Conservation of Mercury Resistance determinants amongst ICE-like mobile bacterial genetic elements: comparative analysis and dissection of function

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Enterobacterial Integrative Conjugative Elements (ICEs) are now detected regularly as carriers of drug resistance and adaptive functions in clinical, animal and environmental bacterial isolates. The elements, which appear as conjugative phage/ plasmid- like hybrids, have a core genetic structure comprising of integrative, regulatory and conjugative transfer functions. Several integrative hotspots within the elements have been characterised where genes from host organisms or transposon-associated elements integrate resulting in evolution of the ICE element itself. One such ICE integrating hotspot, originally characterised in the ICER391, contains a mercury resistance determinant. Bioinformatic analysis of several ICE elements has revealed the conservation of similar mercury resistance determinants amongst ICE elements from a wide geographical origin. Here we compare and characterise this ICER391 mercury resistant determinant and use molecular and bioinformatic analysis to delineate its structure, possible origin and function.

1. Introduction

Hg^{2+} mercury is a toxic heavy metal ranked sixth among the top ten hazardous elements (1). Although amongst the rarest elements (ranked 16^{th}), its levels have risen due to industrial and environmental activities. Anaerobic bacteria are capable of converting inorganic mercury to the more toxic organo-mercuric methylated form (2). As life evolved mercury may have had a major effect on selecting resistance due to its affinity for sulphydryl groups on proteins and hence its antagonistic effect on protein function (3, 4). This may have been the driving force for the emergence of resistance. In nature mercury, in addition to methyl mercury accumulation, can be found as by-products of chemical processing such as seed dressing, gold recovery and it also exists as cinnabar ores which can enter aquatic environments via leaching while there may also be significant reservoirs of dissolved gaseous mercury from reduction processes in soils (5). Resistance to mercury was first reported in the 1960s (6) and many bacteria and Archaea possess resistance determinants that reduce and partition inorganic mercury, Hg^{2+}, to the gaseous, Hg^0 phase. Indeed, five mechanisms of mercury resistance have been identified. 1) Reduced uptake by organisms via effecting cellular permeability, 2) Sequestration of mercury, 3) Demethylation of methyl mercury 4) Mercury methylation and 5) Enzymatic reduction (7). Much work has been carried out on the mercury resistance operons carried on the transposons Tn*21* and Tn*501*, which are utilised extensively as models when comparing other encoded *mer* operons (8).

2. *mer* Resistance Determinants and function of encoded proteins

Resistance to mercury is frequently associated with bacterial mobile genetic elements, is widespread in nature and is often encoded by the genes of the *mer* operon or Hg resistance system. The *mer* operon of the transposons, Tn*501*and Tn*21*, being amongst the most studied of representative systems. Two classes of mercury resistant determinant exist, one offering narrow spectrum resistance to inorganic mercury salts and a second type offering additional resistance to organomercurials (1). Enzymatic detoxification can be carried out by one of two enzymes MerA, a Flavin disulphide oxidoreductase, for inorganic compounds and MerB, a periplasmic organomercury lyase, for organic mercurial compounds. The mechanism for inorganic mercury detoxification involves essentially reductive detoxification via an NADPH-dependent flavin adenine dinucleotide-containing disulphide oxidoreductase which catalyses reduction of inorganic Hg^{2+} ions to Hg^0. This gaseous product is somewhat less toxic and volatilizes through the cell membrane of hosts that carry the determinant. The oxidoreductase is encoded by a conserved *mer*A locus, whose gene product; MerA requires ancillary genes for mercury uptake and transport (*mer*P, *mer*T and *mer*C) and regulation of the process (*mer*R and *mer*D). The transport proteins are generally encoded by *mer*T and *mer*P, however in many systems additional transport activities encoded by *mer*F, *mer*G and *mer*E may be present (4, 5, 9). The MerP protein, an extracellular Hg ion binding protein, and MerT a Hg transport protein have been shown to be associated with the mercury uptake system and they in conjunction with Mer C, E, F, G, when present transport Hg^{2+} to the cytoplasm for oxido-reduction via MerA (see Figure 1). MerE has been reported to be able to transport both inorganic and organic mercuric compounds (10).

