INTRODUCTION

Leaf blight caused by the vascular wilt pathogen Xanthomonas oryzae pv. oryzae (Xoo) is considered to be one of the most serious bacterial diseases in rice, leading to yield losses varying from 20% to 30% in moderately affected, and up to 50% in severely affected paddy fields (Wang et al., 2013; Lu et al., 2014). Generally, the disease can be managed by chemical control or adoption of resistant cultivars. However, their effectiveness is often strain-dependent due to the overreliance on chemical control methods, thus leading to the shift in virulence patterns that determine the pathogen's ability to infect the plant host (Sahu et al., 2018). In addition, the evolutionary adaptations of Xoo to colonize and survive in the low-nutrient xylem vessels further complicate its elimination from infected plants (Yadeta...
and Thomma, 2013). Therefore, it is necessary to develop effective strategies for addressing the challenges posed by interstrain variations and to target the pathogen’s essential cellular processes at a global level.

In the modern post-genomic era, advances in high-throughput experimental techniques have enabled us to understand the pathogenicity of Xoo using various omics data. For example, the comparative genomic study on three different Xoo strains revealed the presence of several gene clusters representing various functions such as avirulence (avr), hypersensitive reaction and pathogenicity (hrp), regulation of pathogenicity factor (rpf), exopolysaccharide production (gum) (Zhu et al., 2000; Wu et al., 2007; Wang et al., 2008; Xu et al., 2015), and extracellular plant cell wall degradation (Aparna et al., 2009; Tayi et al., 2016), which are likely to be associated with pathogenesis. Moreover, transcriptome profiling of wild-type and mutant Xoo strains growing in cell culture or infected rice plants has further unravelled the regulatory functions of some major pathogenicity-specific genes such as the rpfF (Xu et al., 2015) and RaxR (Noh et al., 2016). Similarly, metabolomics analysis of Xoo also identified key metabolites closely related to virulence (Sana et al., 2010). Collectively, these omics studies have stressed the role of different individual components in the complex Xoo virulence network. However, a global understanding of how these individual components interact and respond to the environmental stimuli remains unclear. In this regard, systems biology approaches where such omics data sets can be interrogated in combination with “in silico metabolic modelling” are indispensable, as successfully applied to delineate host–pathogen interactions in plants and humans (Zimmermann et al., 2017; Botero et al., 2018).

The availability of abundant genomic data and efficient bioinformatics tools has made the reconstruction of genome-scale metabolic models (GEMs) of various organisms for analysing their cellular behaviour easier in the recent past (Lakshmanan et al., 2012; 2018; Monk et al., 2014). Because these GEMs capture the genotype–phenotype relationships via a structured collection of genome and biochemical information in the form of metabolic reactions and their associations with genes and proteins in a systematic manner, we can elucidate the physiological behaviour and metabolic states of an organism on various environmental/genetic perturbations (Bordbar et al., 2014). Currently, the complete genome sequences of at least 94 different Xoo strains are available in the GenBank database (Sayers et al., 2019). The genomic and biochemical information that can be obtained from several publicly available data repositories such as KEGG (Kanehisa et al., 2008), MetaCyc (Caspi et al., 2014), and UniProt (Apweiler et al., 2004) has further paved the way to several studies characterizing its virulence and pathogenesis in rice (He et al., 2010; Tripplett et al., 2011; Monk et al., 2014). Moreover, the availability of conveniently accessible software platforms and algorithms for implementing systems biology and omics integration techniques has allowed for the systematic study of plant pathogens and their interactions with hosts (Lakshmanan et al., 2012; Peyraud et al., 2017). Of Xoo strains, the first strain to have its genome completely sequenced was KACC10331 (Lee et al., 2005), which is highly pathogenic and different from other strains in terms of phylogenics, genetic architecture, and degree of virulence. However, Xoo strains mainly share global virulence mechanisms, for example, diffusible signal factor (DSF), xanthan biosynthetic “gum” genes, and xrvA (Feng et al., 2009; He et al., 2010; Noh et al., 2016; Wang et al., 2016). Thus, here we reconstructed a comprehensive GEM of Xoo and validated its growth predictions using cell culture experiments of KACC10331. The model was then used to provide valuable insights on Xoo virulence and its relationship to the global metabolism.

