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Virulence Diversity and Cas gene Cluster Analysis in *Xanthomonas oryzae* pv. *oryzae* Population of India

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ABSTRACT: Exopolysaccharide (EPS) and Xanthomonadin pigment quantification in 39 *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) isolates are investigated in this work, as well as any implications for pathogenicity. With levels ranging from 29 mg to 92 mg, EPS generation, which is essential for the development of the disease and host colonisation, differed among isolates. Longer lesion lengths were connected with certain high EPS producers but not with others, demonstrating the multiple impacts on pathogenicity. When Xanthomonadin pigments were quantified, different levels were found for different isolates, although their combined influence on lesion duration varied. Variations in the presence of *Cas* genes were discovered through the analysis of CRISPR-Cas gene clusters, with some isolates, KPXoo5, KPXoo15, KPXoo26 and KPXoo32 having the entire set and others lacking specific cas genes. These results highlight the genetic variation of *Xoo* strains and highlight the need for more research on the functional effects of Cas gene variants. Overall, this study advances our knowledge of *Xoo* pathogenicity and may have implications for gene-editing studies and crop protection.

Keywords: *Xanthomonas oryzae* pv. *oryzae*, Exopolysaccharide, CRISPR-Cas.

INTRODUCTION

Bacterial blight, caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), is a devastating disease that poses a significant threat to rice production, a staple food for over half of the world's population(Ashwini *et al.,* 2023). As an adaptable and resilient pathogen, *Xoo* employs a repertoire of virulence factors to colonize rice tissues successfully (Salzberg *et al.,* 2008). Among these virulence factors, exopolysaccharides (EPS) and xanthomonadins have emerged as key players, contributing significantly to the pathogenicity and survival of *Xoo* strains.

EPS, complex polysaccharides secreted by *Xoo*, have been recognized for their multifaceted roles in the pathogenic process. These biofilm-forming and immune-modulating molecules are known to enhance bacterial attachment to host tissues and shield *Xoo* from the host's defense mechanisms. Xanthomonadins, on the other hand, are unique pigments produced by *Xoo* strains and have been implicated in various aspects of pathogenicity, including resistance to reactive oxygen species and UV radiation (Coplin and Cook 1990).

Xoo produces a different virulence factor, including, extracellular enzymes, type III effectors and EPS (Liu *et al.,* 2014). During the initial stages of plant pathogen interactions and disease development exopolysaccharide (EPS) helps in facilitate adhesion of bacteria to the host surface (Subramoni *et al.,* 2006). Loss of EPS production has been correlated with loss of virulence in plant pathogens (Coplin and Cook 1990). The role of EPS in the virulence of *Xoo* has been established by a transposon insertion in the gumG homologue of the mutant strain, BXO1002 (Dharmapuri and Sonti 1999). Exopolysaccharide (EPS) production plays a major role in the induction of characteristic symptomsinrice plants (Suryawanshi *et al.,* 2018). EPS is responsible for the adhesion of bacterial cells to substrates and favoring the colonization of the bacteria on plants' surface and internal tissues. The xanthan gum, an anionic complex of EPS, suppresses defense genes and consequently inhibits callose deposition in plants; hence it is an important factor in bacterial pathogenicity.

The ongoing arms race between *Xoo* and rice plants has led to the evolution of sophisticated pathogen recognition and defense mechanisms in both parties. In this intricate molecular battle, *Xoo* employs a suite of virulence factors and immune evasion strategies to colonize rice tissues and establish infection. The Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-Associated (Cas) protein system is a remarkable and versatile microbial immune system found in various bacteria and archaea. This system has garnered considerable attention in recent years due to its potential applications in genome editing and its role in bacterial defense against foreign genetic elements. Within *Xoo*, the presence of CRISPR-Cas systems and the specific Cas gene proteins

involved in these systems have remained relatively unexplored terrain.