Many mercury resistant bacteria are resistant to a broad range of mercuric compounds with the ability to detoxify a range of organic mercuric compounds such as phenylmercuric acetate. The *mer*B gene, which is not always present on *mer* operons and often ancillary, encodes an organomercury lyase, MerB (11), the key enzyme for the detoxification and bioremediation of the organomercurial compound (see Figure 1). Once the substrate is processed by MerB it is ultimately

volatilised by MerA (12). Although there are few differences between Gram-positive and Gram-negative *mer* operons, it has been reported the *mer*B occurs more frequently in Gram-negative operons (13). The active site of MerB contains conserved cysteine residues, which bind Hg at the active site (14). When both MerB and MerA are present, their activity results in broad-spectrum resistance to mercury. In the absence of the *mer*B gene, a periplasmic protein MerG, encoded by the *mer*G gene in Gram-negative organisms can provide resistance to organomercurials, suggesting some redundancy within the system (13)

MerR is a key transcriptional regulator of the *mer* resistant determinant and in the presence of Hg^{2+} binds Hg^{2+} and activates its own transcription as well as that of the other *mer* genes. In the absence of Hg^{2+}, MerR binds tightly to an operator within the *mer* cluster and represses the system (15). MerR binds to the merOP operator region as a dimer. In the presence of $Hg^{2+,}$ a single atom of mercury is bound per dimer, which induces DNA unwinding promoting expression of the operon (16). In some systems, a second regulatory gene, *mer*D, may be present whose product MerD binds to the MerR operator and down-regulates the system (17). It has been postulated that MerD may displace bound Hg-bound MerR from the mer operator allowing new synthesis of fresh MerR able to switch off the induction of the *mer* genes when the external mercury is exhausted (18) thus allowing re-establishment of the repressed state and being energy efficient in terms of gene usage.

Many *mer* operons are found associated with mobile elements and indeed are often transposon encoded. These transposons encoded *mer* systems are frequently of the inducible type and regulated by *mer*D and *mer*R gene products. Several MerA (the key oxidoreductase) variants have been detected in different organisms and it has been suggested that they vary in terms of the mercury removal efficiency, which can be locus and genera specific. This MerA variability amongst different loci has been correlated with binding efficiency between MerA-NADPH complex and Hg^{2+} in resistant bacteria (19). The conservation of the *mer*A locus and the function of the MerA protein make this a key tool for phylogenetic and comparative studies.

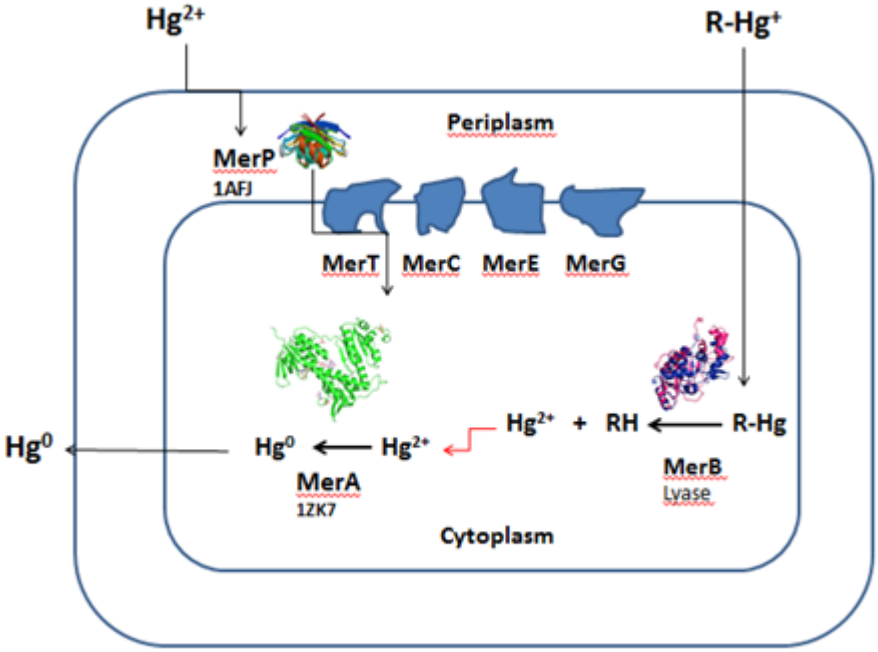

Fig. 1 Summary of the biological processing of inorganic and organo mercuric compounds in Gram-negative bacteria. Inorganic mercury compounds are bound by MerP in the periplasm and transported by MerT (or in some systems other inner membrane transporters e.g. MerC, MerE or MerG to the cytoplasm. Here they undergo oxidoreduction via the Flavin containing MerA oxidoreductase to Hg^0 that is volatile and volatilises from the cell. Organo mercurial compounds $R\text{-}Hg^+$ are detoxified in the cytoplasm via the MerB lyase releasing Hg^{2+}, which is then processed by MerA.