2 | RESULTS AND DISCUSSION

2.1 | Reconstruction of genome-scale metabolic network of Xoo

Xoo strain KACC10331, hereafter referred to as Xoo, has a genome consisting of a circular chromosome of size 4.94 Mb, high GC content (63.7%), and 4,637 open reading frames (ORFs) (Lee et al., 2005). This is one of the highly pathogenic model strains best characterized for its virulence and genetic basis (He et al., 2010; Tripplett et al., 2011; Noh et al., 2016). Hence, we initially reconstructed a draft of the metabolic network of Xoo based on the genome annotations available in biochemical databases, followed by its curation through gap filling, rectification of elemental imbalances, and evaluation of inconsistencies in reaction directionality (see Experimental Procedures). During the gap filling, several enzymatic and transport reactions that were not annotated in KEGG/MetaCyc were identified and newly included according to the BLASTp homology and/or experimental evidence. For example, an acyl-CoA thioester hydrolase (EC 3.1.2.19) required for DSF (and BDSF) synthesis was added based on the report that these quorum-sensing molecules were produced by Xoo (He et al., 2010). Similarly, 2-galactono-1,4-lactone dehydratase (EC 3.1.1.25), -glucosidase (EC 3.2.1.20), and sucrose-phosphate phosphatase (EC 3.1.3.24) reactions were included because uptake of galactose, maltose, and sucrose was observed (Tsuie et al., 2001).

The incorporation of the unique Xoo pathways into the model allowed us to further enhance the model quality. Exopolysaccharide (EPS) production in the form of xanthan is known to be a characteristic of all xanthomonads. Because the Xoo-specific genomic databases have limited coverage on the xanthan biosynthetic pathway, we compiled relevant information from MetaCyc (Caspi et al., 2014) and previous studies on the industrially important xanthan producer Xanthomonas campestris. In addition, xanthomonads exhibit a distinct virulence mechanism involving a quorum-sensing signalling molecule called DSF, which was first reported and characterized in X. campestris pv. campestris (Barber et al., 1997). DSF, chemically cis-11-methyl-2-dodecanoic acid (Wang et al., 2004), is also known to be involved in several regulatory functions in xanthomonads critical for their pathogenicity, including biofilm formation, production of virulence factors, and motility. There are other structural analogues of DSF, designated BDSF (originally identified from Burkholderia cenocepacia) and CDSF.
(obtained from HPLC fraction C), that are known to have similar functional roles to DSF in *X. oryzae* (He et al., 2010). However, to the best of our knowledge, a biosynthesis scheme has been reported for none of the DSFs to date. Therefore, we propose a putative BDSF biosynthetic pathway based on the biochemical and genetic evidence available from the literature, as well as an overall reaction for DSF biosynthesis derived from the best-known stoichiometry for methylated fatty acid biosynthesis, used in model simulations. The resultant Xoo GEM (iXOO673) contains 673 genes, 570 proteins, 839 reactions, and 808 unique metabolites (for more details, refer to Tables S1 and S2). The model is also available as Systems Biology Markup Language (SBML) file (level 3, version 1, http://sbml.org/) (Data S1) and can be downloaded from BioModels database (Glont et al., 2018) with identifier MODEL1912100001.

For the model validation, the steady-state in silico growth rate of iXOO673 was predicted by maximizing the biomass objective function using constraint-based flux analysis (see Experimental Procedures). The model can successfully simulate the cell growth from several carbon sources, including glucose, sucrose, fructose, cellobiose, xylose, mannose, lactose, starch, succinate, and pyruvate (Watanabe, 1963; Tsuge et al., 2001). Moreover, the specific uptake rates of glucose and other complex medium components were calculated along with the specific xanthan production rate (Figure 1a–d) and used to simulate the in silico growth of Xoo during the exponential phase (time intervals 12–18 hr and 18–30 hr). It should be highlighted that the predicted values for the growth rates were highly consistent with the experimental measurements (Figure 1e), thus confirming acceptable model quality.

### 2.2 Metabolic comparison of Xoo with other plant pathogens, *Ralstonia solanacearum* and *Pseudomonas aeruginosa*

In order to gain some insights into the metabolism and virulence functions of Xoo, we compared the central metabolic pathways of the Xoo GEM with those of two other gram-negative pathogens: *Ralstonia solanacearum* GMI1000 and *Pseudomonas aeruginosa* PAO1. While *R. solanacearum* is known to exclusively infect plants, *P. aeruginosa* is an opportunistic human pathogen that is also known to infect plants (Walker et al., 2004). Xoo has a comparatively smaller