Despite the pivotal roles attributed to EPS and xanthomonadins, a comprehensive understanding of their abundance and variation across different *Xoo* strains remains limited. Recognizing the significance of this knowledge gap, this study focuses on quantifying EPS and xanthomonadin production in a diverse set of *Xoo* strains. This study endeavors to bridge this knowledge gap by systematically identifying Cas gene proteins across a diverse set of *Xoo* strains (Mali *et al.,* 2013). Understanding the prevalence and diversity of Cas genes in *Xoo* is not only of fundamental importance for elucidating the molecular strategies employed by *Xoo* strains during infection and has practical implications for crop protection and management. By shedding light on the functional aspects of CRISPR-Cas systems in *Xoo*, this research contributes to our understanding of the intricate host-pathogen interactions in bacterial blight. It may open new avenues for developing targeted strategies to mitigate the impact of this devastating disease on rice agriculture.

MATERIAL AND METHODS

A. Collection of BB diseased leaf samples and isolation of pathogen

A field survey was undertaken in major rice growing regions of Karnataka, India, in June, 2021. A total of 15 samples were collected from different locations in Karnataka. Plants with typical bacterial leaf blight symptoms *viz.*, yellow water-soaked lesions at the margin of the leaf blade, the lesions run parallel along the leaf, presence of bacterial discharge on young lesions early in the morning resembling the milky dewdrop, drying up of leaf blade with white lesions as wavy margin were collected in blotting paper folds, labelled, and then in a paper envelope. The samples were brought to the laboratory and stored at 4°C 5 to isolate the pathogen. A total of 24 *Xoo* isolates were collected from the AICRP-Agriculture Research Station, Gangavathi, Karnataka. The isolates were from different regions of India (Fig. 1) and were sub-cultured periodically. Most *Xoo* isolates were sourced from Karnataka, specifically from the primary regions known for rice cultivation. This studytested all isolates for pathogenicity on susceptible rice variety TN-1. The cultures obtained from re-isolation of the infected tissues were used in all the experiments.

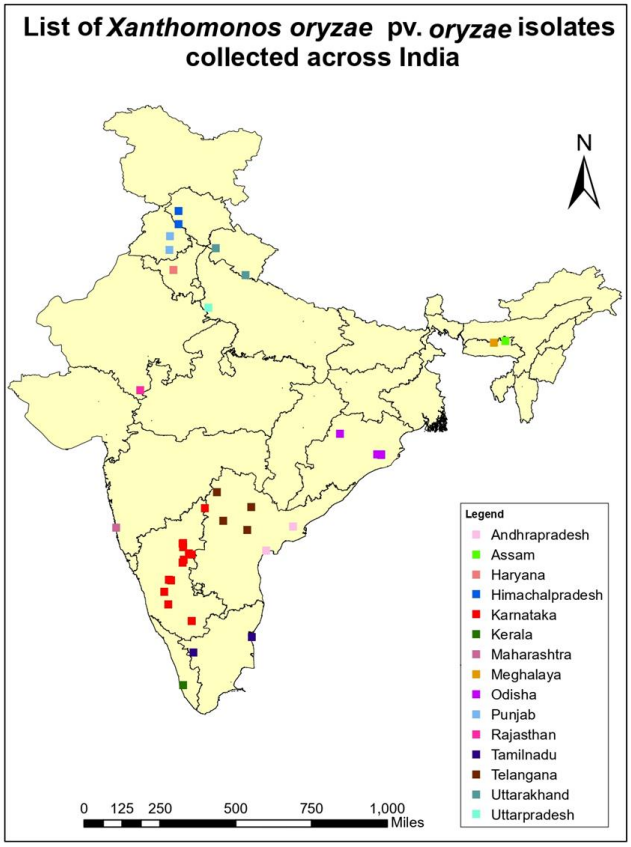

Fig. 1. India map showing the list of *Xanthomonas oryzae* pv. *oryzae* population used in the study.

B. Exopolysaccharide (EPS) quantification

The exopolysaccharide (EPS) measurement was conducted as described by Jeong *et al.* (2008). A single colony of each *Xoo* isolate was inoculated in 40 ml of nutrient broth medium and incubated for 72 h at 28 °C with agitation. The optical density of the bacterial cultures was adjusted to 1.0 at 600 nm with Nutrient Broth. The culture supernatants were transferred into new 50 ml tubes and supplemented with 1.0% potassium chloride (w/v; final concentration). Two volumes of absolute ethanol were added to each solution, and the tubes were placed at -20 °C overnight. The precipitated crude EPS was collected by centrifugation for 30 min at 8,000 g. The EPS pellets were dried at 55 °C for 12 h and the dry weight of each was measured.

