Article

A novel arsenate resistant determinant associated with the ICE pMERPH a member of the SXT/R391 group of mobile genetic elements

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Abstract: ICEpMERPH, the first integrative conjugative element [ICE] of the SXT/R391 family isolated in the United Kingdom and Europe was analyzed to determine the nature of its adaptive functions, its genetic structure and homology to related elements normally found in pathogenic Vibrio or Proteus species. Whole Genome Sequencing of *Escherichia coli* isolate KH802 [which contains the ICE pMERPH] was carried out using Illumina sequencing technology. ICEpMERPH has a size of 110 Kb and has 112 putative open reading frames [ORFs], the "Hotspot regions" of the element were found to contain putative restriction digestion systems, insertion sequences and heavy metal resistance genes that encode resistance to mercury, as previously reported but also surprisingly to arsenate. A novel arsenate resistance system was identified in Hotspot 4 of the element, and unrelated to other SXT/R391 elements. This arsenate resistance system is potentially linked to two genes, orf 69, encoding an organoarsenical efflux MFS transporter-like protein related to ArsJ and orf70, encoding an NAD-dependent glyceraldehyde-3-phosphate dehydrogenase. Phenotypic analysis using isogenic strains of AB1157 with and without the ICE revealed resistance to low levels of arsenate in the range 15-25 mM. This novel, low level resistance may have an important adaptive function in polluted environments, which often contain low levels of arsenate contamination. Bioinformatic analysis on the novel determinant and the phylogeny of ICEpMERPH is presented.

Keywords: Mobile Genetic Elements**,** Integrative Conjugative Elements [ICEs], R391, pMERPH, SXT

1. Introduction

Integrative conjugative elements [ICEs] are a class of bacterial mobile genetic elements characterized by their ability to facilitate their own integration, excision, and transfer from one host bacterial genome to another by a mechanism of site-specific recombination, self-circularization, and conjugative transfer [1]. They behave as mobile genomic islands that encode adaptive functions while encoding mechanisms for their mobility, integration and regulation. One of the largest and most studied families of ICEs are the SXT/R391 family. The SXT/R391 family has >160 members, the elements being identified experimentally or bioinformatically to date [2]. Type 1 elements have been found in a variety of *Vibrio* species as well as in other Gamma-proteobacteria including *Shewanella, Proteus* and *Photobacterium* species [3, 4]. They are distinguished by encoding a Type 1 integrase, which allows site-specific integration into the 5′ end of the essential *prf*C gene [5], however integration restores a functioning *prf*C gene and encodes a new hybrid *Prf*C protein once integrated [5]. Type 2, 3 and 4 ICEs are all inserted at the 3′ end of the multi copy tRNA-Ser gene and have been found in isolates of *Vibrio* species [3, 6]. The ICER391 was the first element of this group discovered in a *Providencia rettgeri* clinical isolate from South Africa, in 1967 [7] and encodes genes for resistance to kanamycin and mercury [3, 8].

Structurally the SXT/R391 family of ICEs contains in general 51 near identical core genes, many of which are involved in integration/excision, conjugative transfer and regulation of the ICEs [9- 13]. In addition to these core genes, all elements contain five hotspots [called HS1-5] and up to five variable regions [called VRI-V] where accessory genes, such as antibiotic resistance genes, heavy metal resistance genes or DNA repair genes, can be found inserted [14, 15].