![Figure 1](image-url)
genome size (4.94 Mb) (Lee et al., 2005) than those of P. aeruginosa (6.3 Mb) (Stover et al., 2000) and R. solanacearum (5.8 Mb), and fewer genes in its central and whole metabolism. This feature might reflect the limitation of Xoo to thrive only in specific ecological niches, which include mostly rice xylem vessels and dead plants in soil (Raskin et al., 2006; Ryan et al., 2011), compared to R. solanacearum (Denny, 2006) and P. aeruginosa. We subsequently compared the unique EC numbers and genes in the central metabolism of these pathogens. Interestingly, the Entner–Doudoroff (ED) pathway of carbon catabolism is conserved in all three pathogens. Taking cues from this, we surveyed nine genera of plant pathogens consisting of >50 strains known to infect plants for the presence of the two key enzymes of the ED pathway: 6-phosphogluconate dehydratase and 2-keto-3-deoxyphosphogluconate aldolase. We found that all genera have strains encoding the ED pathway (Tables S3 and S4). It is also important to note that this pathway has been reported to have close association with the virulence of several mammalian bacterial pathogens (Patra et al., 2012; Diacovich et al., 2017), suggesting that the ED pathway might be a more common feature of gram-negative bacterial pathogens infecting animal and plant hosts than previously thought. Another important observation is that although EC numbers are fairly similar among the three pathogens, Xoo has significantly fewer genes in oxidative phosphorylation and pyruvate metabolisms, possibly indicating the relatively lower importance of these pathways for its survival in the rice xylem niche compared to the other two. In addition, secretion of endo-1,4-β-glucanase/cellulase is an important characteristic for virulence and survival in Xoo (Figure 2) as it has been observed as a common feature in all plant pathogens (Sun et al., 2005; Rajeshwari et al., 2005). Substantiating this, a recent study characterizing the rice leaf microbiome indicated a major role of cellulose in facilitating plant–microbiome interactions at the leaf interface (Roman-reyna et al., 2019).

**FIGURE 2** Metabolic pathways and virulence factor systems associated with Xanthomonas oryzae pv. oryzae (Xoo) pathogenesis. Unique pathways in Xoo related to pathogenic survival and virulence.
The model comparisons also provided cues regarding the nature of pathogenesis exhibited by Xoo. Although three pathogens have different host specificities, the crucial metabolic components contributing to pathogenesis fall under the domain of similar quorum-sensing mechanisms. Each of them produces a distinct group of virulence factors that are part of quorum signalling with several functional roles in their pathogenic lifestyles, including the regulation of biofilm formation, motility, and virulence. For example, members of the Xanthomonas genus mainly produce DSFs, which are fatty acid-like molecules, as their quorum-sensing mediators (He et al., 2010) (Figure 2). P. aeruginosa uses acetylated homoserine lactones (AHLs), whereas R. solanacearum adopts both mechanisms independently to achieve quorum signalling and interspecies communication (Kai et al., 2015; Kumar et al., 2016). Xoo also has a unique signalling pathway mediated by diffusible factor (DF) (Zhou, Huang, et al., 2013), which is produced by a bifunctional enzyme, chorismate lyase (XanB2, EC 4.1.3.40, and EC 4.1.3.45), encoded by rpfK. Note that XanB2 catalyses the hydrolysis of chorismate to 3-hydroxybenzoic acid (3-HBA, DF) or 4-hydroxybenzoic acid (4-HBA). While 3-HBA is involved in the biosynthesis of yellow pigments called xanthomonadins (Figure 2), production of 4-HBA forms the committed step in the biosynthesis of coenzyme Q (CoQ) 8 (Zhou, Wang, et al., 2013). Interestingly, both xanthomonadins and CoQ are associated with epiphytic survival, protection against oxidative damage, and virulence in Xoo (Goel et al., 2001; Park et al., 2009; Zhou, Huang, et al., 2013).

2.3 | Gluconeogenesis, glycogen synthesis, and degradation may contribute to Xoo virulence and plant disease severity during exposure to nitrogen fertilizers