C. Xanthomonadin quantification

With some modification, measuring xanthomonadin pigment was based on the method as described Sahu *et al.* (2018). The cell of the *Xoo* was collected by centrifuging 4 ml broth suspension and was mixed with 1 ml 100 % methanol. The untreated cells were used as the control. The mixtures were further incubated in darkness for 10 min, kept on a rotating shaker followed by centrifugation at 10,000 *g* for 10 min to collect the supernatant. The xanthomonadin pigment was estimated by measuring the absorbance at OD_{445} using an ultraviolet spectrophotometer.

D. Identification of Cas protein regions

"CRISPR Cas Finder" is a bioinformatics tool used to detect and analyse CRISPR-Cas systems in prokaryotic genomes. The genomic sequences of 39 Xoo isolates in FASTA format were uploaded to the CRISPRC as Finder tool.

CRISPR Cas Finder uses a combination of sequence analysis, motif detection, and homology searches to identify CRISPR arrays within the provided sequences. It searches for characteristic sequence motifs that are indicative of CRISPR repeats and spacers. It also identifies potential Cas protein sequences associated with the detected CRISPR arrays. The tool annotates the identified CRISPR arrays and Cas proteins with relevant information about the location of CRISPR arrays, repeat sequences, spacer sequences and putative Cas proteins. The identified CRISPR arrays and Cas proteins can be further analyzed for subtype determination, evolutionary studies and functional predictions.

RESULT AND DISCUSSIONS

A. Exopolysaccharide quantification

The production of exopolysaccharide (EPS) is a characteristic feature of Xanthomonads. A biochemical assay was carried out to assess the EPS accumulation in different isolates. EPS is an important factor in the pathogenicity and virulence of *Xoo*, as it plays a role in disease development and colonization of host plants. The isolates exhibited a range of EPS production levels, ranging from 29 mg to 92 mg. Isolate KPXoo18 shows the highest EPS production, 92 mg, while isolate KPXoo28 exhibits the lowest EPS production, 29 mg. The remaining isolates display varying EPS production levels, indicating diversity in their ability to produce exopolysaccharides.

Some isolates with higher EPS production levels exhibit longer lesion lengths, suggesting a potential correlation between EPS production and lesion development. The isolate KPXoo19 has a relatively high EPS production of 92 mg and a lesion length of 16.8 cm, indicating a possible association between elevated EPS production and increased lesion length. Conversely, some isolates with lower EPS production levels still exhibit longer lesion lengths, suggesting other factors may contribute to lesion development. The isolate, KPXoo25 hada lower EPS production of 38 mg but a relatively long lesion length of 15.6 cm (Fig. 2). It is crucial to emphasize that the association between EPS production and lesion length is not consistently observed across all isolates. This inconsistency suggests that other factors, including genetic diversity and interactions with the host, might also contribute to the development of lesions (Zhou *et al.*, 2013).

The variation in EPS production levels among the isolates suggests genetic diversity or differences in their pathogenicity. Isolates with higher EPS production levels may be more able to form biofilms, adhere to host tissues, and evade the host's immune response, contributing to their pathogenicity. Conversely, isolates with lower EPS production levels may exhibit reduced pathogenicity or virulence. Understanding the variation in EPS production is crucial for studying the disease mechanisms of *Xoo* and developing strategies for disease management and crop protection.

