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Article

Analysis and comparative genomics of the SXT/R391 family ICE, pMERPH

Michael P Ryan [1]*, Shannon Slattery [1] and J Tony Pembroke [1, 2,]

[1] Department of Chemical Sciences, School of Natural Sciences, University of Limerick, Limerick, Ireland
[2] Bernal Institute, University of Limerick, Limerick, Ireland
* Correspondence: Michael.P.Ryan@ul.ie; Tel.: +353-61-234730

Abstract: The aim of this study was to analyse pMERPH, the first integrative and conjugative element (ICE) of the SXT/R391 family isolated in the United Kingdom and to determine its relationship to other members of the SXT/R391 family of ICEs. Whole Genome Sequencing of *Escherichia coli* isolate KH802 (which contains the ICE pMERPH) was performed using Illumina sequencing technology. pMERPH was evaluated by *de novo* assembly of the sequenced genome, via gene prediction and annotation tools, and phenotypic analysis via comparative genomics to other members of the SXT/R391 ICEs. pMERPH has a size of 110 Kb and has 112 predicted ORFs making it one of the bigger SXT/R391 ICE's thus far characterised. The "Hotspot regions" of the element were found to contain putative restriction digestion systems, insertion sequences and heavy metal resistance genes that give resistance to mercury and arsenate. pMERPH is closely related to the SXT-like elements from widely dispersed geographic areas. The sequencing of pMERPH increases the knowledge of the earliest isolated SXT/R391family members and may provide insight on the emergence of such elements.

Keywords: Integrative Conjugative Elements (ICEs), R391, pMERPH, SXT

1. Introduction

Integrative conjugative elements (ICEs) are a class of bacterial mobile genetic elements that are 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-circularisation, and conjugative transfer [1]. They can have a significant influence on the adaptive evolution of bacterial genomes as they allow bacteria to acquire new phenotypic traits and adaptive functions such as resistance to antimicrobial compounds, heavy metals, virulence determinants, metabolic pathways and the ability to resist bacteriophage infection, which bestows host survival [1, 2, 3, 4].

SXT/R391 ICEs are a family of proteobacterial chromosomally integrating mobile genetic elements that consist of four distinct types of elements [5]. By far the largest group of these elements are Type 1 elements, these possess a conserved integrase that mediates site-specific integration into the 5′ end of the *prfC* gene [6, 7]. Type 1 elements have been found in a variety of *Vibrio* species as well as in other Gamma-proteobacterial species including *Shewanella*, *Proteus* and *Photobacterium* species [8]. Type 2, 3 and 4 ICEs are all inserted at the 3′ end of the tRNA-Ser gene and have been found in isolates of *Vibrio* species [5].

The SXT/R391 family of ICEs is one of the largest of the ICE families with >160 elements being identified experimentally or bioinformatically to date [9]. R391 was the first element of this group discovered in a *Providencia rettgeri* clinical isolate from South Africa, in 1967 [10]. The R391 ICE encodes genes that give resistance to kanamycin and mercury [11, 12]. In late 1992, the related SXT^{MO10} element was discovered in one of the initial pandemic *Vibrio cholerae* O139 clinical isolates from Chennai, India [13]. SXT^{MO10} is a 100 kb element that carries genes encoding resistance to sulfamethoxazole, trimethoprim, chloramphenicol, and streptomycin [14].

This family of ICEs contains approximately 51 near identical core genes, many of which are involved in integration/excision, conjugative transfer and regulation of the ICEs [15, 16, 17, 18]. In

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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 without disrupting essential ICE genes [1, 2, 19]. These elements can also promote the mobilisation of non-transmissible genomic islands and virulence plasmids between hosts [20].

The first complete SXT/R391 ICE reported in *Shewanella* species was ICE*Spu*PO1, derived from *S. putrefaciens* W3-18-1, which was isolated from the Pacific Ocean over 15 years ago [18]. pMERPH was the first (and to the best of our knowledge only) environmental SXT/R391 element to be isolated in the United Kingdom. It was identified in 1987 in *Shewanella putrefaciens* that was isolated from the Mersey River and found to encode a mercury resistance operon [21]. In this study the element was sequenced to gain knowledge of the genetic structure of one of the earliest isolated SXT/R391 elements and examine their emergence in a global setting.