SXT/R391 ICEs allow bacteria to adapt and survive in a variety of environmental niches by individually encoding various heavy metal resistance genes, antimicrobial resistance genes and bacteriophage defense genes. ICEs of the SXT/R391 family have been shown to encode resistances to various heavy metals including cobalt [Co], cadmium [Cd], mercury [Hg] and zinc [Zn], which in some cases is related to multi efflux systems as identified in ICE*Spu*PO1 [16]. This ICE encoded efflux pump was found to increase tolerance to divalent metal ions such as cadmium, zinc and cobalt by removing the metal ions from the cells and is a member of the integral membrane protein family [17, 18]. This ICE element was also found to contain a transcriptional regulator for arsenic [but no resistance determinant] and a mercury resistance operon located in variable region IV from ORF 83-88 [16]. Mercury resistance operons have been found in many SXT/R391 ICE elements the most notable, R391 isolated from *Providencia rettgeri* [8] and pMERPH isolated from *Shewanella putrefaciens* [19]. Mercury operons have also been discovered in ICE elements ICE*Vsp*Spa1 and ICE*Eni*Spa1 from marine aquaculture in Spain and ICE*Vsp*Por1 and ICE*Vsp*Por3 in Portugal [20] while several *Proteus mirabilis* isolates from China have also been found to contain mercury resistance determinants [21]. ICE*Sh*95 from a *Shewanella* species and ICEPrSt33672 from *Providencia stuartii* ATCC33772 both clinical isolates were also shown to encode a mercury operon [22, 23]. Complete arsenic operons have not been identified in SXT/R391 ICE elements but have been detected in several other types of unrelated ICE elements. ICE*Sde*3396, from *Streptococcus dysgalactlae* was identified in 2008 and contains a cadmium resistance and an arsenic resistance operon [24]. Several Tn*4371* like ICEs also have been found to contain arsenic resistance operons [25].

In this study, the nucleotide sequence of the SXT/R391 ICEpMERPH, originally identified in *Shewanella putrefaciens* from the River Mersey in 1987 [19], was determined and analyzed. It had previously been shown to encode a mercury resistance operon similar [19] to that encoded by ICER391 [8] but as limited analysis had been subsequently carried out on the element it was believed that this was the only resistance encoded. The aftermath of the industrial revolution left the water quality of the River Mersey poor and it became infamous as one of the most polluted rivers in Europe, both industrial and domestic effluents were discharged without treatment and there were unknown biological impacts resulting from a complex mixture of dangerous substances present in the river [26] at that time.

2. Materials and Methods

2.1. Bacterial strains

The bacterial strains and mobile genetic elements used in this study are listed in Table 1. Strains were stored at − 80 °C in Luria-Bertani [LB] broth containing 50% glycerol. For most experiments, *E. coli* cultures were grown aerobically in LB broth.

Table 1: Genotype of bacterial strains used in this study

Strain	**Genotype**	**Source**
AB1157	F-, *thr-1*, *araC14*, *leuB6*,Δ(*gpt-proA*)62, *lacY1*, *tsx-33*, *qsr'-0*, *glnV44*, *galK2*, *λ-*, *Rac-0*, *hisG4*, *rfbC1*, *mgl-51*, *rpoS396*, *rpsL31* (StrR), *kdgK51*, *xylA5*, *mtl-1*, *argE3*, *thi-1*	*E. coli* genetic stock center (CGSC), Yale University, New Haven, Connecticut, USA
K802	*F-, lacY1 or Δ(cod-lacI)6, glnX44(AS), galK2(Oc), galT22, λ-, e14-, mcrA0, rfbC1, metB1, mcrB1, hsdR2*	NCIMB
ICE	**Genotype**	**Source**

pMERPH	Hg^R	NCIMB

2.2. Phenotypic testing

ICEpMERPH was transferred to *E. coli* strain AB1157 via the method outlined in Murphy and Pembroke, 1995 [27]. Both AB1157 and AB1157ICEpMERPH were then tested for their susceptibility to two arsenic based compounds: Sodium Arsenate dibasic heptahydrate [$Na_2HAsO_4.7H_2O$] and Sodium [Meta] Arsenite [$NaAsO_2$]. A stock solution of 100 mM of Arsenate and 10 mM Arsenite was prepared. Dilutions of Arsenate and Arsenite were prepared using LB medium as a diluent. Initially concentrations of 50 mM, 40 mM, 30 mM 25 mM and 20 mM and 15 mM of Arsenate was tested. Arsenite concentrations of 1- 10 mM were also tested Microtitre plates were incubated overnight at 37 °C in a Biotek ELx808 Ultra microplate reader [Mason Technologies, Dublin], with readings taken every 30 min at 590 nm for the 18 h period.