It is well known that the use of nitrogenous fertilizers exacerbates leaf blight in rice (Mew, 1987). However, reducing their usage may affect the productivity of rice, therefore it is important to decipher the mechanisms underlying the action of nitrogen fertilizers on Xoo virulence. To address this, we simulated growth and virulence using the objective functions described earlier under two distinct conditions: nitrogen-sufficient (NS) and time interval nitrogen-limited (NL). The NS condition uses an unconstrained supply of nitrogen in the form of ammonia, mimicking the use of nitrogen fertilizers, while the NL condition accepts half of the ammonia that is required in the form of ammonia, mimicking the use of nitrogen fertilizers. The NS condition uses an unconstrained supply of nitrogen in the form of ammonia, mimicking the use of nitrogen fertilizers, while the NL condition accepts half of the ammonia that is required in the form of ammonia, mimicking the use of nitrogen fertilizers. The results suggest relatively lower fluxes within the carbon catabolic pathways and higher fluxes in gluconeogenic reactions in the NS condition (Figure 3). Another minor difference between the two conditions is mostly related to the cofactor (NAD/NADP) regeneration routes, depending on the extent of activity of catabolic and anabolic pathways. These results indicate that on exposure to nitrogen fertilizers, Xoo might preferentially divert resources towards the biosynthesis of storage carbon such as glycogen and subsequently degrade them during nutrient starvation. This is especially important for vascular wilt pathogens such as Xoo that are known to mostly colonize xylem vessels, which are poor in nutrients, especially energy-yielding sugars (Yadeta and Thomma, 2013). Moreover, glycogen storage and degradation processes are known to be tightly linked with the colonization and virulence of several bacterial pathogens (Bonafonte et al., 2000; Jones et al., 2008; Bourassa and Camilli, 2009). It should be highlighted that our prediction of the up-regulated gluconeogenic reactions, especially the phosphoenolpyruvate synthase (pps) reaction, is in good agreement with experiments for X. oryzae pv. oryzae (Wang et al., 2007) and X. campestris (Tang et al., 2005). Overall, we show the relationship between the usage of nitrogen fertilizers and gluconeogenesis, glycogen biosynthesis and degradation, thereby hypothesizing that disruption of such pathways in Xoo could be important in reducing the severity of blight during the application of nitrogen fertilizers.

2.4 | DSF regulates several metabolic pathways important for Xoo virulence and survival

GEMs can be used as tools to analyse transcriptomic data and facilitate the identification of key pathways and metabolites around which there is significant differential gene expression. These metabolites, similar to the reporter metabolites (Patil and Nielsen, 2005), can provide cues regarding the functional roles of the transcriptional responses. Here, we used the published microarray data of Xoo wild-type strain and its rpfF mutant (Rai et al., 2012), incapable of the synthesis of DSF, to identify the metabolites with possible roles as mediators of virulence and survival in Xoo. First, we mapped the gene expression data into the model reactions through their gene–protein reaction (GPR) associations, showing differentially expressed reactions distributed across the metabolic pathways (p ≤ 0.05, fold change ≥ 1.3). As expected, the highest fold change (13 times) was found in the reaction catalysed by DSF synthase, encoded by the rpfF gene. Most significantly, up- and down-regulated pathways were identified using the ratio of the difference between numbers of up- and down-regulated genes to the total number of differentially expressed genes. Only pathways with at least two differentially expressed genes are shown as up- or down-regulated in Figure 4. Consistent with literature evidence, xanthan biosynthesis was significantly down-regulated in the rpfF mutant, asserting the regulatory role of DSF, a product of the rpfF gene, on xanthan gum genes. The mapping results also show that several other pathways are significantly differentially expressed in the rpfF mutant, and hence are putatively under the control of DSF regulon. These include the pathways of starch and sucrose metabolism, cell envelope biosynthesis, oxidative phosphorylation, and biotin metabolism, which are down-regulated, and porphyrin and chlorophyll metabolism, and the citrate cycle, which are up-regulated. However, the exact mechanisms
involved in the differential expression of these pathways and their influence on the pathogenesis of Xoo in rice remain to be elucidated. Interestingly, several differentially expressed reactions that do not fall under any known pathways are linked to the survival and virulence of bacterial pathogens. The complete list of such metabolites along with their putative functional roles important for Xoo virulence and survival are provided in Table 1. Furthermore, we observed an up-regulation of XanB2, which is involved in the synthesis of DF (3-hydroxybenzoic acid) in the rpfF mutant, suggesting a possible inverse relationship between DSF and DF signalling. This could

**FIGURE 3** Effect of nitrogen fertilizers on *Xanthomonas oryzae* pv. *oryzae* metabolism. Pathways and reactions with differential flux in nitrogen-sufficient (NS) versus nitrogen-limited (NL) conditions. NS and NL conditions were simulated by unconstraining ammonia uptake and constraining ammonia uptake to 50% of that of the unconstrained value, respectively. Parsimonious flux balance analysis was used to estimate the fluxes in each condition. Fluxes of each reaction are normalized to the respective biomass objective value.
be a strategy employed by Xoo to conserve metabolic resources due to the overlapping roles of DSF and DF signalling, which has been reported earlier in *Xanthomonas* sp. (Poplawsky *et al.*, 1998) and *Lysobacter enzymogenes* (Qian *et al.*, 2013), thus indicating the degree of complexity of regulatory processes in Xoo pathogenesis.