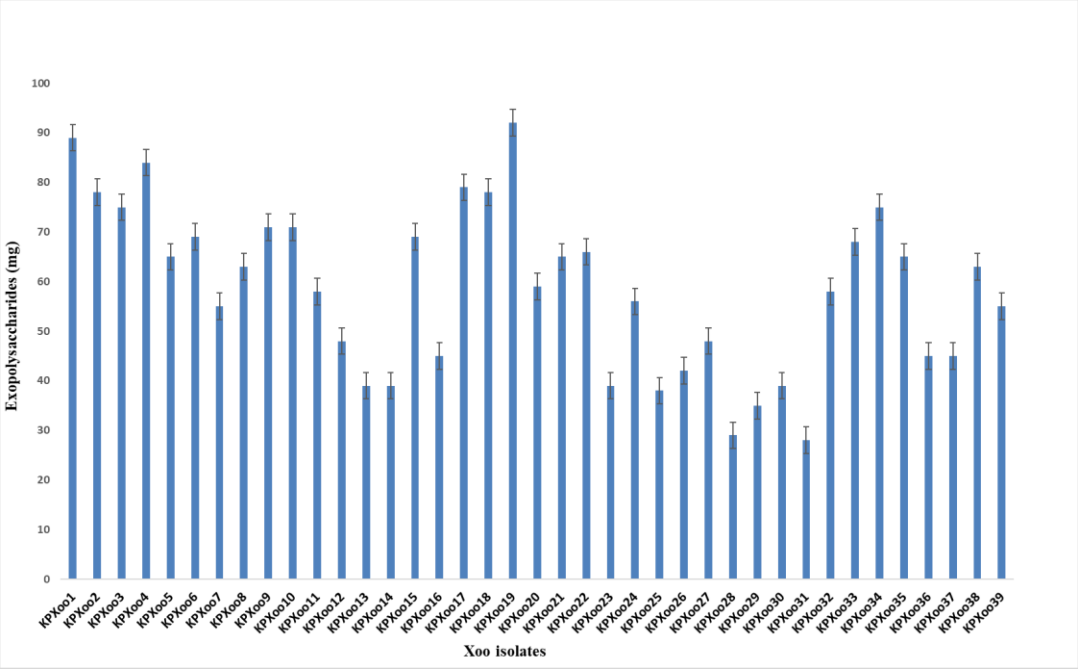

Fig. 2. Quantification of exopolysaccharide produced by *Xoo* isolates.

B. Xanthomonadin quantification

EPS production was measured in milligrams (mg), while the presence of Xanthomonadin pigments was quantified as a numerical value based on the spectroscopic readings. Some isolates exhibited higher EPS production levels and higher Xanthomonadin values, while others have lower values for both parameters. The isolate KPXoo19, has a relatively high EPS production of 92 mg and a Xanthomonadin value of 0.80. It also had a longer lesion length of 16.8 cm. In contrast, isolate KPXoo25 showed lower values for both EPS production (38 mg) and Xanthomonadin content (0.32) but exhibited a relatively long lesion length of 15.6 cm (Fig. 3). These observations suggest that while EPS production and xanthomonadin content may individually play roles in lesion development, their combined effect on lesion length was not consistent across all isolates. This outcome corroborates previous research findings, as reported by Poplawsky *et al.* (2000). It is important to note that other factors, such as genetic variation and host interactions, may also contribute to lesion development like the type three secretion system proteins secreted by the bacterium.

