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Novel Tn4371-ICE like element in *Ralstonia pickettii* and Genome mining for comparative elements

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Abstract

Background

Integrative Conjugative Elements (ICEs) are important factors in the plasticity of microbial genomes. An element related to the ICE Tn4371 was discovered during a bioinformatic search of the *Ralstonia pickettii* 12J genome. This element was analysed and further searches carried out for additional elements.

A PCR method was designed to detect and characterise new elements of this type based on this scaffold and a culture collection of fifty-eight *Ralstonia pickettii* and *Ralstonia insidiosa* strains were analysed for the presence of the element.

Results

Comparative sequence analysis of bacterial genomes has revealed the presence of a number of uncharacterised Tn4371-like ICEs in the genomes of several β and γ-Proteobacteria. These elements vary in size, GC content, putative function and have a mosaic-like structure of plasmid- and phage-like sequences which is typical of Tn4371-like ICEs. These elements were found after a through search of the GenBank database. The elements, which are found in *Ralstonia, Delftia, Acidovorax, Bordetella, Comamonas, Acidovorax, Congregibacter, Shewanella, Pseudomonas Stenotrophomonas, Thioalkalivibrio* sp. HL-EbGR7, *Polaromonas, Burkholderia* and *Diaphorobacter* sp. share a common scaffold. A PCR method was designed (based on the Tn4371-like element detected in the *Ralstonia pickettii* 12J genome) to detect and characterise new elements of this type.

Conclusions

All elements found in this study possess a common scaffold of core genes but contain different accessory genes. A new uniform nomenclature is suggested for ICEs of the Tn4371 family. Two novel Tn4371-like ICE were discovered and characterised, using the novel PCR method described in two different isolates of *Ralstonia pickettii* from laboratory purified water.
Background

Integrative Conjugative Elements (ICEs) carry functional modules involved in their conjugative transfer, chromosomal integration and for control of expression of ICE genes [1]. ICEs are maintained in their host via site-specific integration and establishment at a unique site or sites in their host [2, 3, 4, 5, 6, 7]. ICEs have been discovered in the genomes of various low G+C Gram-positive bacteria, various α, β- and γ-Proteobacteria, and Bacteroides species [8]. The first ICE found was Tn916 from Bacteroides species [8].

One of the best models of ICEs is a family of elements called the R391\SXT family that are found in γ-Proteobacteria. These are interesting elements as over 25 have been found to date in organisms spread across the world. They share a common core scaffold of genes related to integration, excision, transfer and regulation. Different elements can possess different fitness determinants such as antibiotic resistances, heavy metal resistances, and error-prone DNA repair systems [9].

Tn4371 is a 55-kb ICE, which allows its host to degrade biphenyl and 4-chlorobiphenyl. It was isolated after mating between Cupriavidus oxalaticus (Ralstonia oxalatica) A5 carrying the broad-host-range conjugative plasmid RP4 and Cupriavidus metallidurans (Ralstonia metallidurans) CH34. Selection was applied for transconjugants that expressed the heavy metal resistances from CH34 and grew with biphenyl as a sole source of carbon and energy [10]. The transconjugants carried an RP4 plasmid with a 55-kb insert near its tetracycline resistance operon. The insert was shown to transpose to other locations and hence was called Tn4371 [10, 11, 12]. Tn4371 has been sequenced [13] and closely related elements have been found in the genome sequences of a number of bacteria including Ralstonia solanacearum GMI1000, a phytopathogen from French Guyana [14], Cupriavidus metallidurans CH34, a heavy metal resistant bacteria from Belgium [15], Erwinia chrysanthemi 3937, a phytopathogen [16] and Azotobacter vinelandii AvOP, a nitrogen-fixing bacterium isolated from soil in the USA [13, 17]. None of these other elements possessed the biphenyl and 4-chlorobiphenyl degradation genes.
The Tn4371-like ICEs characterised to date are mosaic in structure consisting of Ti-RP4-like transfer systems, an integrase region, plasmid maintenance genes and accessory genes [13]. All the characterised elements integrate into sites on the bacterial genomes with a conserved 5'-TTTTTCAT-3' sequence, termed the \textit{att}B site [11]. Tn4371 transposition most likely involves a site-specific integration/excision process, since the ends of the element can be detected covalently linked as a transfer intermediate [11, 13]. Integration is catalysed by a tyrosine based site specific recombinase related to bacteriophage and ICE family integrases [18].

A small number of putative ICEs have been discovered following sequence analyses of genomes of various low G+C Gram-positive bacteria [19], various \(\alpha\), \(\beta\) and \(\gamma\)-Proteobacteria [20, 21, 22], and \textit{Bacteroides} species [23].

We now report the discovery and comparative analysis of a number of novel uncharacterised Tn4371-like ICEs from several different bacterial species. These elements are also mosaics of plasmid and other genes and posses a common scaffold with apparent hotspots containing insertions of different presumably adaptive genes. Using sequences from the common scaffold a PCR method was developed to discover and characterise new Tn4371-like ICEs in different bacteria. Here we report on the use of this method to discover and characterise two new Tn4371-like ICEs in \textit{Ralstonia pickettii} strains isolated from a purified water system. Furthermore we propose a uniform nomenclature for newly discovered ICEs of the Tn4371 family

\section*{Results and Discussion}

\subsection*{Bioinformatic analysis of Tn4371-like ICEs}

Using bioinformatic analysis tools, searches of the genome databases for elements similar to the Tn4371 element were carried out using the original Tn4371 sequence as a probe. The method used was similar to that used to detect novel members of the R391/SXT family of ICEs in Enterobacteriaceae [22]. In this study novel unreported ICEs closely related to Tn4371 were
discovered in the genome sequences of several different bacteria including the β-proteobacteria, two
elements in *Delftia acidovorans* SPH-1, and a single element *Comamonas testosteroni* KF-1,
*Acidovorax avenae* subsp. citrulli AAC00-1, *Bordetella petrii* DSM12804, *Acidovorax* sp. JS42,
*Polaromonas naphthalenivorans* CJ2 plasmid pPNAP01, *Burkholderia pseudomallei* MSHR346
and *Diaphorobacter* sp. TPSY [Table 1]. Novel elements were also found in the γ-proteobacteria
*Congregibacter litoralis* KT71, *Shewanella* sp. ANA-3, *Pseudomonas aeruginosa* 2192,
*Pseudomonas aeruginosa* PA7, *Pseudomonas aeruginosa* PACS171b, *Pseudomonas aeruginosa*
UCBPP-PA14, *Stenotrophomonas maltophilia* K279a, *Thioalkalivibrio* sp. HL-EbGR7 [Table 2].
The element in *Bordetella petrii* DSM12804 was previously identified but not analyzed in a paper
by Lechner *et al.*, [24]. The elements found in *Delftia acidovorans* SPH-1, *Comamonas testosteroni*
KF-1 and *Bordetella petrii* DSM12804 were also partially characterised along with further
information on the elements in *Cupriavidus metallidurans* CH34 in a paper by Van Houdt *et
al.*, [25]. Geographically all these bacteria were found in different locations in both Europe and the
Americas and were isolated from many different environments including activated sludge, polluted
water and clinical situations [Table 1 and 2]. All elements contained different inserts [containing
accessory genes] in the core backbone except for those found in *Delftia acidovorans* SPH-1 and
*Comamonas testosteroni* KF-1. The size of the newly discovered elements varied from 42 to 70 Kb
and the GC content from 59 to 65% [Table 1 and 2].

