Complete Genome Sequence Reveals Evolutionary Dynamics of an Emerging and Variant Pathovar of *Xanthomonas euvesicatoria*

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Abstract

Xanthomonas, a complex group of pathogens, infects more than 400 plants, which is expanding to new hosts causing serious diseases. Genome-based studies are transforming our understanding on diversity and relationship of host-specific members, known as pathovars. In this study, we report complete genome sequence of a novel pathovar *Xanthomonas axonopodis* pv. *commiphorae* (Xcom) from India. It causes gumming disease of *Commiphora wightii*, a medicinally important plant. Genome-based phylogenetic and taxonomic investigation revealed that the pathovar belongs to *Xanthomonas euvesicatoria* and not *X. axonopodis* as reported earlier. Interestingly, it is a novel host and novel geographic origin for a *X. euvesicatoria* pathovar. A core-genome-based phylogenetic analysis resolved the pathovar complex of this species on the basis of their hosts. Interestingly, this pathovar harbors a unique 35-kb plasmid encoding type III effectors and toxin–antitoxin gene that is absent in other *X. euvesicatoria* pathovars and infects tomato, pepper, rose, onion, philodendron, alfalfa, and citrus plants. The pathovar contains two TAL (transcription activator-like) genes, one on plasmid and another on genomic region with an additional pseudo TAL gene flanked by IS elements in the plasmid. Further, Xcom has acquired a novel set of lipopolysaccharide biosynthesis genes after its divergence from the closely related pathovar that infects rose and supports the role of horizontal gene transfer in hypervariation at this locus in the species. Complete genome sequence of this variant pathovar has provided novel insights into evolution of an emerging pathovar in *Xanthomonas* and will be valuable resource in pathogenomics of *X. euvesicatoria*.

Key words: *Xanthomonas euvesicatoria*, genomics, evolution, pathovar, TAL, complete genome.

Introduction

Xanthomonas is a highly successful pathogen known to infect more than 400 plants in a host-specific manner (Leyns et al. 1984). *Xanthomonas* species and the constituent pathovars have undergone numerous reclassifications because of challenges associated with the conventional methods of bacterial classification (Young et al. 1978; Dye et al. 1980; Van den Mooter and Swings 1990; Vauterin et al. 1995; Hauben et al. 1997; Rademaker et al. 2005). Advent of DNA-based typing and grouping methods provides accurate understanding on species diversity of *Xanthomonas* (Rodriguez et al. 2012; Constantin et al. 2015). In the present scenario, around 150 host-specific members, known as pathovars, are grouped into 33 valid species (List of Prokaryotic names with Standing in Nomenclature [LPSN]; https://creativecommons.org/licenses/by-nc/4.0/.) (Parte 2018), which indicates intra-species diversification as a major phenomenon. Some species like *Xanthomonas citri, Xanthomonas axonopodis*, and *Xanthomonas euvesicatoria* constitute numerous pathovars (Midha and Patil 2014; Constantin et al. 2015; Barak et al. 2016; Bansal et al. 2017). Interestingly, the majority of the pathovars are first reported from India, which is one of the major centers of diversity and major cultivator of host plants. Surprisingly, 19 out of 22 pathovars were found to belong to *X. citri* and not several species as reported earlier (Parkinson et al. 2009; Bansal et al. 2017). These studies indicate that both ecological

and evolutionary investigations are important in systematic understanding the *Xanthomonas* group of pathogens.

With the advent of high-throughput genomics, there is an opportunity to look into diversity and evolution of pathovars and individual isolate at unprecedented details. Now, it is possible to accurately establish species status of new pathovar or an isolate using modern genome-based taxonomic criteria (Meier-Kolthoff et al. 2013; Lee et al. 2016). At the same time, the availability of large number of phylogenomic markers and core-gene content allows to resolve the relationship of a pathovar that forms complex of a particular species. Apart from providing robust phylogeny, comparative studies are leading to the identification of genomic determinants of host specificities and role of horizontal gene transfer in variable gene content. With the emergence of long-read technology, it is now possible to study the highly repetitive transcription activator-like (TAL) proteins that are secreted by type III secretion system to modulate host genes for pathogenicity and role of plasmids in diversification of bacterial isolates (Boch and Bonas 2010). This aspect is particularly relevant in *Xanthomonas* species, which displays high infraspecies diversity (Parkinson et al. 2009; Constantin et al. 2015; Bansal et al. 2017).