2. Results and Discussion

Based on comparative genomics we determined that pMERPH is an SXT/R391-family ICE of the Type 1 variety.. It 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) [22]. All other genes were found in the hotspots and variable regions of the pMERPH genome (Figure 1).

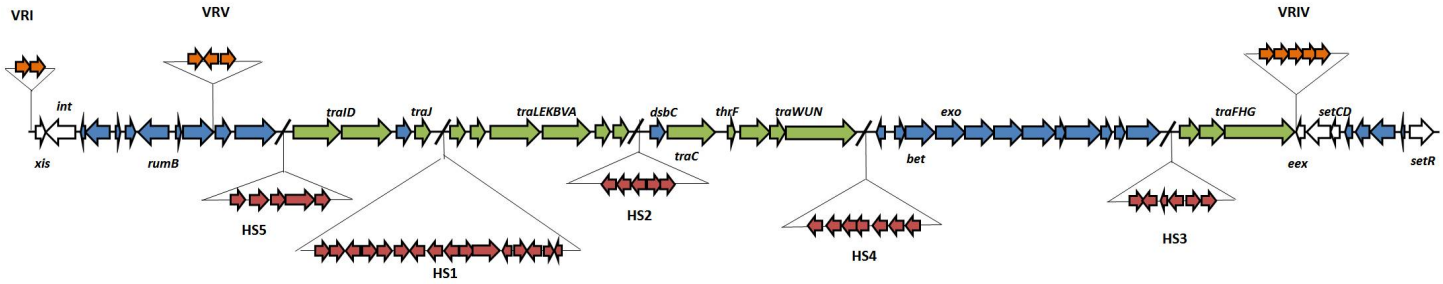

Figure 1: Molecular map of pMERPH, based on annotated nucleotide sequencing, displaying the location of genes associated with the 110 Kb mobile genetic element. Genes coloured purple are associated with excision, integration and control. Genes coloured white are associated with excision, integration and control. Genes coloured green are associated with transfer, all other core genes are coloured blue. Genes associated with hotspot are coloured red and those associated with variable regions are coloured orange.

pMERPH Hotspot 1 (HS1) contains the same 18 gene insertion as previously reported in HS1 of ICE*Mpr*Chn1 (orf32 to orf47) (showing 94% to 100% nucleotide identity across all genes) [23]. 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 [24]. In order to determine if any antimicrobial resistance could be related to this efflux pump a panel of antibiotics (see Materials and Methods) and the antibacterial triclosan were tested against AB1157pMERPH. No increased level of resistance was detected at least with the drug panel used.

pMERPH 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 [14]. Four of the five genes share similarity to those found in HS2 of ICE*Vpa*Can1 [5]. This is suggestive that ICE evolution has involved acquisition of similar genes and their retention over a wide geographical space and their maintenance is

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suggestive of as yet unknown adaptive or survival function. However the lack of functioning characterised 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 [25]. The *mcrB* gene is interrupted by the insertion sequence IS*Pst*2b. IS*Pst*2b is made up of three genes the first encodes an ISL-3 transposase, the second an ArsR-like transcriptional regulator and the third a permeasae [26]. 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 [27]. 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 [28, 29].

The insertion in Hotspot 4 (HS4) contains a predicted seven gene insertion. The first two genes encode for a putative as yet unreported arsenic resistance system that bears similarity to one found in *Pseudomonas aeruginosa* DK2. This detoxification pathway contains a two-gene system composed of *gapdh* and *arsJ*. The *gapdh* gene encodes a predicted glycolytic enzyme, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which is NAD+ dependent. Via this system inorganic As(V) can be transformed into a highly unstable organoarsenical compound called 1-arseno-3-phosphoglycerate (1As3PGA) [30]. 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 [30]. The other five genes in this hotspot encode for a tyrosine phosphatase, a thioredoxin protein, an ArsP like permease protein, an Acr3 like protein and an ArsR family transcriptional regulator. These all appear to be related to a type of arsenic resistance system the function of which is suggestive of a novel as yet uncharacterised mechanism. We examined the functioning of a pMERPH arsenic resistance determinant using AB1157pMERPH and AB1157. From observing the data (see Supplementary Data), in low concentration arsenate environments AB1157pMERPH was able to adapt and continue to grow whereas with AB1157, the arsenate acts as a bacteriostatic agent, that arrests the growth of the bacteria but it does not kill the bacteria. This would make makes strains harbouring pMERPH a more dominant strain in terms of survival in an arsenic contaminated environment. At higher concentrations (25 mM) AB1157pMERPH was found to continue growing whereas AB1157 does not after 18 hours. This adaptive role in arsenic contaminated environments may indeed be the rationale for ICE pMERPH maintenance or acquisition.