**Characterisation of Tn4371-like ICEs in whole genome sequences**

The core structure conserved amongst known Tn4371-like ICEs is presented in Fig. 1. At the *attR*
end of the elements a putative *int* gene [that bears similarities to tyrosine based site-specific
recombinases historically called phage-like integrases [26], possessing the R-H-R-Y tetrad] is found
[Additional file 1]. A phylogenetic study was carried out on all available Tn4371-like *int* genes and
tyrosine recombinases from phages and other ICEs. The phylogenetic tree can be seen in Additional
file 2. These Tn4371-like *int* genes grouped with the *int* genes of ICE*Hin1056*, an ICE from
Haemophilus influenzae and from phages related to the P22 phage. The int gene was found in all characterised elements and was followed by nonconserved ORFs which differed from element to element. These ORFs include putative DNA helicases and nucleases, proteins with β-lactamase domains, similar to RadC DNA repair proteins, putative reductases, transposases of insertion sequences, putative ubiquitin-activating enzymes, putative transcriptional regulators and many different hypothetical proteins whose functions are unknown [Fig. 1, Additional file 3]. These ORF’s were found in differing arrangements in each of the different elements. Polaromonas naphthalahenivorans CJ2 plasmid pPNAP01 contained biphenyl degradation genes in this area of the element and these genes are similar to those found in the original Tn4371 element but are found in a different part of the element. Pseudomonas aeruginosa PACS171b and the second Delftia acidivorans SPH-1 element have an arsenate resistance system located in this region. This system is related to the ars system, and has the genes arsH, arsC, arsB and arsA in the operon in this bacterium. The function of arsH is unknown; however it is necessary for resistance to arsenic in the Yersinia enterocolitica virulence plasmid pYV [27]. The arsC gene encodes a soluble arsenate reductase which reduces intracellular arsenate to arsenite for efflux from the cell [28]. The arsA gene codes for a unique ATPase which binds to the ArsB membrane protein forming an anion transporting arsenite pump [28]. The arsD gene encodes an inducer independent regulatory protein which controls the upper level of operon expression [29]. The second Delftia acidivorans SPH-1 element has genes related to the Mer (Mercury Resistance) operon: merR, merT, merP and merA. The merR gene controls regulation of the operon, merT and merP transport of the mercury ions and merA reduction of the mercury ions [30]. This region also contains a predicted czc [Cd/Zn/Co] efflux system [31, 32]. Czc mediates the inducible resistance to Co^{2+}, Zn^{2+} and Cd^{2+}, the protein products of gens czcA, czcB and czc form a membrane-bound protein complex catalysing an energy dependant efflux of these three metal ions [33]. Following the integrase gene two conserved genes [ORF00013 and ORF00014 in Tn4371] were present in most elements except those in C. litoralis KT71 and Shewanella sp. ANA-3. These
ORF’s are related to proteins encoded by genes located near the transfer origin of *Escherichia coli* F plasmid [Q9WTE4 and Q9S4W2]. Although the function of the first protein is unknown, the second shows similarity to ParB-like nucleases initially identified as a critical element in the faithful partitioning of plasmid DNA during cell division in the absence of selection pressure [34, 35]. Subsequently, a number of similar proteins have been identified in prokaryotes and archaea which carry out the function of segregation of genomic DNA during cell division. ParB homologs are present in almost all eubacteria chromosomes [36].

The next region on all elements contains proteins similar of the XRE [Xenobiotic Responsive Element] family of transcriptional regulators, a putative lipoprotein with a DNA binding domain and a protein of unknown function. The XRE family behave as lambda repressor-like proteins associated with different phages, including *Staphylococcus aureus* phage phi 11 [37] and the *Bacillus subtilis* defective prophage PBSX [38, Fig. 1]. Two different homologues of the XRE were found in different elements one related to that found in the original Tn4371 element (*R. picketti* 12J, *D. acidovorans* SPH-1, *A. avenae* subsp. *citrulli* AAC00-1, *C. testosteroni* KF-1 and *Acidovorax* sp. JS42, *C. litoralis* KT71, *Shewanella* sp. ANA-3, *P. aeruginosa* 2192 and *P. aeruginosa* PA7, *P. aeruginosa* PACS171b, *Thioalkalivibrio* sp. HL-EbGR7 and *B. pseudomallei* MSHR346). A different XRE was found in the remaining elements: *B. petrii* DSM 12804, *S. maltophilia* K279a, *P. aeruginosa* UCBPP-PA14, *Diaphorobacter* sp. TPSY, *P. naphthalenivorans* CJ2 plasmid pPNAP01 and the second element of *Delftia acidovorans* SPH-1.

Following on from the XRE transcriptional regulators, a protein [ORF00035 of Tn4371] was found with similarity to the RdfS excisionase [CAD31514] of ICEMl/SymR7A, the symbiosis island of *Mesorhizobium loti* R7A [39]. Most excisionases, also called recombination directionality factors [RDF’s], share a number of conserved features: they are small [usually <100 amino acids] DNA-binding proteins, that are typically basic with the majority of known RDFs having isoelectric points in the range of pH 8-10 [40]. The size of the ORF00035 protein homologues found in this comparative analysis ranged from 89-98 aa [amino acid] and had pI’s ranging from 8.14 to 9.59.
BlastP scores showed approximately 50% aa identity with the ICEM1SymR7A RdfS, over approximately 55 aa for all of the putative RdfSs discovered in this study [Fig. 1]. No excisionase was found in the second Delftia acidovorans SPH-1 element. The location of this ORF is also of interest as usually excisionases are found close to the integrase gene in most ICEs particularly the SXT/R391 family [41].

The next sequence region of the elements contained plasmid-related genes whose predicted products were related to the RepA [replication] protein of Pseudomonas plasmid pVS1 [BAA96327, 42], plasmid pMLb of M. loti MAFF303099 [NP_109574, 43] and plasmid pEMT8 [CAC94910, 44]; this gene maybe involved in replication of the element [13]. The ParA partition protein of the type Ib family [45] and its associated ParB protein was also found but in all cases the ParB was truncated. Rep and Par proteins have been proposed to act as a stabilisation system for the maintenance of mobile elements in bacterial genomes [19, 36], similar to the toxin-anti-toxin system encoded by ORFs s044 and s045 of the SXT-ICE [46]. Qui et al. found that the P. aeruginosa ICE PAPI-1 contains a homologue of the plasmid and chromosome partitioning gene soj (parA). They demonstrated that deletion of the soj homologue from PAPI-1 resulted in complete loss of PAPI-1 from P. aeruginosa. The mechanism by which the Soj protein promotes PAPI-1 maintenance remains to be elucidated [47]. Similar genes to soj have been found in ICEHin1056 and ICEA [20, 48]. This region was followed by an ORF encoding a conserved hypothetical protein [ORF00040 in Tn4371] whose function is unknown [Fig. 1].

This sequence is followed by a region containing transfer like proteins, the first being a putative conjugation protein TraF related to the pilus assembly proteins of IncP plasmids. This TraF protein is a protease that acts upon the pilus assembly protein TrbC [49]. The second is a putative relaxase-like protein [ORF00041 in Tn4371] that has similarity to the VirD2 protein of Ti plasmids and to the RlxS [relaxase, CAD31511] of ICEM1SymR7A. Transfer and maintenance of ICEM1SymR7A in cells has been shown to be dependent on the relaxase protein RlxS [39, Fig. 1]. A relaxase, usually encoded by the plasmid, recognizes oriT, makes a single-strand DNA break (a nick) in oriT, and
covalently attaches to the 5' end of the nicked DNA strand via a phosphotyrosyl linkage. No helicase domain was found in examining the protein so this indicates that the element may use leading-strand DNA synthesis (rolling-circle replication) from the nicked 3' end to promote strand displacement and single-strand DNA transfer [50, 51].

Following the putative relaxase-like protein is a variable region encoding a number of different ORFs, which vary from element to element; these genes encode putative antibiotic genes, heavy metal resistance pumps and degradative and metabolic enzymes which may have originated by transposition into the element. The sequence between the putative relaxase gene and the first gene of the variable region, in all elements, is similar to the sequence of an area of Tn5 [U00004] indicating that the diversity in this region maybe due to one or a number of Tn5 mediated insertion events. This variable region in the novel ICE in *R. pickettii* 12J encodes a putative set of lipid metabolising genes [Fig. 1]. These are closely related to genes from *Pseudomonas putida* W619 [NZ_AAVY01000010.1] and from the pREC1 plasmid from *Rhodococcus erythropolis* PR4 [NC_007486] [52]. *A. avenae* subsp. citrulli AAC00-1 contained insertion sequences and homologues to general metabolism proteins whose exact functions are unknown. *D. acidavorans* SPH-1 and *C. testosteroni* KF-1 contain a predicted *czc* [Cd/Zn/Co] efflux system [31, 32] in their variable regions. The novel element in *Acidovorax* sp. JS42 contains genes that show similarity to a multidrug resistance pump and insertion sequences [InterPro Scan] in this region. In the variable region in *B. petrii* DSM 12804 there are various proteins that are putatively involved in degradation, however their exact function is unknown. *Burkholderia pseudomallei* MSHR346 has genes that are putatively involved in xenobiotic metabolism; however again their exact function is unknown. *Polaromonas naphthalenivorans* CJ2 plasmid pPNAP01 contains a putative antibiotic resistance pump and metabolism proteins whose role have not been identified. *Diaphorobacter* sp. TPSY contains a predicted *czc* [Cd/Zn/Co] efflux system similar to those in *D. acidavorans* SPH-1 and *C. testosteroni* KF-1. The second *D. acidavorans* SPH-1 contains a copper resistance system Cop related to that of *Pseudomonas syringae*. The genes in this system are laid out in the following order
copSR copABFCD. copSR is a two-component signal transduction system, which is required for the copper-inducible expression of copper resistance [53]. CopA and CopC are abundant periplasmic copper binding proteins, and CopB is associated with copper accumulation in the outer membrane. No specific function for CopD has been determined yet [54]. CopF is involved in the cytoplasmic detoxification of copper ions [55]. In the novel element associated with *Shewanella* sp. ANA-3 the variable region encodes genes that shares similarities with a chloramphenicol efflux pump [InterPro Scan]. *C. litoralis* KT71 and *P. aeruginosa* 2192 have a putative resistance nodulation division (RND) type multidrug efflux pump related to the *mex* system of *P. aeruginosa* [56] and the *oqx* system of *E. coli* plasmid pOLA52 [57] encoded. Apart from antibiotics, the broad substrate range of the Mex efflux systems of *P. aeruginosa* also includes organic solvents, biocides, dyes, and cell signalling molecules [58]. In the ICE of *P. aeruginosa* PA7 this variable region encodes homologs of genes for antibiotic resistance including neomycin/kanamycin resistance, bleomycin resistance, and streptomycin resistance related to the antibiotic resistance genes from Tn5 [U00004]. There are also a set of genes with similarity to the *kdpFABC* system. The KdpFABC complex acts as a high affinity K$^+$ uptake system. In *E. coli*, the complex is synthesized when the constitutively expressed low affinity K$^+$ uptake systems Trk and Kup can no longer meet the cell’s demand for potassium due to external K$^+$ limitation [59]. *Stenotrophomonas maltophilia* K279a had a putative Major Facilitator Superfamily (MFS) efflux pump that usually function as specific exporters for certain classes of antimicrobial agents. This is related to the *emrAB* system from *E. coli* [60]. *P. aeruginosa* UCBPP-PA14 has a predicted *czc* [Cd/Zn/Co] efflux system similar to those in *D. acidovorans* SPH-1 and *C. testosteroni* KF-1. *P. aeruginosa* PACS171b contains a homolog of UspA- the Universal Stress Protein. The UspA protein is important for survival during cellular growth arrest in *E. coli*, but the exact physiological role of the protein is unknown [61]. *Thioalkalivibrio* sp. HL-EbGR7 has a set of genes with approximately 88% aa identity to the putative KdpFABC system in *P. aeruginosa* PA7. This variability is suggestive that this region may be a hotspot for insertion or recombination where insertion clearly does not disrupt or affect the expression of neighbouring
genes. The variation in predicted gene function, size and lack of homology between elements is suggestive of this region contributing a number of different adaptive traits to hosts containing these ICEs.