Recently, a new pathovar of *Xanthomonas* was reported from India and found to cause a serious gummosis in *Commiphora wightii* (Samanta et al. 2013). This plant is cultivated for medicinally important oleo-gum-resin and is native to South Asia. Biochemical and initial phylogenetic analysis has classified it as *X. axonopodis* pv. *commiphorae* (Xcom). In the present study, we report complete genome sequence of this novel pathovar and surprisingly, its genomotaxonomics revealed that Xcom belongs to *X. euvesicatoria* and not to *X. axonopodis*. *Xanthomonas euvesicatoria* is a major pathogen of tomato and pepper, and all other pathovars of this species were reported outside India, mainly in the United States (Potnis et al. 2011; Albuquerque et al. 2012; Barak et al. 2016). In this context, Xcom represents expanded host range and geographic origin of a *X. euvesicatoria* pathovar and has provided us an opportunity to look for the genome dynamics in the emergence of a novel pathovar.

Materials and Methods

Procurement of Bacterial Culture and Revival

The bacterial culture of then defined *X. euvesicatoria* (*axonopodis*) pv. *commiphorae* LMG26789 (Xcom) was procured from the Belgium Coordinated Collections of Microorganisms/LMG and was revived according to the prescribed instructions.

Genomic DNA Isolation and Illumina Sequencing

ZR Fungal/Bacterial DNA Mini-Prep Kit (Zymo Research, Irvine, CA) was used to obtain high-quality bacterial DNA. DNA quantification was performed using Nanodrop 1000 (Thermo Fisher Scientific) and Qubit 2.0 Fluorometric (Invitrogen; Thermo Fisher Scientific). Paired-end Illumina sequencing libraries were prepared with Illumina Nextera XT sample preparation kit (Illumina, Inc., San Diego, CA) in accordance with the manufacturer's recommendations and were sequenced on in-house Illumina Miseq (Illumina) platform with 2 × 250 bp paired-end sequencing kit.

Bacterial Genomic DNA Isolation and ONT MinION Sequencing

Complete bacterial DNA was obtained using the DNeasy Blood & Tissue Kit (Qiagen; 69504) (Qiagen DNeasy, Valencia, CA) and quantified using Nanodrop 1000 and Qubit 2.0. Initial DNA concentration of 4 μg was taken for the shearing of genomic using Covaris g-TUBE (Covaris, Brighton, United Kingdom). Purification of the genomic DNA was again performed with AMPure beads to ensure the concentration remains >1 μg (in 45 μl). DNA end repair was performed using NEBNext Ultra II End repair/dA-Tailing Module (NEB, Ipswich) and again Ampure XP beads (Beckman Coulter) were used to perform the cleanup. The ONT library was prepared using ONT 1D ligation sequencing KIT (SQK-LSK108) with native barcoding kit (EXP-NBD103). The barcode ligation was performed with New England Biolabs/ligase master mix module and purified using 0.45X AMPure beads. Fifty microliters of the pooled DNA sample was used for adapter ligation using T4 DNA ligase.

Prior to MinION Sequencing on Oxford Nanopore MinION Mk1B using MinKNOW (http://community.nanoporetech.com) (Oxford Nanopore Technologies) software (v.1.13.1), quality checks of the flow cell (FLO-MIN106, version R9.4) (Oxford Nanopore Technologies) were performed. The library was combined with the reagents supplied with the Oxford Nanopore and loaded onto the flow cell in accordance with the manufacturer's recommendations with 48-h sequencing procedure, and Albacore v 1.2.1 (Oxford Nanopore Technologies Ltd.) was used to call the bases. The raw fastq reads were filtered based on the minimum length of 2 kb using Filtlong v0.2.0 (https://github.com/rrwick/Filtlong).

Genome Assembly and Annotation Using ONT and Illumina Reads

Unicycler v0.4.4 (Wick et al. 2017) bold mode was used for genome assembly using ONT long reads resulting in complete circular chromosomal and plasmid. The assembled genomes were then error corrected with multiple rounds of pilon v1.22 (Walker et al. 2014) with Illumina short reads. The assembled genome was then checked for the completeness and contamination using CheckM v1.0.11 (Parks et al. 2015) and submitted to NCBI WGS portal and annotated using NCBI Prokaryotic Genome Annotation Pipeline (https://www.ncbi.nlm.nih.gov/genome/annotation_prok/).