2.3. Genome Sequencing and Annotation

The genome of *Escherichia coli* isolate K802 [which contains ICEpMERPH] was sequenced by MicrobesNG [University of Birmingham, Birmingham, UK] using paired-end [insert size between the ends 200-500 bp] HiSeq2000 Illumina technology giving approximately 30-fold coverage. The resulting reads were processed and assembled using MicrobesNG's own automated analysis pipeline. The pMERPH genome was identified amongst 72 contigs by using the BLAST tool to investigate the presence of several different ICER391 [AY090559] ICER997 and ICESXT [AY055428] core scaffold genes [*int, jef, traLEKBVA, setCD*]. The ICEpMERPH sequence was then annotated using the RAST Server [Rapid Annotation using Subsystem Technology] and the Basic Local Alignment Search Tool [BLAST] program at NCBI [28, 29]. Putative functions for all proteins were inferred using the Basic Local Alignment Search Tool [BLAST] [http://ncbi.nlm.nih.gov/BLAST] or InterPro Scan [https://www.ebi.ac.uk/interpro/]. All sequences with arsenic resistant genes were found in the NCBI database [https://www.ncbi.nlm.nih.gov/genbank/]. All molecular maps were created using the RAST Server [Rapid Annotation using Subsystem Technology] and using SnapGene Viewer [https://www.snapgene.com/snapgene-viewer/].

2.4. Phylogenetic Analysis of Core ICE genes

Phylogenetic analysis of ICEpMERPH was performed based on comparison with the concatenated amino acid sequences of 48 SXT/R391 core ICE gene encoded proteins on all previously sequenced whole SXT/R391 elements. These elements are listed in Supplementary Table 1. An unrooted phylogenetic tree was constructed by maximum-likelihood method based on the Poisson correction model using the MEGAX [30]. Bootstrap analysis with 1000 replications was performed to test the reliability of the tree.

2.5. Accession number

ICEpMERPH was submitted to GenBank under accession number **MH974755.**

3. Results

3.1. Phenotypic Testing

On identifying a putative arsenic resistance determinant based on the nucleotide sequencing of ICE pMERPH testing was carried out using AB1157ICEpMERPH and the isogenic strain, AB1157 to determine if resistance was phenotypically present. A range of different arsenate concentrations were tested and resistance was observed relative to isogenic controls including at low levels [15-25 mM] Growth analysis over an 18-hour period [Fig 1] at 25 mM revealed AB1157 was inhibited whereas the presence of the ICEpMERPH allow growth to continue. No resistance to arsenite was detected at any concentration

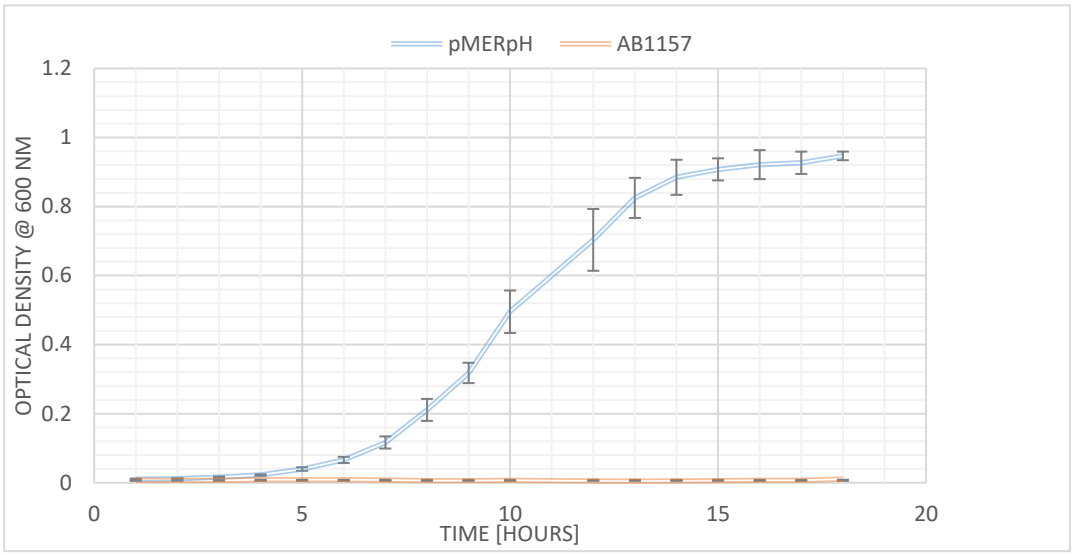

Figure 1: Growth curve of AB1157ICEpMERpH and AB1157 over an 18-hour growth period with 25 mM Arsenate supplemented into the LB Broth culture.