2.5 Metabolic gene essentiality systematically identifies antivirulence targets in Xoo

Gene essentiality in Xoo for its growth in vitro and its pathogenic lifestyle was assessed by constraint-based simulations (see Experimental Procedures). The analysis for growth in minimal medium revealed 24% (161 genes) of the total genes (673 genes) accounted for in the model to be essential. However, the pathogenicity of Xoo is highly dependent on the regulation and expression of several virulence factors that contribute to essential roles for cell growth and its host interactions, such as adhesion to host surfaces, motility, colonization, biofilm formation, and invasion (Wang *et al.*, 2004; Wu *et al.*, 2008). Some of the major players in Xoo metabolism involved in invasion and virulence include EPS, xanthan, DSF, and DF, as discussed before. A multitude of reports have indicated the deletion of genes involved in the formation of these metabolites giving rise to significantly reduced or no virulence in the mutant strains (Dharmapuri and Sonti, 1999; Chatterjee and Sonti, 2002; Zhou, Huang, *et al.*, 2013). Hence, we considered these factors to be essential for a pathogenic lifestyle in Xoo and employed them in combination with the biomass equation as a lumped cellular objective in order to identify crucial genes for invasiveness (ability to colonize and invade the host) and virulence. Figure 5 depicts the distribution of predicted essential genes among various metabolic subsystems in Xoo for invasiveness and virulence, and growth objectives. Of the assigned subsystems, amino acid, cell membrane, and virulence factor biosynthetic pathways harbour the major proportion of the essential genes. Although these genes are considered crucial for the pathogen, targeting them using antimicrobial strategies requires caution as these antimicrobials may also act against the homologous plant genes, detrimentally affecting plant growth and productivity. Therefore, in the first stage of our analysis we performed a BLASTp search with the essential gene list for growth or invasiveness objective against the proteome of *Oryza sativa* (japonica cultivar group) (taxid: 39,947) in which highly stringent cut-off scores for the E value (>0.05) and percentage identity (<20%) were used to filter the BLASTp output to yield target genes highly nonhomologous to rice genes. Among the 53 resultant targets, seven genes were reported to be essential and two to be nonessential in the gene deletion studies performed on *Xanthomonas* sp. (Yan and Wang, 2011; Keshri *et al.*, 2013) (Table S5). In the second stage, we could identify the genes that are vital for virulence factor production (see Experimental Procedures) and subject them to BLASTp homology search to retrieve nonhomologous genes to rice. It should be noted...
TABLE 1  
Key metabolites and their corresponding reactions affected by DSF regulation with possible roles in Xanthomonas oryzae pv. oryzae (Xoo) survival and virulence

| Reaction(s) | Enzyme/transporter protein(s) | Key metabolite(s) | Putative functional role in Xoo survival and virulence | References |
|-------------|--------------------------------|------------------|------------------------------------------------------|------------|
| CLPNS       | Cardiolipin synthase          | Cardiolipin      | Tolerance against acidic and osmotic stress in bacteria| Ohniwa et al. (2013); Romantsov et al. (2009) |
| FCLT        | Ferrochelatase                | Iron, protoheme  | DSF mutant of Xoo is shown to exhibit reduced virulence in iron-deficient conditions; a mutant of Xanthomonas citri subsp. citri defective in protoheme biosynthesis did not cause citrus canker| Chatterjee and Sonti (2002); Laia et al. (2009) |
| TRE6PS, TRE6PP | Trehalose 6-phosphate synthase, trehalose-phosphatase | Trehalose 6-phosphate, trehalose | Osmoprotectant, interference with plant carbon metabolism | Stryvold and Strom (1991); Müller et al. (1998) |
| PMANM       | Phosphomannomutase            | Mannose-1-phosphate | Precursor for lipopolysaccharide and xanthan biosynthesis | Koplin et al. (1992) |
| PTRCabc     | Putrescine transport via ABC system | Putrescine | Required for virulence in several bacteria | Wortham et al. (2010); Wu et al. (2012); Jelsbak et al. (2012) |
| 4AHD2       | 4a-hydroxytetrahydrobiopterin dehydratase | Tetrahydrobiopterin, 6,7-dihydrobiopterin | Tetrahydrobiopterin is a cofactor for nitric oxide synthases; nitric oxides are known to protect bacteria from oxidative stress | Tayeh and Marletta, (1989); Gusarov et al. (2009) |
| XYLt2, 14BGGH | D-xylose transport via proton symport, endo-1,4-β-D-glucanase/cellulase | Xylose, cellobiose | Carbon source from lignocellulose degradation; DSF is known to regulate the activity of cellulases and xylanases | Sun et al. (2005); Rajeshwari et al. (2005) |