Genealogical Analysis

ClonalFrameML (Didelot and Wilson 2015) analysis was used to generate phylogenetic tree using genome alignment obtained using MAUVE v2.3.1 (Darling et al. 2004) and maximum likelihood tree was obtained using PhyML v3.1 (Guindon et al. 2010). CFML analysis was carried out using MAUVE alignment and PhyML tree, performing 100 simulations.

LPS Cassette and TAL Genes Analysis

Full-length LPS cassettes were retrieved from genomes using *etfA* and *metB* conserved genes and annotated using PROKKA 1.11 (Seemann 2014). Easyfig 2.2.2 was used to generate figure (Sullivan et al. 2011). Further, TAL genes analysis on complete genome was carried out using AnnoTALE (Grau et al. 2016) software.

Results

Complete Genome Sequence of Xcom

Complete genome sequencing of Xcom was performed using Oxford Nanopore MinION and Illumina Miseq platform (see Materials and Methods). Xcom has a circular chromosomal DNA of 4.8 Mb and one plasmid of 35 kb with NCBI accession numbers: CP031059 and CP031060, respectively. The genome coverage obtained is 124× with 4,203 numbers of coding sequences and 53 tRNAs. GC content for the genomic and plasmid regions are 65.2% and 62.5%, respectively. Further, its completeness and contamination for the genome were found to be 99.64% and 0%, respectively.

As we have a complete genome sequence, highly repetitive *tal* genes could be easily fetched. TAL effectors are transkingdom remote controls of gene expression, which once inside the plant cell nucleus, induces the transcription of eukaryotic genes in a sequence-specific manner (Boch and Bonas 2010). Interestingly, with complete genome, *tal* gene profile of the Xcom was found to be unique in having two *tal* genes, one on the genomic (Xcom_11830) and one on the plasmid (Xcom_21330) region, in addition to a pseudo *tal* gene (Xcom_21360) in the plasmid region. Both the copies of *tal* genes in plasmid are surrounded by transposable elements. Interestingly, the chromosomal *tal* gene is also flanked by a transposable element. Apart from *tal* genes, the plasmid also encodes for non-TAL effectors and type II toxin–antitoxin genes. The unique plasmid also harbors a cluster of nine hypothetical genes and an acetyltransferase gene.

Taxonogenomic and Genealogical Investigation

Taxonogenomic analysis (using OrthoANI and dDDH) including type strains of phylogenetic groups of *X. axonopodis* depicted that Xcom belongs to *X. euvesicatoria* (orthoANI value of 98.72% and dDDH value of 88.9%) much above the cutoff for species delineation of 96% and 70%, respectively, and not to *X. axonopodis* (orthoANI value of 93.14% and dDDH value of 49.6%) (fig. 1). Hence, now onwards, we will refer the pathovar as *X. euvesicatoria* pv. *commiphorae* LMG26789 (Xcom).

Xanthomonas euvesicatoria is a complex species consisting of pathovars infecting asterid, rosid, and monocot hosts (supplementary table 1, Supplementary Material online). Hence, we carried out genealogical investigation to understand the evolution of diverse range of pathovars (fig. 2). Interestingly, strains from asterids hosts, which are native to America, formed a distinct clonal group (CG-I); and strains infecting rosids, including Xcom and monocots hosts, formed CG-II, while *Xanthomonas alfalfae* was not included in CGs. Interestingly, hosts of CG-II strains are mainly native to Asia (except *Philodendron*, known to be originated in South America).