3.2. Full Sequence analysis

ICEpMERPH can be classified as a Type 1 SXT/R391 ICE based on comparative genomics of the newly determined nucleotide sequence. Analysis of the annotated sequence revealed the ICEpMERPH has 112 Open Reading Frames [ORFs] and follows the conserved synteny for "typical" Type 1 R391/SXT elements [Figure 1]. Fifty-one of these ORFs were predicted to code for the core scaffold of SXT/R391 elements [genes related to integration, excision, conjugative transfer and regulation] [3]. All other genes were found in the hotspots and variable regions of the ICEpMERPH genome [Figure 2].

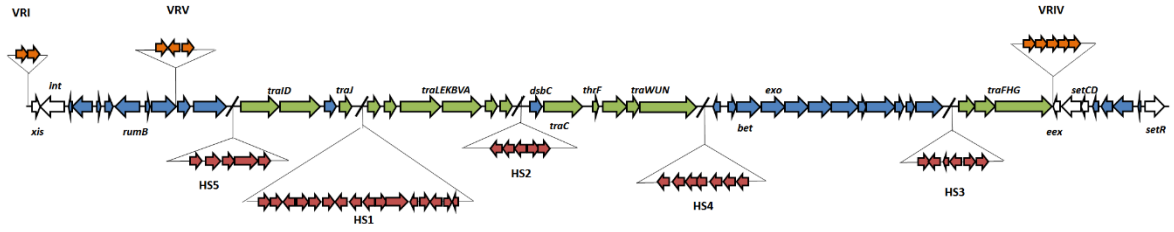

Figure 2: Molecular map of pMERPH displaying the location of genes associated with the 110 Kb mobile genetic element. Genes colored white are associated with excision, integration and control. Genes colored green are associated with transfer, all other core genes are colored blue. Genes associated with hotspot are colored red and those associated with variable regions are colored orange.

ICEpMERPH Hotspot 1 [HS1] contains the same 18 gene insertion as previously found in HS1 in ICE*Mpr*Chn1 [orf32 to orf47] [showing 94% to 100% nucleotide identity across all genes] [31]. The functions of most of these genes are unknown but predicted to encode hypothetical genes with no known functional homologs. There are several predicted transposases and predicted low level homology to a three-component efflux pump that could possibly confer a multidrug resistance phenotype. This putative efflux system shares similarities to the AcrAB efflux system [32]. In order to determine if any antimicrobial resistance could be related to this efflux pump a panel of antibiotics and the antibacterial triclosan were tested against AB1157ICEpMERPH. No increased level of resistance was detected at least with the drug panel used (Data not shown).

ICEpMERPH Hotspot 2 [HS2] contains 5 genes also of unknown function. The first three genes are highly similar to those found in HS2 of ICE*Vch*Mex01 [15]. Four of the five genes share similarity to those found in HS2 of ICE*Vpa*Can1 [4]. This is suggestive that ICE evolution has involved acquisition of similar genes and their retention over a wide geographical space and their maintenance is suggestive of adaptive or survival function. However, the lack of functioning characterized homology makes this still speculative.

The insertion in Hotspot 3 [HS3] is made up of six predicted ORFs. The hotspot encodes an interrupted mcrBC-like restriction digestion system. This system was originally discovered in *Escherichia coli* K-12 [33]. The *mcrB* gene is interrupted by the insertion sequence IS*Pst2*b. IS*Pst2*b is made up of three genes the first encodes an ISL-3 transposase, the second an ArsR-like transcriptional regulator and the second permease [34]. The function of this insertion is unknown. This structure is found also in Tn*6516* originating from *Achromobacter* spp and in ICE*Hs*1 an ICE found in *Histophilus somni* that encodes for antimicrobial resistance and metal tolerance [35]. Following this insertion is the rest of truncated *mcrB* gene and the whole *mcrC* gene. ICE*Pmi*Jpn1 containing the uninterrupted mcrBC restriction digestion system [36, 37].