3. Variants and Mosaics of *mer* operons

Many types and variations of *mer* determinants have been shown to exist. In a study of faecal bacteria (9) found 185 strains carrying nine polymorphic *mer* loci. *mer*B was only present occasionally suggesting these strains encoded narrow spectrum mercury resistance. Some strains additionally lacked *mer*D suggesting a lack of downregulation within this particular system while others lacked *mer*C perhaps suggesting an altered uptake system. Mindolin *et al*., found 29 *mer* determinants amongst environmental isolates of which nine were related to Tn*21*, twelve were related to Tn*5053*, four to Tn*5041* and one to Tn*5044* (20). Sequencing revealed that many of these determinants were chimeras of recombination events between various *mer*-encoding elements. These data support the hypothesis that *mer* operons in general are genetic

mosaics with insertions and deletions of functional genes before and after the *mer*A gene possibly via the participation of *chi* recombination sites (9). Indeed, the prototype *mer* transposon Tn*21* appears to have evolved by the insertion of an In2 ancestor, which lacked IS*1353* into the *urf2M* gene of a hypothetical mercury resistance transposon, to generate Tn*21*. Such an event may also have generated Tn*1696* associated with plasmid R1033, where In4 inserted at the *res* site of a Tn*5036*-like *mer* transposon (21, 22, 23).

4. ICE SXT/R391 Elements and *mer* operons

Integrative conjugative elements (ICEs) are a class of bacterial integrating elements (24, 25, 26) that transfer widely amongst bacterial hosts carrying adaptive genes. The SXT/R391 group of enterobacterial ICEs are amongst the best studied of such elements (27, 28) Sequencing of the ICE R391 (29) an integrative conjugative element (30) revealed it contained a mercury resistance determinant similar in structure to Tn*21*. The element, which was one of the first ICEs sequenced, contained other adaptive functions such as antibiotic resistance and an unusual UV sensitising function (27, 31). Many other members of the SXT/R391 group have since been sequenced making data available for comparative and genetic analysis.

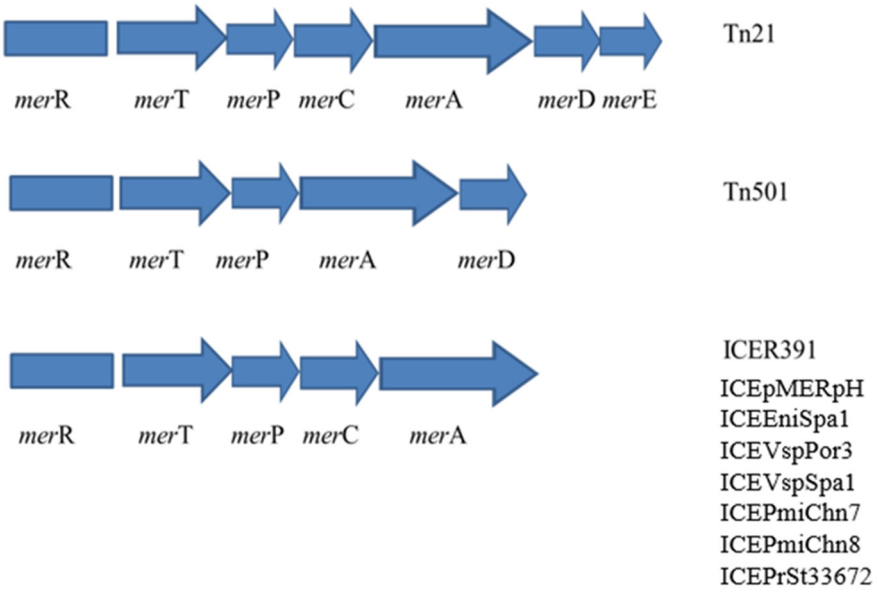

Fig. 2 Comparison between the structures of prototype *mer* operons associated with Tn*21*, Tn*501* and *mer* operon associated with the prototype SXT/R391 ICER391 and other ICE group elements. *merR* and *merD* encode regulatory sequences, *merA* encodes the detoxifying oxido reductase, while *merT*, *merP* and *merE* encode transport proteins (see Figure1) (1, 10, 29).