Variations in Lipopolysaccharide Biosynthetic Gene Clusters

Lipopolysaccharide (LPS) locus in genus *Xanthomonas* is hypervariable at species and pathovar level (Patil et al. 2007). *Xanthomonas euvesicatoria* pathovar displays hypervariability in LPS biosynthetic gene cluster (supplementary fig. 1, Supplementary Material online). *Xanthomonas perforans* and *X. euvesicatoria*, both infecting asterids from CG I, are having diverse LPS gene clusters. In contrast, *X. perforans* and *X. axonopodis* pv. *dieffenbachiae* that infect asterids and rosids, respectively, have same LPS cassette. At the same time, *X. euvesicatoria* and *X. alfalfa* strain GEV-Rose-07 from CG II are having identical LPS. Moreover, Xcom shares half of its cassette with these pathovars, while the other half of Xcom LPS cassette is showing homology with *Xanthomonas albelineans.*

Discussion

Earlier, genome-based investigation has revealed that five known pathovars (*X. axonopodis* pv. *dieffenbachiae* strain LMG12749, *X. axonopodis* pv. *allii* CFBP6369, *X. alfalfae* strain GEV-Rose-07, *X. axonopodis* pv. *citrumelo* F1, and *X. perforans*) of *Xanthomonas* belonged to species *X. euvesicatoria* (Barak et al. 2016). These were misclassified into three different species, that is, *X. axonopodis*, *X. alfalfa*, and *X. perforans*, and some were reported as *Xanthomonas* sp. Originally, Xcom was also classified as *X. axonopodis* pathovar based on traditional approaches and limited sequence information (Samanta et al. 2013). However, similar to the case of other pathovars, genome-based taxonomy and phylogenetic analysis clearly established Xcom as a new pathovar of *X. euvesicatoria*. Hence, genome-based studies are critical in understanding the complex group of pathogens and particularly