that these essential genes represent theoretical antiblight targets; gaining additional knowledge about their possible virulence properties would further help us narrow down the more promising targets. Therefore, we lastly sought to analyse all the resultant genes essential for the pathogenic lifestyle of Xoo and their potential predisposition to virulence. This can be done using several online tools, including Vicmpred (Saha and Raghava, 2006) and MP3 (Gupta et al., 2014), allowing us to predict the possible virulence properties of the genes and proteins based on amino acid composition and patterns (Zheng et al., 2012). The shortlisted genes could be considered as the most promising antiblight targets because most of them are overrepresented in xanthan and xanthomonadin biosynthesis pathways known to be associated with Xoo virulence (Figure 5 and Tables S6–S8).

Methods to combat plant diseases in the past have mainly relied on chemical control, but it has been proven that such methods induce water pollution (Özkara et al., 2016) and are not effective, as pathogens build up antibiotic resistance to pesticides gradually and grow to be stronger and harder to control (Chandra et al., 2017). In the search for effective alternatives and environmentally friendly solutions to overcome rice leaf blight caused by Xoo, we reconstructed a genome-scale metabolic model of Xoo, characterized its pathogenicity, and identified potential antivirulence targets by resorting to metabolic network comparison, constraint-based flux analysis, and transcriptome data integration. The ED pathway, gluconeogenesis, and glycogen biosynthesis and degradation pathways were highlighted in our analyses as key central metabolic pathways potentially contributing to Xoo virulence. Moreover, through integration of relevant transcriptomic data we revealed the possible metabolic routes through which the virulence factors, especially DSF and DF, contribute to Xoo pathogenesis. Finally, using systematic analysis involving gene essentiality and a rice host homology search, we proposed experimentally testable antibacterial targets that could be crucial in controlling the disease. Emerging techniques such as host-induced gene silencing (HIGS) involving small interfering RNA molecules can be used to tackle Xoo by inhibiting the potential antivirulence target genes proposed here (Machado et al., 2018). In future, the current in silico model guided framework can be further extended by including a comprehensive genome-scale model of O. sativa, iOS2164 (Lakshmanan et al., 2015) and its leaf microbiome for characterizing their interactions with Xoo and the host. As such, this will allow us to systematically devise new strategies to effectively control leaf blight in rice.

3 | EXPERIMENTAL PROCEDURES

3.1 | Bacterial strain, media, and batch growth

Xoo was obtained from the Korean Agricultural Culture Collection (KACC, South Korea) and stored in 20% glycerol at -80 °C before use. Sequential seed-cultures in nutrient broth (NB) were used to perform the main culture. The main batch culture was prepared in.
modified glucose medium comprising 5 g/L glucose, 6 g/L beef extract, 2 g/L (NH₄)₂SO₄, 2 g/L MgSO₄.7H₂O, and 2 g/L KH₂PO₄ in deionized water. The conditions of batch culture were maintained at 200 rpm and 28 °C. At every 6-hr interval, the culture broths were used to monitor the Xoo growth, glucose consumption, and xanthan production.

3.2 Estimation of optical density, glucose, and xanthan

During fermentation, the optical density (OD) of the broth was measured by UV spectrophotometry (Genesys 6, Thermo Scientific) at 600 nm. To quantify glucose concentration in the medium, 1 ml of broth was centrifuged at 5,000 rpm for 10 min and filtered with 0.22 μm polytetrafluoroethylene (PTFE) filter. Then, 100 μl of filtered medium was lyophilized and dryness samples were derivatized using the following procedure: 50 μl of pyridine containing methoxyamine hydrochloride (20 mg/ml, vol/vol) was added to the dried sample and kept at 30 °C for 90 min. Subsequently, 50 μl N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) was added and heated for 30 min at 37 °C. After filtration with 0.22 μm PTFE filter, the samples were analysed with gas chromatography time-of-flight mass spectrometry (GC-TOF-MS). Xanthan production was determined by Li et al. (2016) with 10 ml of cell-free broth. The supernatant was precipitated with 20 ml ethanol and centrifuged at 5,000 rpm for 10 min. The precipitates were dried at 60 °C for 24 hr and weighed to calculate the xanthan gum yield.