Following this variable region is encoded a putative transcriptional regulator protein TraR and a homologue of the type IV coupling protein TraG [similar to those in IncP plasmids]. TraG is responsible for DNA transfer during conjugation and is a putative DNA binding protein [62]. Interestingly the gene order of this region and the order of genes preceding it are also suggestive of an insertion [of the variable region just discussed] into a primordial transfer module.

The putative DNA binding gene traG is followed by a group of genes encoding proteins [TrbBCDEJLFGI] with similarity to the mating-pair formation [mpf] apparatus or type IV secretion system closely related to IncP and Ti plasmids. This system presumably mediates the DNA transfer of the ICE to recipient cells [63, 64]. These genes show similarity to those required for conjugative transfer of the Agrobacterium Ti plasmid, pNGR234a and RP4, except that two genes, trbK and trbH, found on these plasmids are missing [65]. In the Tn4371-like elements the gene order was trbBCDEJLFGI in all the characterised elements found in this study and similar to the molecular organisation in ICEMISymR7A [19, Fig. 1]. The TrbB, TrbC, TrbE, TrbG, and TrbL proteins are involved in the creation of the mpf apparatus, TrbC is involved in pilus formation and TrbE displays ATPase activity [65].

The novel ICEs detected in this study are integrated into various locations in the genomes of the host bacteria where they were discovered. In Acidovorax sp. JS42 other partial copies of Tn4371-like elements were also found in addition to the full element reported here. Two elements were discovered and characterised in D. acidovorans. A further partial element was found in B. petrii this however lacked the intTn4371 gene. This situation is similar to that found in R. metallidurans CH34 and indicates that duplication or multiple insertions of the elements occur in bacteria. Near complete copies of Tn4371-like elements were also found in Burkholderia ambifaria AMMD and Burkholderia multivorans ATCC17616, where both were found to lack the Tn4371-
like integrase gene suggesting that the elements may no longer be mobile. New elements were also found in *Ralstonia solanacearum* MolK2 and a second element in *Diaphorobacter* sp. TPSY, these share similarities in the stabilisation and transfer regions of the element to Tn4371-like elements but they have a different integrase region not related to the *int*<sub>Tn4371</sub> gene.

All of the elements reported here [Table 1 and 2] appear to share a common scaffold or backbone that is approximately 24 kb in size containing a 1.5 kb integrase gene; an 8.5 kb replication/stability gene cluster and a 14 kb conjugal transfer/mating pair formation cluster [Fig. 1]. A visual representation of this can be seen in Figs. 2, 3, 4 and 5 where the various sequences were aligned for comparison, the core scaffold identified and ‘adaptive’ genes highlighted which vary from element to element.

Bioinformatic comparisons were performed between the genes that make up the core scaffold region of the ICE and these ranged from the highly conserved *traG* gene, with 84 to 96% aa identity, *trbE* gene, with 76 to 94% aa identity, and the *parA* gene, with 90 to 97% aa identity, to the less-conserved *traR* gene, with 53 to 84% aa identity. On average the genes that we ascribed to the core showed > 75% aa identity and were also related by gene order. All gene numbers and a basic description of the genes are included in Additional file 3.

**Defining the Tn4371 family of ICEs and nomenclature**

These elements have been classed as ICEs as we believe at this moment in time this is the best terminology currently available. They follow all the criteria of ICEs having integration and transfer modules, possessing an excisionase gene and having genes and gene layout (*rdfS*, *rlxS* and the *trb* genes) similar to other ICEs namely ICEMISym<sup>R7A</sup>. The original element can also excise from bacterial chromosome and form a circular intermediate [9], however the element has not been shown to transfer between different bacteria, and this could be due to the original element lacking the *trbD* gene [13].
Although the elements identified in this study are not identical, they share a similar core backbone that, in our view, warrants their inclusion into the Tn4371 ICE family. All encode a related integrase, related maintenance and transfer genes and the gene order of homologous genes are similar, if one were to remove variable inserted regions which differ from element to element. We propose that any ICE that encodes an integrase gene closely related to \(\text{int}_{\text{Tn4371}}\), defined as over 70% protein homology and that has similar maintenance and transfer genes be considered part of the Tn4371 family of ICEs.

Given the number of Tn4371-like elements discovered in this study, it seems sensible to name newly described ICEs of the Tn4371 family with a uniform nomenclature. We propose adapting the system used for naming transposons described by Roberts et al., [66]. This system is a website [http://www.ucl.ac.uk/eastman/tn/] based system which assigns Tn numbers in sequence e.g. Tn6033, Tn6034, etc and the elements were then called ICE\(_{\text{Tn4371}6033}\), ICE\(_{\text{Tn4371}6034}\), etc to distinguish that they are ICEs of the Tn4371 family. The names assigned to the elements discovered in this study are listed in Table 1 and 2. This system was chosen as other systems such as that used by Burrus et al., [8] for naming members of the SXT\(\text{\textbar}391\) family of ICEs are not regulated and can differ between laboratories leading to confusion.

**Tn4371-like ICE detection and molecular characterisation**

Following the discovery of the widespread nature of Tn4371-like ICEs in the genomes of many new organisms, PCR primers were designed to amplify important genes of the core scaffold to aid in the rapid identification of new Tn4371-like elements. We tested this on a culture collection of fifty-eight *Ralstonia pickettii* and *Ralstonia insidiosa* strains from various environments and geographic locations. The PCR primers were based on conserved consensus sequences of core genes identified from all the elements identified in this study and those reported previously.

The results in Fig. 6 show the genes encoding a putative integrase \([\text{int}]\), the putative stabilisation system \([\text{repA, parA, parB}]\), a homologue to the DNA transfer protein \([\text{traG}]\) and a putative pilus
assembly and synthesis protein [trbI]. DNA sequencing of the four amplicons in the tester strains demonstrated that Tn4371-like sequences exist in the genome of *R. pickettii* ULM001. While this data clearly demonstrates the presence of Tn4371-like elements in tester strains the possibility of multiple elements in such strains cannot be excluded, although out sequencing of resulting amplicons is suggestive of only one element.

Three of the fifty-eight *Ralstonia* isolates, ULM001, ULM003 and ULM006 [which were laboratory purified water isolates from different locations in France] showed positive amplification for *int* 

Tn4371 integrase gene when tested with the intFor1 and intRev1 primer pair in PCR amplification [Table 3]. Sequencing revealed that the ULM001 *int* gene showed 85% and 99% nucleotide identity to the Tn4371 *int* gene and ICE\textsubscript{Tn4371,6033} *int* gene, respectively. The RepAF and RepAR primers also amplified the *repA* gene and the *parA* gene in ULM001, ULM003 and ULM006. Sequencing these amplicons revealed that in ULM001 the repA and parB genes were present and showed 88% and 99% nucleotide identity to the *RepA* and *ParA* genes from Tn4371 and ICE\textsubscript{Tn4371,6033} respectively. A *traG* Tn4371 homolog was also detected in ULM001, ULM003 and ULM006 following PCR amplification. Sequencing revealed that the ULM001 *traG* Tn4371 gene showed 91% and 89% nucleotide identity to *traG* from Tn4371 and ICE\textsubscript{Tn4371,6033} respectively. TrbIF and TrbIR primers were used to amplify the *trbI* gene in ULM001 and ULM003 while no amplification occurred in ULM006. Sequencing showed that the ULM001 amplicon was a homolog, which had 88% and 99% nucleotide identity to the *trbI* gene from Tn4371 and ICE\textsubscript{Tn4371,6033} respectively. The absence of a *trbI* gene amplicon in ULM006 may indicate a deleted gene or truncated element in this strain. The use of these primer sets has thus revealed the presence of two new elements, which can then be further characterised. The ICEs detected in this study from *Ralstonia pickettii* were named ICE\textsubscript{Tn4371,6043} and ICE\textsubscript{Tn4371,6044} using the nomenclature system described above, a general map of the elements can be seen in Fig. 6.

