

Distribution of horizontally transferred heavy metal resistance operons in recent outbreak bacteria

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Mankind is confronted by the outbreaks of highly virulent and multi-drug resistant pathogens. The outbreak strains often belong to well known disease associated species such as *Salmonella*, *Klebsiella* and *Mycobacterium*, but even normally commensal and environmental micro-organisms may suddenly acquire properties of virulent bacteria and cause nosocomial infections. The acquired virulence is often associated with lateral exchange of pathogenicity genomic islands containing drug and heavy metal resistance determinants. Metal ions are used by the immune system of macro-organisms against bactericidal agents. The ability to control heavy metal homeostasis is a factor that allows the survival of pathogenic microorganisms in macrophages. In this paper we investigate the origin of heavy metal resistance operons in the recent outbreak strains and the possible routes which may lead to acquisitions of these genes by potentially new pathogens. We hypothesise that new outbreak micro-organisms appear intermittently on an intersection of the non-specialized, genetically naïve strains of potential pathogens and virulence factor comprising vectors (plasmid and/or phages) newly generated in the environmental microflora. Global contamination of the environment and climate change may also have an effect towards the acceleration and appearance of new pathogens.

Despite the availability of complete genome sequences of multiple pathogens and the profound knowledge we encompass on both epidemiological and molecular aspects of bacterial pathogenicity, the sudden outbreaks that arise from new highly virulent pathogens do not cease to take mankind by surprise. The last striking outbreak was caused by an enterohemorrhagic *Escherichia coli* in Germany in 2011, where hundreds of people were affected. The first outbreak strain TY2482 was isolated at Hamburg-Eppendorf University Medical Center on May 25th and sequenced at the Beijing Genomics Institute in Shenzhen on 2nd of June¹. Isolation and sequencing of the other outbreak associated strains followed and a number of genome comparative studies were published²⁻⁴ which served as a demonstration of an impressive increase in the level of sequencing technologies and their relevance to clinical studies. The studies showed a connection between the new outbreak *E. coli* and an earlier

outbreak strain isolated in Africa ten years ago. However, the origin and mechanisms of emergence of the outbreak variants of the usually harmless *E. coli* remained generally unclear⁵⁻⁷ even upon the immense analysis.

A number of genomic islands (GIs) comprising of possible virulence determinants were identified in TY2482, and at least one of these possessed a plasmid associated mercury resistance operon⁸. Heavy metal resistance genes are often found in pathogenicity genomic islands (PAIs) of different highly virulent micro-organisms⁹⁻¹⁴. These are utilized by virulence micro-organisms for heavy metal sensing and removal which aid against the exploitation of a transition metal such as copper by mammalian immune defences¹⁵. Copper is known to be an essential toxicity component used by macrophages for killing pathogens within phagosomes¹⁵⁻¹⁷. However, the most frequent heavy metal resistance genes reported to be associated with virulence GIs and plasmids are the ones that make up mercury resistance operons. Schottel et al reported that in a collection of some 800 antibiotic-resistance plasmids isolated from clinical *E. coli*, 25% carried mercury resistance determinants¹⁸. The latter serves as an indication that these determinants are frequent in plasmids and may also be associated with microbes which confer resistance to antibiotics. In fact, the *mer* operons are highly versatile¹⁹ and it has recently been demonstrated experimentally that the sensitivity of mercury-sensing regulators may be re-directed by mutagenesis to sense other heavy metal pollutants²⁰. The role of the *mer* operons in pathogenicity remains unclear, but their prevalence in PAIs suggests that they may be important factors which pathogens may use for alternative functions such as transport and detoxification of antibiotics and other detrimental compounds. Furthermore, the roles of mercury resistance genes in bacterial resistance towards clinical disinfectants have also been reported²¹. In relation to the latter, an acquired antiseptic and disinfectant resistance of *Acinetobacter baumannii* was associated with the arsenic and mercury resistance operons¹⁴ which could possibly be of plasmid origin.

In this work we traced down the distribution of heavy metal resistance operons associated with mobile genomic regions. Initially, a total of 17,984 GIs were identified by SeqWord Sniffer program in 1,639 bacterial chromosomes and plasmids⁸. The GIs that showed DNA sequence similarity with the virulence plasmids pSB102, R478 and pSD_88^{9,10,22} comprising of multiple heavy metal resistance operons and also shared compositional similarity with GIs of enterohemorrhagic *E. coli*, were selected for a detailed analysis. From the analysis it was found that GIs sharing both sequence and compositional similarity were distributed among distant bacteria of different taxonomic orders (Fig. 1A). The latter finding implicates that these GIs are possibly associated with a wide range of plasmids. Plasmids may exchange transposable elements with the chromosomes or sometimes the whole plasmid may be integrated into a bacterial genome. Having been fixed on the chromosome, the mobile element undergoes amelioration, a process that smoothes out differences in oligonucleotide usage patterns of the host chromosome and that of the acquired element²³. Fig. 1B shows the results of a stratigraphic analysis of GIs, represented as linked nodes. The stratigraphic method calculates the distances in OU patterns between the GIs and host chromosomes and determines their relative time of acquisition. In Fig. 1B the nodes which are depicted by a lighter colour show higher levels of compositional similarity to the host chromosomes than those depicted by a darker colour, which probably still resemble the composition of their donor genomes. The colours of nodes are linked with the acquisition periods. Recent acquisitions are distant from the hosts in terms of composition they therefore have a darker colour. A colour in this case means that these GIs have not lost their specific original composition yet. Overtime these GIs get affected by the mutational pressures of their new hosts, they thus start to ameliorate and resemble the patterns of the host organisms. The lighter GIs in Fig. 1B are ancient acquisitions, which have been in the host chromosomes for longer, hence the