3.3 Estimation of complex medium components

Cells and supernatants from complex medium cultures were collected at every 6-hr time interval to analyse the time-course metabolite profiles. Briefly, 5 ml broth was centrifuged at 5,000 rpm for 30 min and the supernatant was used for analysis of medium components and free amino acids. Dried medium was derivatized as explained earlier and analysed by GC-TOF-MS. Free amino acid analysis of broth was performed with an L-8500 Amino Acid Analyzer.

FIGURE 5 Prediction of antimicrobial targets in Xanthomonas oryzae pv. oryzae (Xoo). (a) Gene essentiality workflow to systematically predict the antimicrobial targets in Xoo. (b) Metabolic pathways to which genes essential for growth and virulence belong. (c) Metabolic pathways to which potential antimicrobial targets belong.
(Hitachi) with an ion exchange column (Hitachi HPLC packed column #2622PF, 4.6 × 60 mm) and UV detector (VIS1 570 nm, VIS2 440 nm).

3.4 Setting up GC-TOF-MS and Xoo dry cell component measurements

The GC-TOF-MS analyses were performed using a Leco TOF Pegasus III mass spectrometer (Leco) operating in electron ionization (EI) mode (70 eV) with a 7890A GC system (Agilent). The column was 30 m in length, with 0.25 mm i.d. and 0.25 μm film thickness (DB-5MS, J & W Scientific). The carrier gas was helium at a constant flow of 1.5 ml/min. A total of 1 μl of the derivatized sample was injected in a split mode (5:1). The oven temperature was sustained at 75 °C for 2 min and then increased to 300 °C at a rate of 15°C/min. The temperature was then held at 300 °C for 3 min. The injector and ion source temperatures were 250 and 230 °C, respectively. The acquisition rate was 20 scans/s, with a mass scan range of m/z 45–1,000. The sample was identified with RT and fragment patterns of STD and commercial library. After centrifugation of 50 ml cultures incubated for 12, 30, and 60 hr, cells were rinsed with phosphate-buffered saline (PBS) three times and lyophilized. In order to extract cellular components of Xoo, 1 ml methanol was added to dried cells and the contents were smashed with a mixer mill for 1 min (30 Hz). Water and chloroform were sequentially added to the samples and vortexed for 2 min. Subsequently, the samples were centrifuged, filtered, and analysed as explained in the previous sections.

3.5 Processing GC-TOF-MS data

The GC-TOF-MS analysis files were converted to netCDF format using ChromaTOF software (LECO). The MS data were processed using the Metalign software package (http://www.metalign.nl). The resulting data were exported to Excel (Microsoft) using SIMCA-P + 12.0 software (Umetrics). The data sets were log-transformed, autoscaled (unit variance scaling), and mean-centered in a column-wise fashion. In the partial least squares discriminant analysis score plots, variables (VIP > 1, p < .05) were selected as significantly differential metabolites. The metabolites were matched with the references and the NIST05 MS Library (NIST/EPA/NIH, 2005). Statistical calculations were performed with SPSS v. 21.0 for Windows.

3.6 Reconstruction of the Xoo genome-scale metabolic model

Xoo-specific reaction lists from the KEGG (Kanehisa et al., 2008) and MetaCyc (Caspi et al., 2014) databases were retrieved and merged to obtain an initial draft reaction network. The metabolite and reaction nomenclature was used according to the BiGG database (Schellenberger et al., 2010) wherever possible in order to facilitate comparisons with other published models. Initial model building and refinement started with the removal of generic and duplicate multistep reactions and rectification of elementary imbalances for the remaining reactions. Whereas MetaCyc provides information regarding the reaction reversibility, no such information is available in KEGG. Hence, the reactions obtained from MetaCyc were retained and those obtained from KEGG were modified for their reversibility according to the literature evidence, wherever available. Gene-protein-reaction (GPR) associations were included manually for each reaction by performing a thorough check on the enzyme subunit and isoenzyme related information. The model was further refined using gap filling based on literature and enzyme sequence homology evidence obtained through BLASTp, together with information from databases such as UniProt (Apweiler et al., 2004). Several nongene-associated biochemical and extracellular transport reactions were added based on the Escherichia coli K-12 MG1655 model, iAF1260 (Feist et al., 2007), and experimental evidence to complete the metabolic reconstruction by closing the network gaps.