**The attL and attR region of Tn4371 ICEs**
Analysis of hosts harbouring Tn4371-like elements indicated that integration occurred at an 8-bp attB site generating attL and attR element chromosomal junctions [11, Fig. 7a]. An alignment of the first and last 200bp of the elements analysed in this study with Tn4371-like element from previous studies showed the attL site had a sequence of TTTTC/TA/GT and attR had a sequence of TTTTC/TA/GT for some bacteria, while others had no direct repeats. These alignments can be seen in Additional file 4. The exact sequence of the direct repeat for each element is presented in Table 4. The absence of direct repeats in some of these elements may mean that they are no longer mobile.

Tn4371 has been shown to excise from the RP4 plasmid in Ralstonia eutropha forming a circular extrachromosomal intermediate [10, Fig. 7a] as a transfer intermediate. The strains in which we detected Tn4371-like elements were examined to see if they also excised forming extrachromosomal intermediates [CirIm] using a PCR assay that allowed amplification across the circular junction but which would not amplify if the element were integrated. Primer LE1 is specific to integrated Tn4371-like ICE DNA at the attL left-end where as primer RE1 is specific to integrated Tn4371-like ICE at the attR right-end [Fig. 7a, Table 3]. Both primers are oriented towards the Tn4371- like ICE junctions, and PCR product will be generated only if the respective left and right ends [attL and attR sites] excise from the chromosome and circularise [CirIm], reconstituting attP [attachment locus on the element]. A model of integration and excision of the ICE can be seen in Fig. 7a. PCR products of ~220-bp were obtained from ICE_{Tn4371}6043 [ULM001] and ICE_{Tn4371}6044 [ULM003] [Fig. 7b.], indicating that a circular extrachromosomal form of the element is present in these cells, while no PCR product was obtained from ULM006 [Fig. 7b]. The sequencing of the attP region of ICE_{Tn4371}6043 gave an attL region of TTTTTCAT and an attR region of TACTTTTTT. This rapid amplification across the circular attP junction can also be utilised for the rapid identification of Tn4371-like elements. It is possible that the PCR may have picked up tandems of the element if those happened to be intermediates in "transposition".
Conclusion

Tn4371-like ICEs are found in a wide range of γ-proteobacteria and β-proteobacteria from both clinical and environmental sources. These types of bacteria are known for their large metabolic repertoires and these elements could potentially be a source of acquisition of adaptive functions for these organisms. The discovery of the Tn4371-like ICEs in the P. aeruginosa strains, S. maltophilia K279a and B. pseudomallei MSHR346 are the first reports of these elements found in human pathogens. This along with the discovery of putative antibiotic resistance genes in their genomes indicates that these elements may have an impact in clinical situations. The discovery and characterisation of novel Tn4371-like elements as reported here adds significantly to the repertoire of such elements and helps define the core scaffold of such elements. It is clear that these elements are highly adaptable and may contribute significantly to the metabolic capabilities of their host. This study increases the knowledge available about these elements adding data on eighteen new elements to the five already known. A new nomenclature system for Tn4371-like elements was designed to avoid confusion in the naming of these elements. The primer system used to detect and characterise the Tn4371-like ICEs in Ralstonia pickettii ULM001 and ULM003 could be adapted and used for other bacterial species for the rapid screening of such elements.

Methods

Bacterial strains and growth conditions

The strains used in this study are shown in Table 5. All the strains were stored at -20 °C in Nutrient Broth [Biolab, Budapest, Hungary] with 50% glycerol. Isolates were grown aerobically on Nutrient Agar [Biolab, Budapest, Hungary] and incubated overnight at 30 °C.

Molecular analysis of genes of Tn4371-like ICEs
PCR primers were designed based on the conserved aligned scaffold common to all ICEs characterised in this study and from the consensus sequence of the *Ralstonia pickettii* 12J Tn4371 ICE using the Primer 3 program [67, http://frodo.wi.mit.edu/]. All primers are listed in Table 5. The cycling conditions were as follows: initial denaturation (98 °C, 2 min); 35 cycles consisting of denaturation [98 °C for 15 s], primer annealing [T<sub>A</sub> [estimated primer annealing temperature], 1 min], and extension [72 °C, 1 min/kb]; followed by a final extension step [72 °C, 10 min]. Amplification was carried out with a GC buffer [in a total reaction of 100 µL containing 0.2 mM deoxynucleoside triphosphates, 100 pmol of each primer, 8 µL of genomic template DNA, and 3 units of Phusion polymerase [New England Biolabs, UK]. Amplification was carried out using a GeneAmp 2400 Thermocycler. Bacterial DNA for PCR amplification was extracted according to Ausubel *et al.* [68]. Amplicons to be sequenced were directly purified from the PCR reaction by the NucleoSpin Extract II kit [Macherey-Nagel, Düren] according to the manufacturer’s instructions. Sequence analysis was performed by Euorfins-MWG [Germany] using both the forward and reverse primers listed in Table 3.

**Bioinformatic Analysis of the Tn4371-like ICEs in genomes**

All analysed DNA sequences were retrieved from the GenBank database [http://www.ncbi.nlm.nih.gov]. DNA and protein sequences similar to Tn4371 [13, AJ536756] were detected within the NCBI nonredundant nucleotide and protein databases [http://www.ncbi.nlm.nih.gov] via BLASTP and BLASTN analysis using the original Tn4371 sequence as a probe [69]. Assembly and comparison with other Tn4371-like sequences was performed with the Artemis Comparison Tool [ACT] [70, http://www.sanger.ac.uk/Software/ACT]. The complete DNA sequences were also manually annotated to verify the deposited sequence. The similarity of proteins encoded by the element was determined as % aa identities over the entire protein to its Tn4371 equivalent via BLASTP. Unknown ORFs were analysed using InterProScan [http://www.ebi.ac.uk/InterProScan/ 71] to locate motifs
or domains where similarity with known proteins was low or absent. Size and total % GC content was determined using the GC-Profile program [72, http://tubic.tju.edu.cn/GC-Profile/]. Phylogenetic and molecular evolutionary analyses were conducted using genetic-distance-based neighbour-joining algorithms within MEGA version 4.0 [73, http://www.megasoftware.net/]

Nucleotide sequence accession numbers

The DNA sequences described in this article have been assigned the accession numbers listed in Table 3.

Author’s Contributions

MRP was responsible for conception of the study, experimental design, data collection, and analysis and preparation of the manuscript. JTP and CCA participated in experimental design, data analysis and preparation of the manuscript. All authors read and approved the final manuscript.

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References:

1. Toussaint A, Merlin C: Mobile elements as a combination of functional modules. 
   Plasmid 2002, 47: 26-35.

2. Burrus V, Pavlovic G, Decaris B, Guédon G: Conjugative transposons: the tip of the iceberg. Mol Microbiol 2002, 46: 601-610.
3. Churchward G: **Conjugative transposons and related mobile elements.** In *Mobile DNA II*. Edited by Craig NL, Craigie R, Gellert M, Lambowitz ML. Washington DC: American Society for Microbiology; 2002: 177-191.

4. Merlin C, Mahillon J, Nesvera J, Toussaint A: **Gene recruiters and transporters: the modular structure of bacterial elements.** In *The Horizontal Gene Pool: Bacterial Plasmids and Gene Spread*. Edited by Thomas CM. Newark: Hardwood Academic Publishers; 2000: 363-408.

5. Smith CJ, Tribble GD, Bayley DP: **Genetic elements of Bacteroides species: a moving story.** *Plasmid* 1998, 40: 12-29.

6. Hochhut B, Waldor MK: **Site-specific integration of the conjugal Vibrio cholerae SXT element into prfC.** *Mol Microbiol* 1999, 32: 99-110.

7. Osborn MA, Bolter D: **When phage, plasmids, and transposons collide: genomic islands, and conjugative- and mobilizable-transposons as a mosaic continuum.** *Plasmid* 2002, 48: 202-212.

8. Burrus V, Waldor MK: **Shaping bacterial genomes with integrative and conjugal elements.** *Res Microbiol* 2004, 155: 376-386.

9. Burrus V, Marrero J, Waldor MK: **The current ICE age: Biology and evolution of SXT-related integrating conjugal elements.** *Plasmid* 2006, 55: 173-183.
10. Springael D, Kreps S, Mergeay M: Identification of a catabolic transposon, Tn4371, carrying biphenyl and 4-chlorobiphenyl degradation genes in Alcaligenes eutrophus A5. *J Bacteriol* 1993, **175**: 1674-1681.