The insertion in Hotspot 5 (HS5) codes for the putative type I restriction-modification system (RM) hdsRMS [31] similar to that found in ICE*Vch*Mex01 [14]. These systems carry out DNA modification, recombination, and repair and 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. [31] These genes may confer protection against bacteriophage infection, as was demonstrated for other ICEs of the SXT/R391 family [32]. In both cases there is a gene inserted between *hdsS* and *hdsR*, in pMERPH this is an anti-codon nuclease of unknown function.

pMERPH contains no insertions in Variable Regions II and III as has been reported in other ICEs. 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, Δ*hip*A mutants of R391 demonstrate that the ICE shows a 12 fold increase in loss from the host when compared to the wild-type [33]. VRIV contains a five gene mercury resistance system *merRTPCA*. *merR* encodes a regulatory sequences, *merA* encodes a detoxifying oxido-reductase, while *merC*, *merT* and *merP* encode transport proteins. This system is also found in R391, ICE*Pmi*Chn8 and ICE*Pmi*Chn9 [34, 35, 36]. VRV contains four genes that share homology with VRV of ICE*Val*A056-2. The potential function of this variable region is unknown.

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A phylogenetic tree (Figure 2) 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 ICE pMERPH clustered with ICE*Apl*2 which was an ICE discovered in *Actinobacillus pleuropneumoniaei* MIDG3553 which was isolated from the pneumonic lung of a pig [37]. These results show the wide geographic spread of SXT/R391 like elements.

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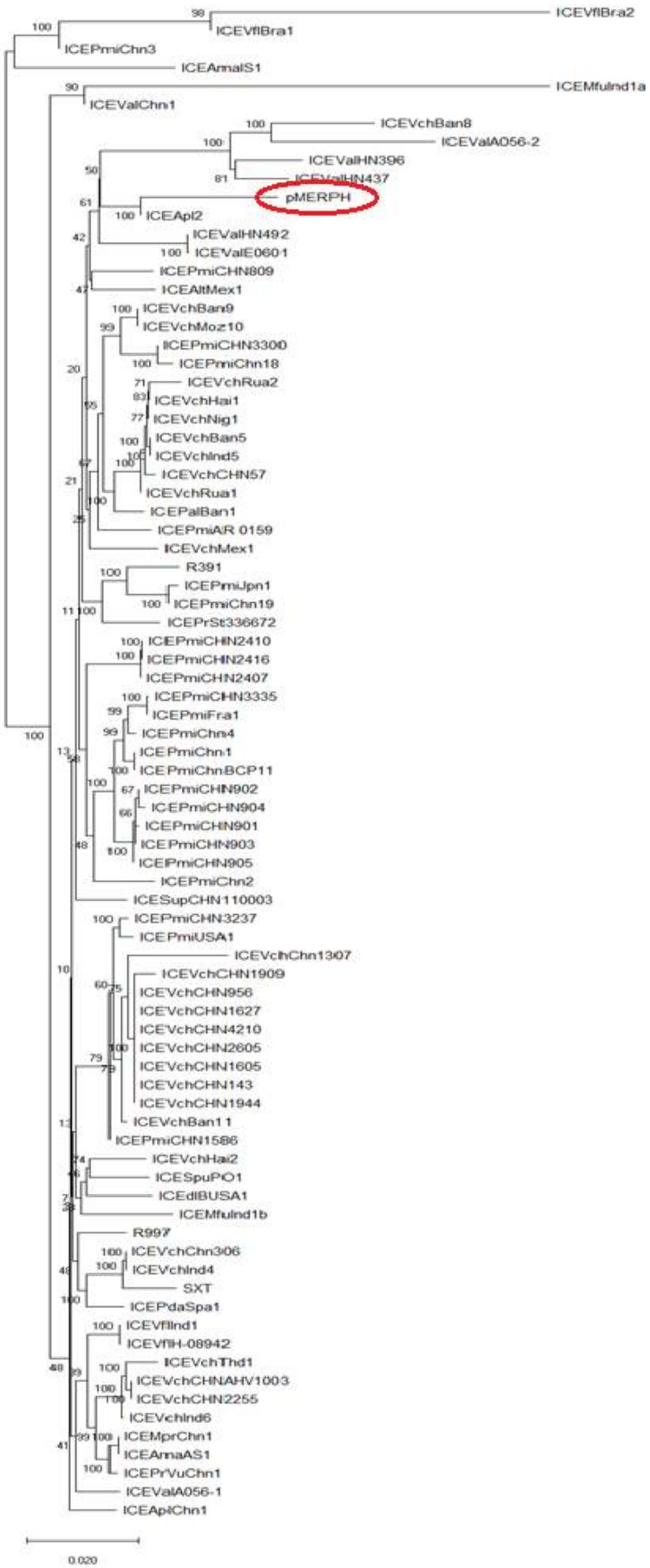

