichia coli. J Bacteriol. 1991; 173; 2864–2871. https://doi.org/10.1128/jb.173.9.2864-2871.1991 PMID: 1708380