Osborn *et al* , characterised the control of a *mer* determinant from the ICEpMERPH (then thought to be a plasmid but since found to be a member of the SXT/R391 ICE family) (23, 33, 34) and demonstrated that the *mer* promoter (termed PTCPA) could be repressed by MerR, which was shown to be inducible by Hg^{2+} ions. This indicated that the regulation was similar to classic *mer* determinants such as the Tn*21* elements. However, sequence determination also revealed that the ICE determinant was quite divergent showing less than 60% similarity with known determinants at that time, while phylogenetic analysis revealed that the ICEpMERPH encoded one of the most divergent Gram-negative determinants characterised (32). Here we compare the structure of ICE SXT/R391 encoded *mer* determinants known to date utilising bioinformatics and phylogenetic analysis.

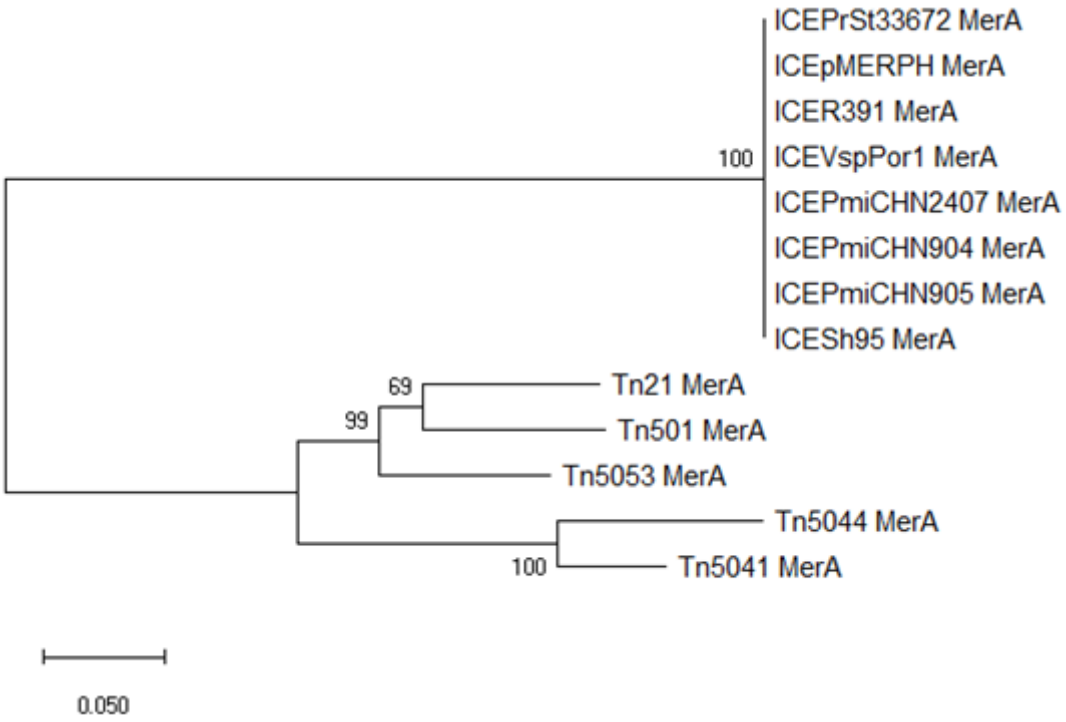

Fig. 3 Comparative phylogenetic analysis of the MerA oxidoreductase protein present within a number of SXT/R391-like ICE elements and prototype transposon expressing elements Tn*21*, Tn*501* and homologs. The analysis was carried using the MEGA phylogenetic analysis programme (35)

Analysis of the MerA protein from ICER391 identified similar proteins in several other complete ICE SXT/R391 elements in the Genbank nucleotide database (these can be seen in Table 1). Further analysis of the MerA protein from these ICE SXT/R391 elements indicated that they are related to *mer* operons found on plasmids in several Gamma proteobacterial species including *Paraglaciecola agarilytica*, *Glaciecola* sp. and *Salmonella enterica*. These *mer* operons are associated with transposons or transposon related sequences. Phylogenetic analysis indicated that all MerA proteins (Figure 3) from ICE SXT/R391 elements grouped closely together while those from the archetypical *mer* operons (Figure 3) grouped separately indicating the divergence of SXT/R391 *mer* operons. All *mer* operons from R391/SXT elements currently known possessed the same gene synetey as can be seen in Figure 2.