11. Merlin C, Springael D, Toussaint A: Tn4371: a modular structure encoding a phage-like integrase, a *Pseudomonas*-like catabolic pathway and RP4/Ti-like transfer. *Plasmid* 1999, **41**: 40-54.

12. Springael D, Diels L, Mergeay M: Transfer and expression of PCB-degradative genes into heavy metal resistant *Alcaligenes eutrophus* strains. *Biodegradation* 1994, **5**: 343-357.

13. Toussaint A, Merlin C, Monchy S, Benotmane MA, Leplae R, Mergeay M, Springael D: The biphenyl- and 4-chlorobiphenyl-catabolic transposon Tn4371, a member of a new family of genomic islands related to IncP and Ti plasmids. *Appl Environ Microbiol* 2003, **69**: 4837-4845.

14. Boucher CA, Barberis PA, Trigalet AP, Demery DA: Transposon mutagenesis of *Pseudomonas solanacearum*: Isolation of Tn5-induced avirulent mutants. *J Gen Microbiol* 1985, **131**: 2449-2457.

15. Mergeay M, Houba C, Gerits J: Extrachromosomal inheritance controlling resistance to cadmium, cobalt and zinc ions: evidence from curving in a *Pseudomonas*. *Arch Int Physiol Biochim* 1978, **86**: 440-442.
16. Kotoujansky A, Lemattre M, Boitard P: **Utilization of a thermosensitive episome bearing transposon Tn10 to isolate Hfr donor strains of Erwinia carotovora subsp. chrysanthemi.** *J Bacteriol* 1982, **150**: 122-131.

17. Burgess BK, Jacobs DB, Stiefel EI: **Large-scale purification of high activity Azotobacter vinelandii nitrogenase.** *Biochim Biophys Acta* 1980, **614**: 196-209.

18. McGrath BM, O'Halloran JA, Piterina AV, Pembroke JT: **Molecular tools to detect the IncJ elements: a family of integrating, antibiotic resistant mobile genetic elements.** *J Microbiol Meth* 2006, **66**: 32-42.

19. Burrus V, Pavlovic G, Decaris B, Guédon G: **The ICESt1 element of Streptococcus thermophilus belongs to a large family of integrative and conjugative elements that exchange modules and change their specificity of integration.** *Plasmid* 2002, **48**: 77-97.

20. Sullivan JT, Trzebiatowski JR, Cruickshank RW, Gouzy J, Brown SD, Elliot RM, Fleetwood DJ, McCallum NG, Rossbach U, Stuart GS, Weaver JE, Webby RJ, de Bruijn FJ, Ronson CW: **Comparative sequence analysis of the symbiosis island of Mesorhizobium loti strain R7A.** *J Bacteriol* 2002, **184**: 3086-3095.

21. Mohd-Zain Z, Turner SL, Cerdeño-Tárraga AM, Lilley AK, Inzana TJ, Duncan AJ, Harding RM, Hood DW, Petö TE, Crook DW: **Transferable antibiotic resistance elements in Haemophilus influenzae share a common evolutionary origin with a diverse family of syntenic genomic islands.** *J Bacteriol* 2004, **186**: 8114-8122.
22. Pembroke JT, Piterina AV: A novel ICE in the genome of *Shewanella putrefaciens* W3-18-1: comparison with the SXT/R391 ICE-like elements. *FEMS Microbiol Lett* 2006, **264**: 80-88.

23. Xu J, Mahowald MA, Ley RE, Lozupone CA, Hamady M, Martens EC, Henrissat B, Coutinho PM, Minx P, Latreille P, Cordum H, Van Brunt A, Kim K, Fulton RS, Fulton LA, Clifton SW, Wilson RK, Knight RD, Gordon JI: Evolution of Symbiotic Bacteria in the Distal Human Intestine. *PLoS Biol* 2007, **5**: e156.

24. Lechner M, Schmitt K, Bauer S, Hot D, Hubans C, Levillain E, Locht C, Lemoine Y, Gross R: Genomic island excisions in *Bordetella petrii*. *BMC Microbiol* 2009 **9**: 141.

25. Van Houdt R, Monchy S, Leys N, Mergeay M: New mobile genetic elements in *Cupriavidus metallidurans* CH34, their possible roles and occurrence in other bacteria. *Antonie Van Leeuwenhoek* 2009 **96**: 205-226.

26. Nunes-Düby SE, Kwon HJ, Tirumalai RS, Ellenberger T, Landy A: Similarities and differences among 105 members of the Int family of site-specific recombinases. *Nucleic Acids Res* 1998, **26**: 391-406.

27. Ryan D, Colleran E: Arsenical resistance in the IncHI2 plasmids. *Plasmid* 2002, **47**: 234-240.

28. Ji G, Silver, S: Reduction of arsenate to arsenite by the ArsC protein of the arsenic resistance operon of *Staphylococcus aureus* plasmid pI258. *Proc Natl Acad Sci USA* 1992, **89**: 9474-9478.
29. Wu J and Rosen BP: The *arsD* gene encodes a second trans-acting regulatory protein of the plasmid-encoded arsenical resistance operon. *Mol Microbiol* 1993, 8: 615-623.

30. Nascimento AM, Chartone-Souza E: Operon mer: bacterial resistance to mercury and potential for bioremediation of contaminated environments. *Genet Mol Res* 2003, 2: 92-101.

31. van der Lelie D, Schwuchow T, Schwidetzky U, Wuertz S, Baeyens W, Mergeay M, Nies DH: Two-component regulatory system involved in transcriptional control of heavy-metal homoeostasis in *Alcaligenes eutrophus*. *Mol Microbiol* 1997, 23: 493-503.

32. Grosse C, Grass G, Anton A, Franke S, Santos AN, Lawley B, Brown NL, Nies DH: Transcriptional organization of the *czc* heavy-metal homeostasis determinant from *Alcaligenes eutrophus*. *J Bacteriol* 1999, 181: 2385-2393.

33. Nies DH, Nies A, Chu L, Silver S: Expression and nucleotide sequence of a plasmid-determined divalent cation efflux system from *Alcaligenes eutrophus*. *Proc Natl Acad Sci U S A*. 1989, 86: 7351-7355.

34. Austin S, Ziese M, Sternberg N: A novel role for site-specific recombination in maintenance of bacterial replicons. *Cell* 1981, 25: 729-736.

35. Gerlitz M, Hrabak O, Schwab H: Partitioning of broad host range Plasmid RP4 is a complex system involving site-specific recombination. *J Bacteriol* 1990, 172: 6194-6203.
36. Bignell C, Thomas CM: **The bacterial ParA-ParB partitioning proteins.** *J Biotechnol* 2001, **91**: 1-34.

37. Das M, Ganguly T, Chattoraj P, Chanda PK, Bandhu A, Lee CY, Sau S: **Purification and characterization of repressor of temperate S. aureus phage phi11.** *J Biochem Mol Biol* 2007, **40**: 740-748.

38. McDonnell GE, McConnell DJ: **Overproduction, isolation, and DNA-binding characteristics of Xre, the repressor protein from the Bacillus subtilis defective prophage PBSX.** *J Bacteriol* 1994, **176**: 5831-5834.

39. Ramsay JP, Sullivan JT, Stuart GS, Lamont IL, Ronson CW: **Excision and transfer of the Mesorhizobium loti R7A symbiosis island requires an integrase IntS, a novel recombination directionality factor RdfS, and a putative relaxase RlxS.** *Mol Microbiol* 2006, **62**: 723-734.

40. Lewis JA, Hatfull GF: **Control of directionality in integrase-mediated recombination: examination of recombination directionality factors (RDFs) including Xis and Cox proteins.** *Nucleic Acids Res* 2001, **29**: 2205-2216.

41. O'Halloran JA, McGrath BM, Pembroke JT: **The orf4 gene of the enterobacterial ICE, R391, encodes a novel UV-inducible recombination directionality factor, Jef, involved in excision and transfer of the ICE.** *FEMS Microbiol Lett* 2007, **272**: 99-105.
42. Heeb S, Itoh Y, Nishijyo T, Schneider U, Keel C, Wade J, Walsh U, O’Gara F, Haas D: Small, stable shuttle vectors based on the minimal pVS1 replicon for use in gram-negative, plant-associated bacteria. *Mol Plant Microbe Interact* 2000, **13**: 232-237.

43. Kaneko T, Nakamura Y, Sato S, Asamizu E, Kato T, Sasamoto S, Watanabe A, Idesawa K, Ishikawa A, Kawashima K, Kimura T, Kishida Y, Kiyokawa C, Kohara M, Matsumoto M, Matsuno A, Mochizuki Y, Nakayama S, Nakazaki N, Shimpo S, Sugimoto M, Takeuchi C, Yamada M, Tabata S: Complete genome structure of the nitrogen-fixing symbiotic bacterium *Mesorhizobium loti*. *DNA Res* 2000, **7**: 331-338.