3.7 Generation of the biomass objective function

Simulating the GEM using flux balance analysis involves setting up a cellular objective function that is often represented by an equation composed of stoichiometric amounts of components involved in biomass formation (Feist and Palsson, 2010). The biomass equation of Xoo was derived based on the information obtained from both experiments and published data. As the availability of Xoo biomass-specific literature is limited, compositions from similar gram-negative bacteria were partially used. The amino acid composition of biomass was based on our GC-MS data of Xoo cell extract. RNA composition was used according to X. campestris pv. campestris B100 (Schatschneider et al., 2013). DNA composition was set according to the GC% of Xoo. Lipid and fatty acid compositions, soluble cell fraction, and intracellular carbohydrate compositions mostly comprising glycogen as well as the ATP cost for the growth-associated maintenance (GAM) requirement were assumed to be similar to the E. coli K-12 MG1655 biomass (Feist et al., 2007). The nongrowth-associated ATP maintenance (NGAM) was assumed to be similar to the maintenance costs of X. campestris pv. campestris (Jarman and Pace, 1984).

3.8 Model simulation, gene essentiality, and other bioinformatics analysis

FBA was performed on the Xoo reconstructed network to maximize biomass objective function, with constraints such as uptake rates of glucose and other complex medium components, and xanthan production rate. FBA simulations were performed using the COBRA toolbox (Schellenberger et al., 2011) with the Gurobi 5 optimization solver (Gurobi Optimizer Inc., 2014). The FBA linear programming (LP) problem for biomass maximization can be mathematically represented as follows:
\[
\text{max } Z = \sum_j c_j v_j 
\]
\[
\text{s.t. } \sum_j S_j v_j = 0 \text{ for metabolite } i
\]
\[
v_j^{\text{min}} \leq v_j \leq v_j^{\text{max}} \text{ for reaction } j
\]

where \(S_j\) is the stoichiometric coefficient of metabolite \(i\) in reaction \(j\), \(v_j\) represents the flux through reaction \(j\), \(v_j^{\text{min}}\) and \(v_j^{\text{max}}\) respectively represent the lower and upper constraints on the flux through reaction \(j\), and \(Z\) is the biomass objective function, where \(c_j\) denotes the weight of each biomass precursor present in the objective function.

Simulations of NS and NL conditions were performed using a variant of FBA that uses the LP formulation shown above along with an additional objective for parsimonious enzyme usage (pFBA) (Lewis et al., 2010). The resultant flux solutions were normalized to unit biomass production rates in order to facilitate the comparison of relative fluxes in various pathways across the NS and NL conditions.

The conditional essential genes of Xoo were identified by gene essentiality analysis using FBA in the COBRA toolbox as described by Joyce et al. (2006). Whereas the biomass objective function was used for essential gene prediction for growth in medium, the essential genes for the pathogenic lifestyle were predicted using an objective function that included biomass plus virulence factors, including xanthan, DSFs, and DF. Genes whose knockouts yielded an objective function value less than or equal to 5% of the wild-type objective value were considered to be essential. In order to identify potential antimicrobial targets in Xoo, the essential genes obtained from model simulation with biomass as the objective function were further subjected to a BLASTp homology search against the rice proteome to discard the essential genes in Xoo that are homologous to rice gene sequences.

ACKNOWLEDGEMENTS
This work was supported by the Biomedical Research Council of the Agency for Science, Technology and Research (A*STAR), Singapore, and the Next-Generation BioGreen 21 Program (SSAC, No. PJ01334605), Rural Development Administration, Republic of Korea.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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TABLE S2 Metabolites of iXOO673
TABLE S3 BLASTp results of the key ED pathway enzyme, 6-phosphogluconate dehydratase
TABLE S4 BLASTp results of the key ED pathway enzyme, 2-dehydro-3-deoxy-phosphogluconate aldolase
TABLE S5 List of genes essential for growth and non-homologous to rice genes
TABLE S6 Pathogenicity prediction of genes essential for growth and virulence using VICMpred tool
TABLE S7 Pathogenicity prediction of genes essential for growth and virulence using MP3 tool
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DATA S1 SBML file of iXOO673

How to cite this article: Koduru L, Kim HY, Lakshmanan M, et al. Genome-scale metabolic reconstruction and in silico analysis of the rice leaf blight pathogen, Xanthomonas oryzae. Molecular Plant Pathology. 2020;21:527–540. https://doi.org/10.1111/mpp.12914