Table 1 ICE's of the SXT/R391 group containing *mer* determinants, associated with different bacterial hosts, relative accession number, isolation location, size and accessory genes of the ICE. Hg^r: Mercury resistance, Tc^r: Tetracycline resistance, RMS: Restriction modification system, Co/Zn/Cd; Cobalt, Zinc, Cadmium resistance. Km^r; Kanamycin resistance.

Bacteria	ICE	Environment	Location Year	Accessory genes	Accession Number	Size (kb)	Ref.
Providencia rettgeri	ICER391	Clinical/ human faeces	South Africa 1967	Km^r, Hg^r,	AY090559	88	29
Pseudomonas putrefaciens	ICEpMERPH	Marine Estuary	Mersey 1987	Hg^r	Z49196	109	N/A
Enterovibrio nigricans VA8	ICE*Eni*Spa1	Marine aquaculture (Fish)	Spain 2008	Hg^r	HE577626	15 (partial sequence)	36
Vibrio splendidus V69	ICE*Vsp*Por3	Marine aquaculture (Fish)	Portugal 2007	Tc^r, Hg^r, RMS	HE646881	27 (partial sequence)	36
Vibrio splendidus ZF2	ICE*Vsp*Spa1	Marine aquaculture (Fish)	Spain 2009	Tc^r, Hg^r	HE577630	26 (partial sequence)	36
Proteus mirabilis 09MAS2410	ICE*Pmi*Chn8	Clinical	China 2008-2015	Hg^r, Amp^r RMS, Co/Zn/Cd resistance,	KX243406	93	37
Proteus mirabilis 09MAS2416	ICE*Pmi*Chn9	Clinical	China 2008-2015	Hg^r, Amp^r RMS, Co/Zn/Cd resistance	KX243407	94	37

Providencia stuartii ATCC33672	ICE*PrSt*33672	Unknown	Unknown	Hg^r	CP008920	76	38

5. Observations on ICE-like *mer* operons

In terms of conservation, the *mer*C gene is present in Tn*21* and in the ICER391; however, the gene appears to be dispensable as it is not present in Tn*501* yet both Tn*21* and Tn*501* confer the same level of Hg^{2+} resistance (1, 39). While the prototype *mer* operons associated with Tn*21* and Tn*501* possess a second regulatory gene *mer*D this is absent in ICER391. MerD binds to the operator region of *mer* operons as does *mer*R but more weakly. As suggested the presence of MerD may control the re-repression of the operon once mercury is exhausted or all the detoxification has been completed (18). The presence of the *mer*D gene on Tn*21* and Tn*501* may thus add to their efficiency and be somewhat of an energy saving device in terms of limiting wasteful gene expression. Its absence in ICER391 thus may suggest that it is more of a utility operon and that the subtleties of expression are not needed. Equally not having extra genes may be energy efficient if not absolutely needed in this case. The absence of *mer*B associated with any SXT/R391 ICE characterised to date indicates that the encoded operon handles inorganic mercury with no ability for organo mercury detoxification. Indeed, *mer*B seldom occurs in gram-negative bacteria (1) in which ICE-like elements of the SXT/R391 group predominate.

Eight SXT/R391 ICE elements (Table 1) have been shown by nucleotide sequence determination to contain *mer* determinants. Interestingly the *mer* determinants for all these ICE elements is similar in terms of gene synteny (Figure 2). Comparative bioinformatics analysis indicates that the *mer* determinant is very similar and that the *mer* determinant is located within ICE variable region 4 in all cases. This itself is unusual given the global distribution of the SXT/R391 ICE elements that contain the *mer* operon, ranging from the ICE R391 detected in South Africa, ICEpMERPH detected in the Mersey in the UK, marine determinants detected in Spain and Portugal and ICEs detected in China, illustrative of the global distribution of ICE elements (Table 1). In addition, the date of isolation of SXT/R391 ICEs ranges from 1969 (ICER391) to 2015 (ICE*Pmi*Chn9).

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