44. Gstalder ME, Faelen M, Mine N, Top EM, Mergeay M, Couturier M: Replication functions of new broad host range plasmids isolated from polluted soils. *Res Microbiol* 2003, **154**: 499-509.

45. Gerdes K, Moller-Jensen J, Bugge Jensen R: Plasmid and chromosome partitioning: surprises from phylogeny. *Mol Microbiol* 2000, **37**: 455-466.

46. Dziewit L, Jazurek M, Drewniak L, Baj J, Bartosik D: The SXT conjugative element and linear prophage N15 encode toxin-antitoxin-stabilizing systems homologous to the tad-ata module of the *Paracoccus aminophilus* plasmid pAMI2. *J Bacteriol* 2007, **189**: 1983-1997.

47. Qiu X, Gurkar AU, Lory S: Interstrain transfer of the large pathogenicity island (PAPI-1) of *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA* 2006, **103**: 19830-19835.
48. Marenda M, Barbe V, Gourgues G, Mangenot S, Sagne E, Citti C: A new integrative conjugative element occurs in *Mycoplasma agalactiae* as chromosomal and free circular forms. *J. Bacteriol* 2006, **188**: 4137-4141.

49. Haase J, Lanka E: A specific protease encoded by the conjugative DNA transfer systems of IncP and Ti plasmids is essential for pilus synthesis. *J Bacteriol* 1997, **179**: 5728-5735.

50. Byrd DR, Matson SW: Nicking by transesterification: the reaction catalysed by a relaxase. *Mol Microbiol* 1997, **25**: 1011-1022.

51. Lanka E, Wilkins BM: DNA processing reactions in bacterial conjugation. *Annu Rev Biochem* 1995, **64**: 141-169.

52. Sekine M, Tanikawa S, Omata S, Saito M, Fujisawa T, Tsukatani N, Tajima T, Sekigawa T, Kosugi H, Matsuo Y, Nishiko R, Imamura K, Ito M, Narita H, Tago S, Fujita N, Harayama S: Sequence analysis of three plasmids harboured in *Rhodococcus erythropolis* strain PR4. *Environ Microbiol* 2006, **8**: 334-346.

53. Mills SD, Jasalavich CA, Cooksey DA: A two-component regulatory system required for copper-inducible expression of the copper resistance operon of *Pseudomonas syringae*. *J Bacteriol* 1993, **175**: 1656-1664.

54. Cha JS, Cooksey DA: Copper resistance in *Pseudomonas syringae* mediated by periplasmic and outer membrane proteins. *Proc Natl Acad Sci USA* 1991, **88**: 8915-8919.
55. Mergeay M, Monchy S, Vallaeyts T, Auquier V, Benotmane A, Bertin P, Taghavi S, Dunn J, van der Lelie D, Wattiez R: *Ralstonia metallidurans*, a bacterium specifically adapted to toxic metals: towards a catalogue of metal-responsive genes. *FEMS Microbiol Rev* 2003, 27: 385–410.

56. Kohler T, Michea-Hamzehpour M, Henze U, Gotoh N, Curty LK, Pechere JC: Characterization of *MexE-MexF-OprN*, a positively regulated multidrug efflux system of *Pseudomonas aeruginosa*. *Mol Microbiol* 1997, 23: 345-354.

57. Hansen LH, Johannesen E, Burmolle M, Sorensen AH, Sorensen SJ: Plasmid-encoded multidrug efflux-pump conferring resistance to olaquindox in *Escherichia coli*. *Antimicrob Agents Chemother* 2004, 48: 3332-3337.

58. Papadopoulos CJ, Carson CF, Chang BJ, Riley TV: Role of the MexAB-OprM Efflux Pump of *Pseudomonas aeruginosa* in Tolerance to Tea Tree (*Melaleuca alternifolia*) Oil and Its Monoterpene Components Terpinen-4-ol, 1,8-Cineole, and α-Terpineol. *Appl Environ Microbiol* 2008, 74: 1932-1935.

59. Siebers A, Altendorf K: K\(^+\)-translocating *Kdp*-ATPases and other bacterial P-type ATPases. In: Alkali cation transport systems in prokaryotes. Edited by Bakker EP. CRC Press, Boca Raton, Florida. 1993: 225-252.

60. Furukawa H, Tsay JT, Jackowski S, Takamura Y, Rock CO: Thiolactomycin resistance in *Escherichia coli* is associated with the multidrug resistance efflux pump encoded by *emrAB*. *J Bacteriol* 1993, 175: 3723-3729.
61. Nachin L, Nannmark U, Nyström T: **Differential roles of the universal stress proteins of *Escherichia coli* in oxidative stress resistance, adhesion, and motility.** *J Bacteriol* 2005, **187**: 6265-6272.

62. Gomis-Ruth FX, de la Cruz F, Coll M: **Structure and role of coupling proteins in conjugal DNA transfer.** *Res Microbiol* 2002, **153**: 199-204.

63. Schroder G, Lanka E: **The mating pair formation system of conjugative plasmids-A versatile secretion machinery for transfer of proteins and DNA.** *Plasmid* 2005, **54**: 1-25.

64. Lawley D, Klimke WA, Gubbins MJ, Frost LS: **F factor conjugation is a true type IV secretion system.** *FEMS Microbiol Lett* 2003, **224**: 1-15.

65. Li PL, Everhart DM, Farrand SK: **Genetic and sequence analysis of the pTiC58 trb locus, encoding a mating-pair formation system related to members of the type IV secretion family.** *J. Bacteriol* 1998, **180**: 6164-6172.

66. Roberts AP, Chandler M, Courvalin P, Guédon G, Mullany P, Pembroke T, Rood JI, Smith CJ, Summers AO, Tsuda M, Berg DE: **Revised Nomenclature for Transposable Genetic Elements.** *Plasmid* 2008, **60**: 167-173.

67. Rozen S, Skaletsky HJ: **Primer3 on the WWW for general users and for biologist programmers.** In *Bioinformatics Methods and Protocols: Methods in Molecular Biology.* Edited by Krawetz S, Misener S. Totowa, NJ: Humana Press; 2000: 365-386
68. Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K: *Current Protocols in Molecular Biology*. John Wiley & Sons, New York; 1997

69. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ: **Basic local alignment search tool.** *J Mol Biol* 1990, **215**: 403-410.

70. Rutherford K, Parkhill J, Crook J, Horsnell T, Rice P, Rajandream MA, Barrell B: **Artemis: sequence visualization and annotation.** *Bioinformatics* 2000, **16**: 944-945.

71. Zdobnov EM, Apweiler R: **InterProScan-an integration platform for the signature-recognition methods in InterPro.** *Bioinformatics* 2001, **17**: 847-848.

72. Gao F, Zhang CT: **GC-Profile: a web-based tool for visualizing and analyzing the variation of GC content in genomic sequences.** *Nucleic Acids Res* 2006, **34**: W686-W691.

73. Tamura K, Dudley J, Nei M, Kumar S: **MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0.** *Mol Biol Evol* 2007, **24**: 1596-1599.

74. Konstantinidis KT, Isaacs N, Fett J, Simpson S, Long DT, Marsh TL: **Microbial diversity and resistance to copper in metal-contaminated lake sediment.** *Microb Ecol* 2003, **45**: 191-202.

75. Walcott RR, Fessehaie A, Castro AC: **Differences in pathogenicity between two genetically distinct groups of Acidovorax avenae subsp. citrulli on cucurbit hosts.** *J Phytopathol* 2004, **152**: 277-285.
76. Schleheck D, Knepper TP, Fischer K, Cook AM: Mineralization of individual congeners of linear alkylbenzenesulfonate by defined pairs of heterotrophic bacteria. *Appl Environ Microbiol* 2004, **70**: 4053-4063.

77. Haigler BE, Wallace WH, Spain JC: Biodegradation of 2-nitrotoluene by *Pseudomonas* sp. strain JS42. *Appl Environ Microbiol* 1994, **60**: 3466-3469.

78. von Wintzingerode F, Schattke A, Siddiqui RA, Rösick U, Göbel UB, Gross R: *Bordetella petrii* sp. nov., isolated from an anaerobic bioreactor, and emended description of the genus *Bordetella*. *Int J Sys Evol Microbiol* 2001, **51**: 1257-1265.

79. Jeon CO, Park W, Ghiorse WC, Madsen EL: *Polaromonas naphthalenivorans* sp. nov., a naphthalene-degrading bacterium from naphthalene-contaminated sediment. *Int J Syst Evol Microbiol* 2004, **54**: 93-97.

80. Coates JD, Weber KA, Scherer M, Achenbach LA: The Diverse Microbiology of Anaerobic Fe(II) Oxidation. American Geophysical Union, Fall Meeting 2007, abstract #B22D-01.

81. Saltikov CW, Cifuentes A, Venkateswaran K, Newman DK: The *ars* detoxification system is advantageous but not required for As (V) respiration by the genetically tractable *Shewanella* species strain ANA-3. *Appl Environ Microbiol* 2003, **69**: 2800-2809.