The insertion in Hotspot 5 [HS5] codes for the putative type I restriction-modification system [RM] hdsRMS [38, 39] similar to that found in ICE*Vch*Mex01 [15]. These systems carry out DNA modification, recombination, and repair. They are composed of three polypeptides: R [restriction endonuclease], which recognizes and cuts specific DNA sequences; M [modification], which methylates the same sequence to inhibit DNA cleavage and protect the host cell against invasion of foreign DNA; and S [specificity], which determines the specificity of both R and M. [39]. These genes may confer protection against bacteriophage infection, as was demonstrated for other ICEs of the SXT/R391 family [38]. In both cases there is a gene inserted between *hdsS* and *hdsR*, in ICEpMERPH this is an anti-codon nuclease of unknown function.

ICEpMERPH contains no insertions in Variable Regions II and III. The element does however have insertions in Variable Region I, IV and V [VRI, VRIV, VRV]. The insertion in VRI is structurally identical to that found in the ICE R391. VR1 contains three genes including a putative hipAB-like TA system. This system improves stability of the element when integrated into the bacterial chromosome, ΔhipA mutants of R391 indicate that the ICE shows a 12-fold increase in loss from the host when compared to the wild-type [40]. VRIV contains a five-gene mercury resistance system *merRTPCA*. *merR* encodes a regulatory sequence, *merA* encodes a detoxifying oxido-reductase, while *merC*, *merT* and *merP* encode transport proteins. This system is also found in R391, ICE*Pmi*Chn2410 and ICE*Pmi*Chn2416 [8, 21, 41]. VRV contains four genes that share homology with VRV of ICE*Val*A056-2. The potential function of this variable region is unknown [42].

A phylogenetic tree [Figure 3] was constructed based on the concatenated amino acid sequences of all SXT-R391 core proteins for all published core genome sequences of these elements. The ICEpMERPH clustered with ICE*Ap*l2, which was an ICE, discovered in *Actinobacillus pleuropneumoniaei* MIDG3553 that was isolated from the pneumonic lung of a pig [43]. These results show the wide geographic spread of SXT/R391 like elements.