Figure 2: Phylogenetic tree from the maximum-likelihood analysis of the core concatenated proteins of 85 SXT/R391 ICEs.

3. Materials and Methods

3.1 Genome Sequencing and Annotation

The genome of *Escherichia coli* isolate K802 (which contains pMERPH) 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 R391 (AY090559) and SXT (AY055428) core scaffold genes (*int*, *jef*, *traLEKBVA*, *setCD*). The pMERPH sequence was then annotated using the RAST Server (Rapid Annotation using Subsystem Technology) and the Basic Local Alignment Search Tool (BLAST) program at NCBI [38, 39]. 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/). pMERPH was submitted to GenBank under accession number MH974755.

3.2 Phylogenetic Analysis of Core ICE genes

Phylogenetic analysis was performed based on the concatenated amino acid sequences of 48 SXT/R391 core genes encoded proteins on all 85 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 [40]. Bootstrap analysis with 1000 replications was performed to test the reliability of the tree.

3.3 Phenotypic testing

pMERPH was transferred to *E.coli* strain AB1157 via the method outlined in Murphy and Pembroke, 1995 [41]. Both AB1157 and AB1157pMERPH were then tested for their susceptibility to low arsenic based compounds: Sodium Arsenate dibasic heptahydrate and Sodium (Meta) Arsenite to determine if the newly identified putative *ars* operon in Hotspot 4 could provide resistance to arsenic compounds. A stock solution of 100 mM of both Arsenate and Arsenite was prepared. Dilutions of both Arsenate and Arsenite were prepared by using LB Broth as a diluent in 50 ml incubation tubes. Overnight broth cultures of AB1157pMERPH and AB1157 were added to the tubes, 50 µl in each. Initially concentrations of 50 mM, 40 mM, 30 mM and 20 mM of both Arsenate and Arsenite were tested. Lower range concentrations of Arsenate were also tested (15 mM, 20 mM and 25 mM) were tested in triplicate over an 18- hour incubation period at 37°C at 200 rpm. Optical density was measured at 600 nm for each sample.

Both strains were also tested against a panel of antibiotics using the EUCAST and /or CLSI disk diffusion methods. This list of antibiotics can be seen in Supplementary Data.

3. Conclusions

pMERPH was the first (and so far only reported) environmental SXT/R391 element identified in the United Kingdom and has not been previously sequenced. This element contains features found in a variety of SXT/R391 elements from around the globe confirming and illustrating the mosaic nature of these elements. pMERPH demonstrates that gene sequences are emerging all over the globe that appear to have at one time been acquired by primordial SXT/R391 ICE elements. Many of these sequences are uncharacterised and do not appear to have functional homologs that have as yet been characterised such that the adaptive function remains obscure and requires further characterisation. The sequencing of pMERPH increases the knowledge of the earliest isolated SXT/R391 elements and provides insight on the emergence of these elements globally.

Supplementary Materials: Supplementary materials can be found at www.mdpi.com/xxx/s1.

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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Conflicts of Interest: The authors declare no conflict of interest.

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