82. Fuchs BM, Spring S, Teeling H, Quast C, Wulf J, Schattenhofer M, Yan S, Ferriera S, Johnson J, Glöckner FO, Amann R: Characterization of a marine
gammaproteobacterium capable of aerobic anoxygenic photosynthesis. *Proc Natl Acad Sci USA* 2007, **104**: 2891-2896.

83. Hanna SL, Sherman NE, Kinter MT, Goldberg JB: **Comparison of proteins expressed by* Pseudomonas aeruginosa* strains representing initial and chronic isolates from a cystic fibrosis patient: an analysis by 2-D gel electrophoresis and capillary column liquid chromatography-tandem mass spectrometry. *Microbiology* 2000, **146**: 2495-2508.

84. Brodinova NS, Baskakova NV, Moroz AF, Vertiev IuV, Mokrievich NM: [Exotoxin A production during *Pseudomonas aeruginosa* PA-7 cultivation in Martin's broth]. *Zh Mikrobiol Epidemiol Immunobiol* 1984, **4**: 22-26. [Article in Russian]

85. Crossman LC, Gould VC, Dow JM, Vernikos GS, Okazaki A, Sebaihia M, Saunders D, Arrowsmith C, Carver T, Peters N, Adlem E, Kerhornou A, Lord A, Murphy L, Seeger K, Squares R, Rutter S, Quail MA, Rajandream MA, Harris D, Churcher C, Bentley SD, Parkhill J, Thomson NR, Avison MB: **The complete genome, comparative and functional analysis of* Stenotrophomonas maltophilia reveals an organism heavily shielded by drug resistance determinants. *Genome Biol* 2008, **9**: R74.

86. Lee DG, Urbach JM, Wu G, Liberati NT, Feinbaum RL, Miyata S, Diggins LT, He J, Saucier M, Déziel E, Friedman L, Li L, Grills G, Montgomery K, Kucherlapati R, Rahme LG, Ausubel FM: **Genomic analysis reveals that Pseudomonas aeruginosa virulence is combinatorial. *Genome Biol* 2006, **7**: R90.

87. Hayden HS, Gillett W, Saenphimmachak C, Lim R, Zhou Y, Jacobs MA, Chang J, Rohmer L, D'Argenio DA, Palmieri A, Levy R, Haugen E, Wong GK, Brittnacher MJ, Burns JL,
Miller SI, Olson MV, Kaul R: Large-insert genome analysis technology detects structural variation in *Pseudomonas aeruginosa* clinical strains from cystic fibrosis patients. *Genomics* 2008, 91: 530-537.

88. Sorokin DY, van den Bosch PL, Abbas B, Janssen AJ, Muyzer G: Microbiological analysis of the population of extremely haloalkaliphilic sulfur-oxidizing bacteria dominating in lab-scale sulfide-removing bioreactors. *Appl Microbiol Biotechnol* 2008, 80: 965-975.
Figure Legends

Fig 1: Common core scaffold of Tn4371-like ICEs (in blue) and above inserted genes present in R. pickettii ICE\textsubscript{Tn4371}6033 (in yellow).

Fig 2: Use of the Artemis comparison tool to analysis Tn4371-like ICE sequences of Tn\textsubscript{4371}, R. pickettii 12J, both elements from D. acidovorans SPH-1 and C. testosteroni KF-1. All ICEs analysed shared extensive sequence homology, and general gene order. Arrows on top delimit the functional regions whose order is well conserved in all Tn\textsubscript{4371} -like ICEs.

Fig 3: Use of the Artemis comparison tool to analysis Tn\textsubscript{4371}-like ICE sequences of Tn\textsubscript{4371}, P. aeruginosa 2192, P. aeruginosa PA7, P. aeruginosa UCBPP-PA14 and P. aeruginosa PACS171b. All ICEs analysed shared extensive sequence homology, and general gene order. Arrows on top delimit the functional regions whose order is well conserved in all Tn\textsubscript{4371} -like ICEs.

Fig 4: Use of the Artemis comparison tool to analysis Tn\textsubscript{4371}-like ICE sequences of Tn\textsubscript{4371}, Shewanella sp. ANA-3, C. litoralis KT71, S. maltophilia K279a and Thioalkalivibrio sp. HL-EbGR7. All ICEs analysed shared extensive sequence homology, and general gene order. Arrows on top delimit the functional regions whose order is well conserved in all Tn\textsubscript{4371} -like ICEs.

Fig 5: Use of the Artemis comparison tool to analysis Tn\textsubscript{4371}-like ICE sequences of Tn\textsubscript{4371}, A. avenae subsp. citrulli AAC00-1, Acidovorax sp. JS42, B. petrii DSM12804, Diaphorobacter sp. TPSY and P. naphthalenivorans CJ2 plasmid pPNAP01. All ICEs
analysed shared extensive sequence homology, and general gene order. Arrows on top delimit
the functional regions whose order is well conserved in all Tn4371-like ICEs.

Fig 6: Amplification of genes of the putative Tn4371-like ICE ICE_{Tn43716043} in *Ralstonia pickettii* strain ULM001 (a laboratory purified water isolate). A scheme of the amplified genes is shown above the 0.7% agarose gel of the PCR products generated with the primers listed in Table 2. Open white arrows denote ORFs of the *Ralstonia pickettii* ICE, and small black arrows represent the relative location of primers. Lanes M1 and M2 contain 200-10000 bp molecular size markers (Bioline Hyperladder I), respectively. The lanes and the product sizes are as follows: Lane 1, *int* gene and flanking bases (1035 bp); Lane 2 *RepA* gene (1657 bp), Lane 3 *traG* gene (1483 bp); Lane 4 *trbI* gene (1597 bp).

Fig 7: A) Schematic representation of Tn4371 excision and insertion into the *R. pickettii* chromosome. Primer LE1 and RE1 are the primers for detection of the circular form of the element. B) Agarose gel of *attP* of ICE_{Tn43716043} and ICE_{Tn43716044}. Lanes M contains 200-10000 bp molecular size markers (Bioline Hyperladder I), Lane 1 ULM001, Lane2 ULM002, Lane 3 ULM006.
| Tn4371-like Elements | Size | %GC Content | Location | Environment | Accessory Genes | Reference | Name | Accession Number |
|----------------------|------|-------------|----------|-------------|----------------|-----------|------|------------------|
| Ralstonia pickettii 12J | 54121 bp | 64.63 | USA | Copper-contaminated sediment from a lake | Lipid metabolism | [74] | ICE_Tn4371_6033 | CP001068 |
| Acidovorax avenae subsp. citrulli AAC00-1 | 59844bp | 63.12 | USA | Watermelon | Insertion Sequences metabolism | [75] | ICE_Tn4371_6036 | NC_008752 |
| Delftia acidovorans SPH-1 | 57901 bp | 63.66 | Germany | Activated sludge | czc metal resistance pumps | [76] | ICE_Tn4371_6037 | NC_010002 |
| Comamonas testosteroni KF-1 | 52455 bp | 63.77 | Switzerland | Activated sludge | czc metal resistance pumps | [76] | ICE_Tn4371_6038 | NZ_AA0100000 |
| Acidovorax sp. JS42 | 53489 bp | 62.88 | USA | Groundwater | Multidrug resistance pump Insertion Sequences | [77] | ICE_Tn4371_6039 | NC_008782 |
| Bordetella petrii DSM12804 | 47191 bp | 63.73 | Germany | River sediment | Aromatic compounds metabolism | [78] | ICE_Tn4371_6040 | NC_010170 |
| Burkholderia pseudomallei MSHR346 | 49278 bp | 62.21 | Australia | Melioidosis patient | metabolism | N/A | ICE_Tn4371_6064 | CP001408 |
| Paraburkholderia naphthalenivorans CJ2 plasmid pPNAP01 | 70106 bp | 62.89 | USA | Coal-tar-waste contaminated site | Biphenyl degradation | [79] | ICE_Tn4371_6065 | CP000530 |
| Diaphorobacter sp. TPSY | 49020 bp | 65.30 | USA | Soil | czc metal resistance pumps | [80] | ICE_Tn4371_6066 | CP001392 |
| Delftia acidovorans SPH-1 | 66755 bp | 64.94 | Germany | Activated sludge | Various types of metal resistance pumps | [76] | ICE_Tn4371_6067 | NC_010002 |
| Tn4371-like Elements | Size   | % GC Content | Location | Environment                       | Accessory Genes                                | Reference | Name                        | Accession Number |
|----------------------|--------|--------------|----------|-----------------------------------|-----------------------------------------------|-----------|-----------------------------|------------------|
| *Shewanella* sp. ANA-3 | 45233 bp | 59.43        | USA      | Arsenate treated wood pier        | Multidrug resistance pump                      | [81]      | ICE_Tn43716034              | NC_008577        |
| *Congregibacter litoralis* KT71 | 50661 bp | 59.52        | North Sea | Ocean-surface water               | RND type multidrug efflux pump                 | [82]      | ICE_Tn43716035              | NZ_AAOA01000008  |
| *Pseudomonas aeruginosa* 2192 | 48538 bp | 62.62        | USA      | Cystic fibrosis patient           | RND type multidrug efflux pump                 | [83]      | ICE_Tn43716041              | NZ_AAKW01000024  |
| *Pseudomonas aeruginosa* PA7 | 55287 bp | 52.38        | Argentina | Clinical wound isolate           | Multiple antibiotic resistance genes            | [84]      | ICE_Tn43716042              | NC_009656        |
| *Stenotrophomonas maltophilia* K279a | 43509 bp | 62.76        | UK       | Blood infection                   | Multidrug resistance pump                      | [85]      | ICE_Tn43716068              | AM743169         |
| *Pseudomonas aeruginosa* UCBPP-PA14 | 43172 bp | 65.55        | USA      | Burn patient                      | czc metal resistance pumps                      | [86]      | ICE_Tn43716069              | CP000438         |
| *Pseudomonas aeruginosa* PACS171b | 42156 bp | 64.12        | USA      | Cystic fibrosis patient           | Arsenate resistance pumps                       | [87]      | ICE_Tn43716070              | EU595746         |
| *Thioalkalivibrio* sp. HL-EbGR7 | 42540 bp | 64.95        | Unknown  | Bioreactor removing sulfide from biogas | Potassium transporter system                    | [88]      | ICE_Tn43716071              | CP001392         |
| Genes | Size (bp) | Primers | Tm (°C) | R. pickettii 12J Position | Accession no. |
|-------|-----------|---------|--------|--------------------------|---------------|
| CirIm | ~220      | RE1 GCATGGAAGACTTGACAG  
                  LE1 GAGCTTGAGTTTTGCCACG | 54 | N/A | N/A | FM244490 |
| int   | 1035      | intFor1 TTTTGAGTTTTGCACG  
                  intRev1 GAGAGCATCGATGCTTTCC | 61.7 | 2715201 | 2716235 | FM244486 |
| RepA, ParA, ParB | 1657 | RepAF GAGACTACCAGCGCCTCAAG  
                          RepAR ACGTGTTTCATGAGGACTTCTCC | 55 | 2734598 | 2736255 | FM244487 |
| traG  | 1483      | traGF GTTCGAGTGTTCTTTTCTTC  
                          traGR GAAATTGCTGTCCGCTTAGTAG | 61 | 2757179 | 2758661 | FM244488 |
| trbI  | 1597      | trbIF AACTGACCATGAGCCAGGAC  
                          trbIR AAAGCTCCTCAAGGCGGAAG | 62 | 2767516 | 2769113 | FM244489 |
**Table 4: Direct repeats at the ends of each element**