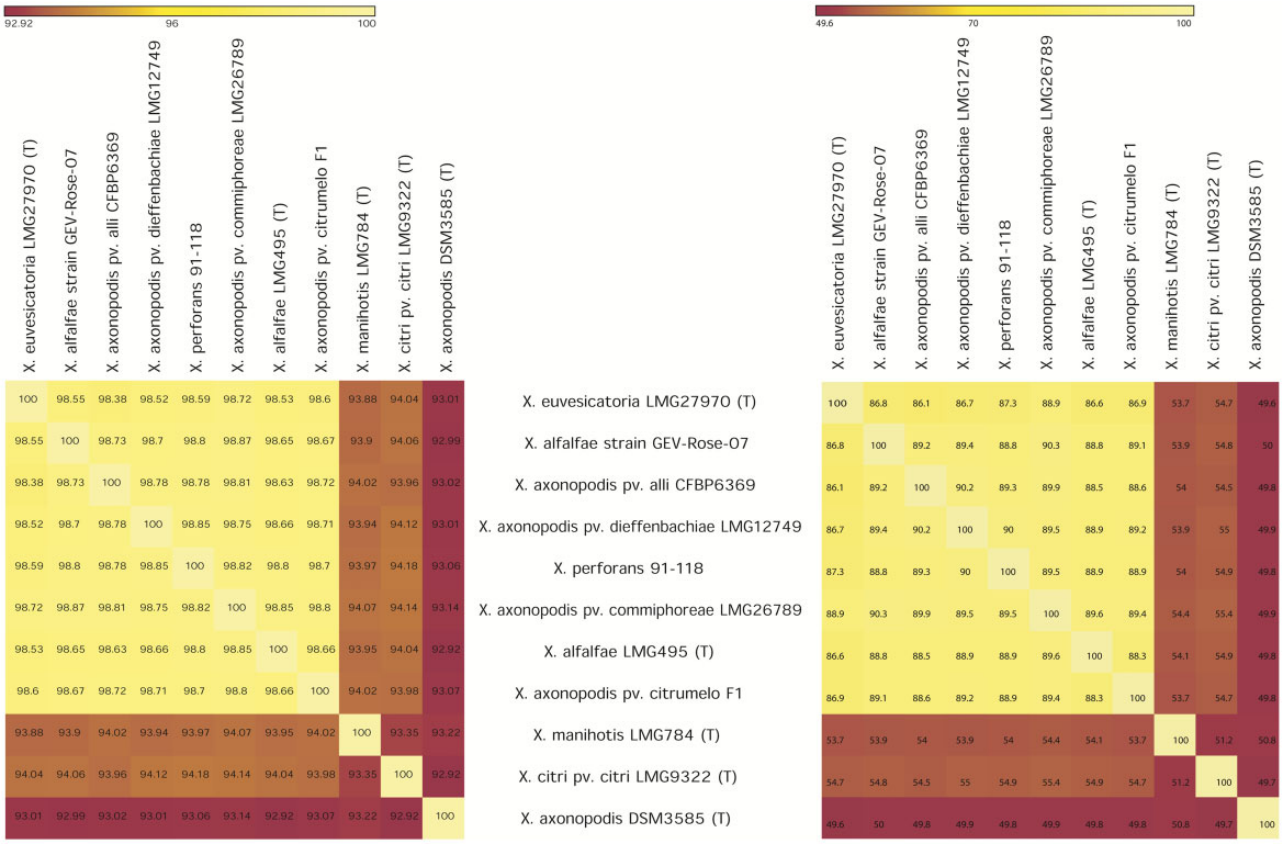

Fig. 1.—Heatmap displaying orthoANI (right hand side) and dDDH (left hand side) values showing Xcom belongs to *X. euvesicatoria* based on >96% ANI and >70% dDDH cutoff values.

pathovar-rich species, such as *X. euvesicatoria, X. axonopodis*, and *X. citri*.

In an earlier genome-based study, we have reported that 19 pathovars, previously classified into three different species, belonged to one species, that is, *X. citri* (Bansal et al. 2017). All these pathovars were first reported from India, in the last century and believed to have spread to other parts of the world (Parkinson et al. 2009). In contrast, all the other pathovars of *X. euvesicatoria* were first reported outside India and Xcom will be the first pathovar from *X. euvesicatoria* known from India. *Xanthomonas euvesicatoria* and *X. citri* are evolutionary diverse species in the genus. Hence, it is surprising that a new pathovar that infects a host that is native to India belongs to *X. euvesicatoria*. This provided us an opportunity to carry out a complete genome-based investigation on diversity and evolution of *X. euvesicatoria* pathovars by including an ecologically variant pathovar.

Initial core-genome phylogeny revealed that Xcom is closely related to pathovar that infects rose. Interestingly, the rose pathovar was first reported in 2004 from Texas and Florida regions of North America (Huang et al. 2013). The present analysis has also suggested that the two recently reported rose and Xcom pathovars are from a rapidly diversifying lineage and might have originated from a common ancestor that has evolved into pathovars infecting other rosids, such as *Commiphora*, rose, alfalfa, and citrus. Apart from phylogeny, it is also important to look into the dynamic variations that play role in the evolution of Xcom. We were successful in using short reads from Illumina platform and long reads from nanopore sequencer in obtaining complete genome of Xcom pathovar. Complete genome sequence is particularly relevant for new and emerging pathogens such as Xcom, as we can also study plasmids, repetitive TAL proteins, and hypervariable/dynamic genomic regions known in *Xanthomonas*.

Complete genome sequence allowed us to identify a unique plasmid in Xcom that harbors one complete and one pseudo TAL genes surrounded by IS elements. TAL proteins are secreted by *Xanthomonas* pathovar using type III secretion system to regulate host genes. However, due to modular structure of *tal* genes, they are nearly impossible to get assembled using short sequencing reads. Among the available complete genomes of *X. euvesicatoria*, only *X. perforans* LH3 (Richard et al. 2017) was found to be have a TAL gene on the plasmid. Interestingly, none of the other complete genomes such as *X. euvesicatoria* 85-10, *X. euvesicatoria*