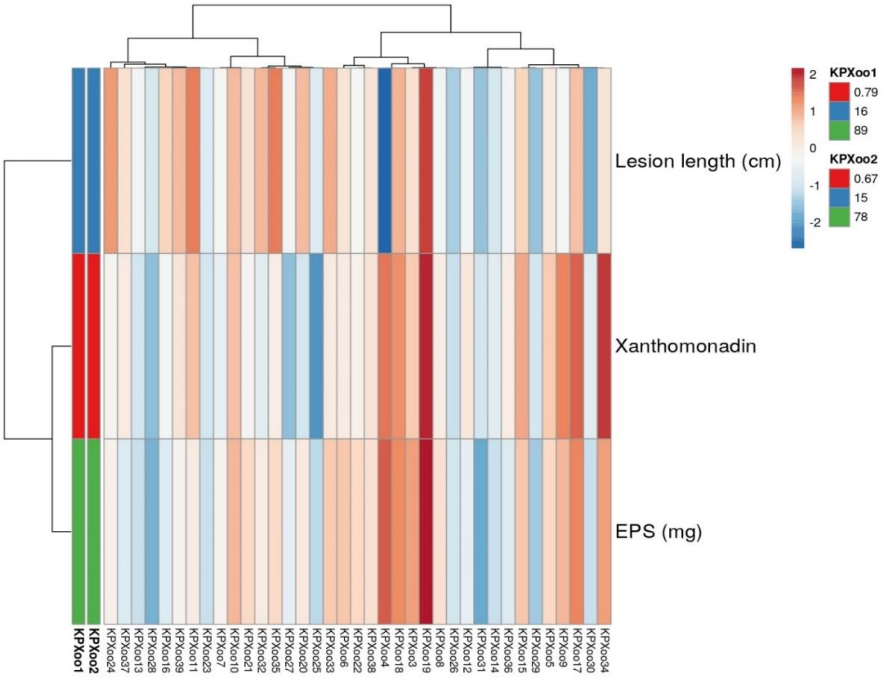

Fig. 3. Heat map showing the quantification of exopolysaccharide and xanthomonadin and its effect on lesion development.

C. Cas genes cluster analysis in Xoo

CRISPR Cas Finder identified the presence and absence of various cas genes in different *Xoo* isolates, KPXoo5, KPXoo15, KPXoo17, KPXoo26, KPXoo32, and KPXoo35. These cas genes are associated with the CRISPR-Cas immune system, which plays a role in the defense against foreign genetic elements, such as phages and plasmids. The isolates KPXoo5, KPXoo15, KPXoo26 and KPXoo32 possess the complete set of cas genes, *Cas1*, *Cas2*, *Cas3*, *Cas4*, *Cas5*, *Cas7*, and *Cas8*. The presence of the full CRISPR-Cas system suggests that these *Xoo* isolates likely have an active and functional immune response mechanism against foreign genetic elements. The isolates, KPXoo17 and KPXoo35 lack the *Cas4* gene which is involved in spacer acquisition and adaptation (Fig. 4). The absence of *Cas4* might indicate a potential difference in their ability to adapt to new phage or plasmid invasions compared to the other isolates.

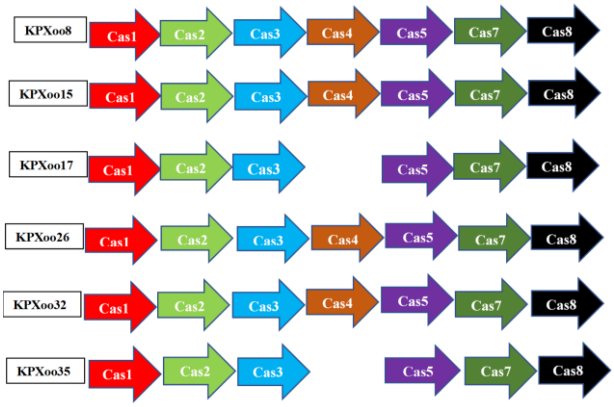

Fig. 4. Cas gene profiling among the *Xoo* isolates.

The absence of *Cas4* in KPXoo17 and KPXoo35 may affect their ability to acquire new spacers and adapt to evolving threats. However, the rest of the cas genes are present, indicating that they can still utilize the existing spacers for defense. The variation in the presence or absence of cas genes among different *Xoo* isolates highlights the genomic diversity within this bacterial species. Such diversity can be attributed to horizontal gene transfer and adaptation to local environments. The presence or absence of cas genes in different *Xoo* isolates signifies their potential variations in the CRISPR-Cas immune systems. These systems play a role in defending against foreign genetic elements, and the diversity observed among these isolates warrants further study to determine their functional implications and adaptability mechanisms. The findings regarding the functional Type I-C CRISPR-Cas system in *Xoo,*show this system's significance, implications and potential applications in gene editing (Liu *et al.*, 2021).

CONCLUSIONS

The production of exopolysaccharide (EPS) and quantification of Xanthomonadin pigments were assessed to understand their roles in pathogenicity. The results showed that EPS production levels varied among isolates, potentially influencing lesion development. *Xoo* isolates with higher EPS production tended to have longer lesions, suggesting a correlation between EPS production and virulence. Regarding the CRISPR-Cas immune system, it was shown that certain *Xoo* isolates had a comprehensive complement of cas genes, indicating the presence of an operational and effective defence mechanism against exogenous genetic elements. Nevertheless, it is noteworthy that two isolates exhibited the absence of the Cas4 gene, a crucial component in spacer acquisition and adaptation. This genetic variation may affect their capacity to counteract novel challenges effectively.

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Conflict of Interest. None.

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