| Tn437I-like Elements                                      | Direct repeats |
|-----------------------------------------------------------|----------------|
| Ralstonia pickettii 12J                                   | TTTTTTCAT      |
| Shewanella sp. ANA-3                                      | TTTTTTAT       |
| Congregibacter litoralis KT71                             | TTTTTTAT       |
| Acidovorax avenae subsp. citrulli AAC00-1                 | TTTTTTCAT      |
| Delftia acidovorans SPH-1                                 | TTTTTTCAT      |
| Comamonas testosteroni KF-1                               | TTTTTTAT       |
| Pseudomonas aeruginosa 2192                               | TTTTTTAT       |
| Pseudomonas aeruginosa PA7                                 | TTTTTTGT       |
| Stenotrophomonas maltophilia K279a                        | TTTTTTGT       |
| Pseudomonas aeruginosa PACS171b                            | TTTTTTAT       |
| Diaphorobacter sp. TPSY                                    | TTTTTTCAT      |
| Delftia acidovorans SPH-1                                 | TTTTTTCAT      |
| Acidovorax sp. JS42                                        | NP             |
| Bordetella petrii DSM12804                                 | NP             |
| Thioalkalivibrio sp. HL-EbGR7                              | NP             |
| Burkholderia pseudomallei MSHR346                          | NP             |
| Polaromonas naphthalenivorans CJ2 plasmid pPNAP01          | NP             |
| Pseudomonas aeruginosa PA14                                 | NP             |

NP, Not Present
| Strain          | Source                                                                 |
|----------------|------------------------------------------------------------------------|
| *R. picketti*  | JCM5969, NCTC11149, DSM6297, CIP73.23                                   |
|                | CCUG3318                                                               |
| *R. picketti*  | CCM2846                                                                |
|                | CCUG18841                                                              |
| *R. insidiosa* | ATCC4199                                                               |
| *R. insidiosa*| LMG21421                                                               |
| *R. picketti*  | ULC193, ULC194, ULC277, ULC297, ULC298, ULC224, ULC421                 |
|                | Microbiology laboratory of Limerick Regional Hospital                   |
|                | (Cystic Fibrosis Patients)                                             |
| *R. picketti*  | ULI785, ULI788, ULI790, ULI791, ULI796, ULI798, ULI800, ULI801, ULI804, ULI806, ULI807, ULI818, ULI159, ULI162, ULI165, ULI167, ULI169, ULI171, ULI174, ULI181, ULI187, ULI188, ULI193 |
|                | Isolated from various Millipore Purified water systems (Ireland)       |
| *R. insidiosa* | ULI821, ULI797, ULI785, ULI181, ULI794, ULI185, ULI166, ULI189, ULI784, ULI163, ULI795 |
|                | Isolated from various Millipore Purified water systems (Ireland)       |
| *R. picketti*  | ULM001, ULM002, ULM003, ULM004, ULM005, ULM006                         |
|                | Isolated from various Millipore Purified water systems (France)        |
| *R. picketti*  | ULM007, ULM008, ULM009, ULM010, ULM011                                 |
|                | Isolated from various Millipore Purified water systems (Ireland)       |
| *R. insidiosa* | ULM008, ULM009                                                          |
|                | Isolated from various Millipore Purified water systems (Ireland)       |
Additional Files

Additional file 1:
Title: Alignment of the conserved domains among the site-specific recombinases of the tyrosine integrase family
Description: Alignment of the conserved domains among the site-specific recombinases of the tyrosine integrase family from phages, conjugative transposons, plasmids and other sources. R (Arginine) being in Domain I and H (Histidine)-R-Y (Tyrosine) in Domain II.

Additional file 2:
Title: Phylogenetic tree of the Integrase proteins from Tn4371-like integrases available on the GenBank database and other Phage and ICE integrases.
Description: Phylogenetic tree of the Integrase proteins from available Tn4371-like integrases available on the GenBank database and other Phage and ICE integrases. Cluster analysis was based upon the neighbour joining method. Numbers at branch-points are percentages of 1000 bootstrap resamplings that support the topology of the tree. The scale bar represents 0.2 substitutions per nucleotide position.

Additional file 3:
Title: Gene numbers for genes in the elements discovered in this study
Description: The gene numbering for genes of the elements discovered in this study. Genes with yellow background are the scaffold genes of the element.

Additional file 4:
Title: Alignment of the first/last 200bp of Tn4371-like ICEs using ClustalW.
Description: **Fig S1a**: Alignment of the first 200bp of Tn4371-like ICEs using ClustalW.  
**Fig S1b**: Alignment of the last 200bp of I Tn4371-like ICEs using ClustalW.
|                | Integration | Putative Stabilisation System | Accessory Genes | Putative Type IV Secretion System |
|----------------|-------------|-------------------------------|-----------------|-----------------------------------|
| **Tn4371**     |             |                               |                 |                                   |
|                | 6500        | 13000                         | 19500           | 26000                             |
|                | 32500       | 39000                         | 45500           | 52000                             |
| **Shewanella sp. ANA-3** |             |                               |                 |                                   |
|                | 6500        | 13000                         | 19500           | 26000                             |
|                | 32500       | 39000                         |                 |                                   |
| **C. litoralis KT71**        |             |                               |                 |                                   |
|                | 6500        | 13000                         | 19500           | 26000                             |
|                | 32500       | 39000                         | 45500           |                                   |
| **S. maltophilia K279a**     |             |                               |                 |                                   |
|                | 6500        | 13000                         | 19500           | 26000                             |
|                | 32500       | 39000                         |                 |                                   |
| **Thioalkalivibrio sp. HL-EbGR7** |             |                               |                 |                                   |
|                | 6500        | 13000                         | 19500           | 26000                             |
|                | 32500       | 39000                         |                 |                                   |
Figure 6
Additional files provided with this submission:

Additional file 1: additional file 1.pdf, 34K
http://www.biomedcentral.com/imedia/1771188492903409/supp1.pdf
Additional file 2: additional file 2.pdf, 41K
http://www.biomedcentral.com/imedia/9664021112903410/supp2.pdf
Additional file 3: additional file 3.xls, 146K
http://www.biomedcentral.com/imedia/1462018404290341/supp3.xls
Additional file 4: Additional File 4.pdf, 79K
http://www.biomedcentral.com/imedia/3141479903245721/supp4.pdf