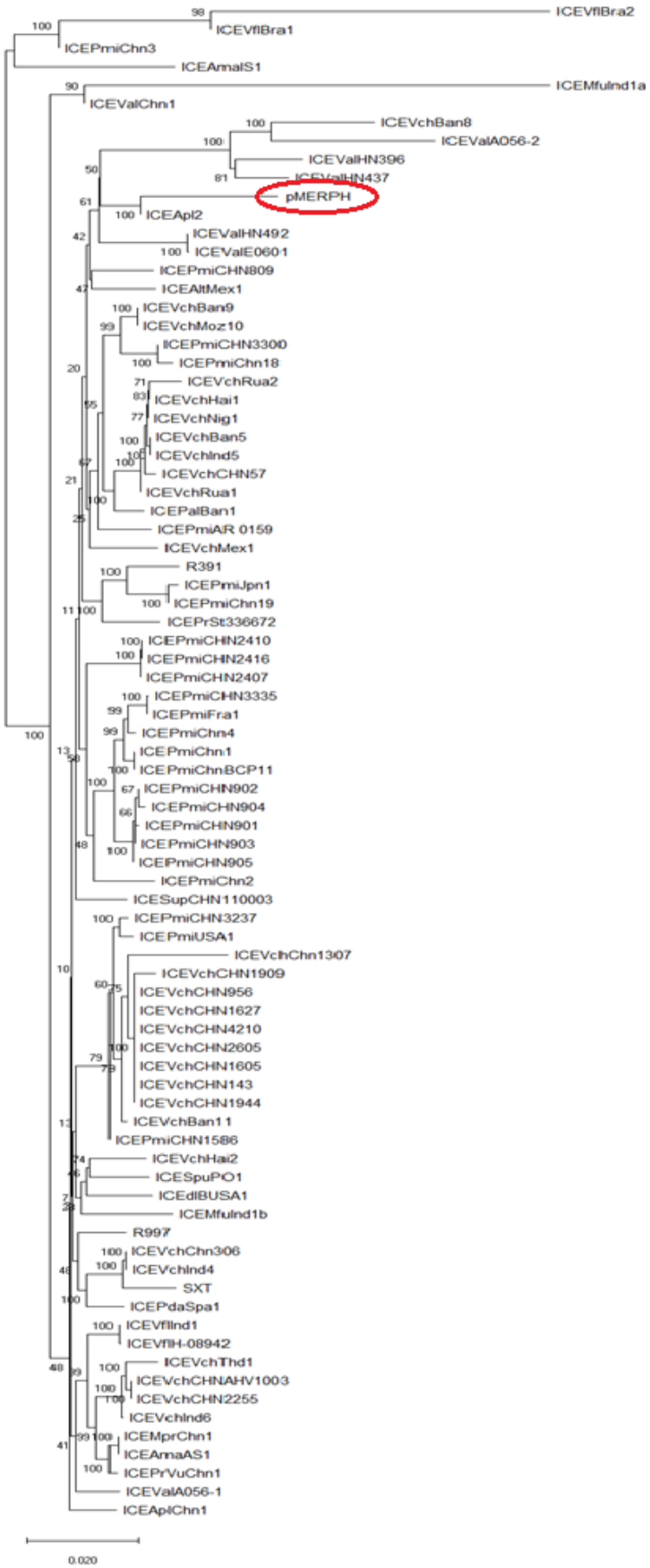

Figure 3: Phylogenetic tree from the maximum-likelihood analysis of the core concatenated proteins of 85 SXT/R391 ICEs including the newly annotated ICEpMERPH.

3.3. Genetic basis for Arsenic Resistance encoded by pMERPH

The insertion in Hotspot 4 [HS4] contains a predicted seven gene insertion. The first two genes encode for a putative arsenic resistance system that bears similarity to that found in *Pseudomonas aeruginosa* DK2. This detoxification pathway contains a two-gene system composed of *gapdh* and *arsJ*. The gene gapdh encodes a predicted glycolytic enzyme, glyceraldehyde-3-phosphate dehydrogenase [GAPDH], which is NAD+ dependent. Via this system inorganic As[V] is transformed into the highly unstable organoarsenical compound 1-arseno-3-phosphoglycerate [1As3PGA] [44]. 1As3PGA can be expelled from the cell by an efflux permease, ArsJ, where it rapidly dissociates into inorganic As[V] and 3-phosphoglycerate [3PGA] due to its short half-life in the natural environment [44].

These genes on ICEpMERPH are followed by a tyrosine phosphatase, and a thioredoxin protein. The function of the tyrosine phosphatase is unknown. In some arsenic resistance systems thioredoxin is frequently used in conjunction with ArsC enzymes [such the ArsC of *Staphylococcus aureus* pI258] as an electron source [45]. The next gene is an ArsP that codes for a methylarsenite [MAs[III]] efflux permease that extrudes trivalent organoarsenicals from cells, conferring resistance [46]. The next gene codes for an Acr3-like protein. Acr3 is an arsenite efflux pump can pump inorganic arsenite from cells. Acr3 proteins are found in bacteria, archaea, fungi and some plant species [47]. The final gene is this hotspot codes for an ArsR family transcriptional regulator. No *arsC* gene or similar arsenic reductase genes could be found in this hotspot. Near identical hotspots have been found in unidentified SXT/R391 ICEs in *Vibrio parahaemolyticus* species isolated from diseased seafood in Vietnam and Cambodia. This is first time to our knowledge that a system such as this has been identified in an SXT/R391 ICE mobile genetic element.

5. Conclusions

A novel arsenate resistant determinant was identified in ICEpMERPH. This is the first identified arsenate resistance determinant found in SXT/R391 ICE mobile genetic elements. This novel system gives the element an adaptive advantage that allows for survival in a heavy metal polluted environment and allows the spread of the element through the environment.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1,, Table S1: Identified ICESXT/R391 family members with complete genome sequence available,

Author Contributions: MPR designed the study, assisted with analyzing the data, and wrote the manuscript. SS provided assistance in designing experiments, preformed the experiments, assisted with analyzing the data, and contributed to the editing of the manuscript. JTP designed the study, assisted with analyzing the data, and contributed to the editing of the manuscript.

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