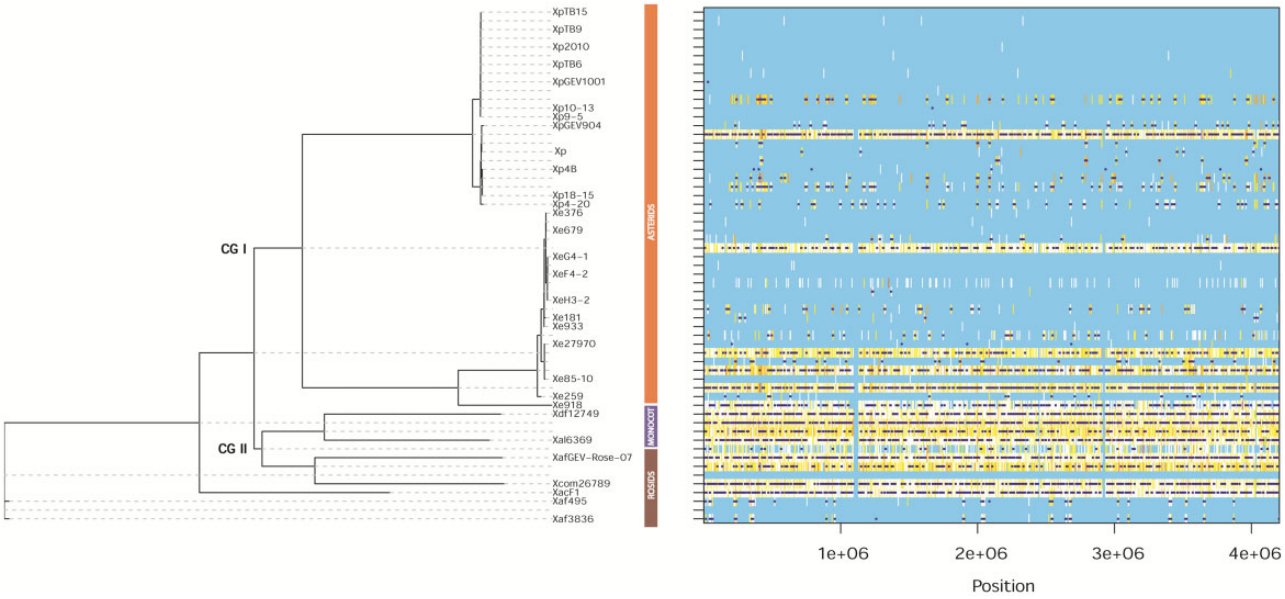

Fig. 2.—Genealogical analysis of recombination and mutation events of *X. euvesicatoria* pathovars as depicted by ClonalFrameML. Strains isolated from asterids or monocots or rosids are indicated along with the tree. Here, the variations detected by comparing each clade with its most recent common ancestor are depicted in the graph. Substitutions are represented by vertical lines and recombination events by dark blue horizontal bars. Light blue vertical lines represent no substitution and white lines refer to nonhomoplasic substitutions. Any other color represents homoplastic substitutions with increase in homoplasy associated with increase in degree of redness from white to red. Xp, *X. perforans*; Xe, *X. euvesicatoria*; XafGEV-Rose-07, *X. alfalfa* strain GEV-Rose-07; Xdf12749, *X. axonopodis* pv. *dieffenbachiae* LMG12749; Xal6369, *X. axonopodis* pv. *alli* CFBP6369; XacF1, *X. aconopodis* pv. *citrumelo* F1; Xcom26789, *X. euvesicatoria* pv. *commiphorae* LMG26789.

LMG930, and *X. perforans* 91-118 were found to have TAL genes. To the best of our knowledge, this is the first report of genome sequence of an *X. euvesicatoria* pathovar encoding a *tal* gene on the chromosome. At the same time, the presence of a complete and a pseudogene of TAL protein surrounded by IS elements suggests on-going dynamics mediated through a unique plasmid.

In *X. euvesicatoria* pathovars, LPS gene clusters are hypervariable (Potnis et al. 2011). LPS is both a major virulence factor and a pathogen-associated molecular pattern (Patil et al. 2007). The acquisition of a novel 17.3-kb LPS gene cluster is associated with a host-specific variation in *X. perforans* that infects only tomato compared with *X. euvesicatoria* that infects both tomato and pepper. LPS variation also seems to play a major role in the evolution of Xcom, as half of the LPS gene cluster is novel while other half is similar to that of closely related pathovar that infects rose. This suggests that the acquisition of a new set of LPS genes has happened after divergence of rose and Xcom pathovars from the common ancestor.

Overall, our genome-based investigation allowed us to clarify the taxonomic status of Xcom and its relationship with the other pathovars of *X. euvesicatoria*. The complete genome sequence and findings will be valuable in developing unique markers for identification and surveillance of this novel pathogen. At the same time, identification of a unique plasmid with type III effectors and both TAL and non-TAL along with toxin–antitoxin genes suggests its evolution as a major pathogen through dynamic variation.

Supplementary Material

Supplementary data are available at *Genome Biology and Evolution* online.

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Author Contributions

K.B. and S.K. performed whole-genome sequencing and submission to NCBI and analysis. K.B. drafted manuscript with inputs from P.B.P. and S.K. P.B.P. conceived and participated

in its design with inputs from K.B. All the authors read and approved the manuscript.

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