resemblance of the patterns. As illustrated, the heavy metal resistance GI of the strain *E. coli* 55989 depicted as the central node in the cell *b2* (Fig. 1B), appears to be an older insert as compared to the mercury resistance operon of *E. coli* TY2482 (Fig. 1B, cell *b3*). The former GI shares strong sequence and compositional similarity with the plasmid R478 isolated from *Serratia marcescens* in 1969¹⁰, while the latter is similar to the fragment of the plasmid pSD_88 isolated from *Salmonella enterica* in 2011²². *E. coli* TY2482 caused the outbreak in Germany in 2011 while *E. coli* 55989 appeared as the causative agent of a similar outbreak in Central African Republic in the late 1990s. Comparison of their genomes showed 99.8% identity and it was logical to conclude that TY2482 and 55989 shared a common ancestor²⁴. In contrast to commensal *E. coli* and pathogenic *Shigella* which share a variety of newly acquired virulence associated GIs, the genomes of TY2482 and 55989 comprise mostly of old uniform GIs which they inherited from their common ancestor except for those comprising mercury resistance operons. Their ancestor is believed to have been a genetically naïve organism which managed to avoid the major fluxes of gene exchange between enterobacteria for a very long time⁸. The virulent TY2482 which is thought to have evolved from this hypothetical ancestor acquired pathogenicity determinants such as Shiga-toxin encoding prophage; multi-drug and heavy metal resistance operons from both pathogenic enterobacteria²⁴ and environmental bacteria⁸. Fig. 1 shows that similar and much older mercury resistance GIs are harboured by β -Proteobacteria of *Acidovorax* and *Alicyclophilus* genera, which very likely were the initial hosts of these mobile genetic elements as far as we could see. These mobilomes were at later stages disseminated to other distant microorganisms such as mycobacteria through *Frankia/Nocardia* (cells *d1-e4* in Fig. 1) or directly from β -Proteobacteria (*c4-c5*). These elements also dispersed to *Pseudomonas* (*d4*), thereafter to the soil α -Proteobacteria of genera *Ochrobacterium*, *Methylobacterium*, *Arthrobacter*, *Xanthobacter*, *Oligotropha* and *Acidiphilum* (*d5*); and also Acidobacteria (*b5-c6*). It is an intriguing question whether the distribution of these GIs played a role in the evolution of pathogenic *Mycobacterium* or in the pathogenicity development of opportunistic nosocomial pathogens of *Ochrobacterium* and *Arthrobacter*. The most recent insertions of these GIs were found in virulent *E. coli* TY2482, 55989 and UMNO26; *Salmonella enterica* Typhi, Dublin and Newport; *Acinetobacter baumannii* AYE; in a clinical isolate *Pseudomonas aeruginosa* UCBPP-PA14 and a fish pathogen *Aeromonas salmonicida* A449. It is remarkable that the metal resistance operons are more frequently identified in recently acquired GIs of this group (Fig. 1). This observation is in consistence with the discovery by Ramaiah and De that in a period of 5 years (1997 to 2003) the number of microorganisms isolated from sea water which were tolerant to 10 ppm of HgCl₂ increased from none to 75% or even 95% of CFU in the polluted zones of Mumbai²⁵. The general environmental pollution may be behind the activation of these dormant GIs and making the genes which increase tolerance to heavy metals and other pollutants to be on demand by a great range of environmental bacteria. Having reached potentially pathogenic bacteria, these genes get adjusted towards resistance against drugs and antibacterial immune responses^{18, 26}.

The map of relations between GIs in Fig. 1 illustrates possible paths for the distribution of PAIs supported by the compositional similarity between GIs and sharing of similar DNA sequences identified by blastn. It was reported that when compositional similarity reaches 75% and above the likelihood to find homologous DNA sequences in a pair of GIs rapidly increases. The latter statement is in support of the common ancestry hypothesis⁸. However, it has to be noted that GIs which show strong compositional similarity may possess different fragments, only few of these contain matching sequences.

The figure suggests that *Klebsiella* (cell *b3*) could possibly acquire GIs from *Salmonella* and *Acinetobacter*. It could probably be that the latter had truly happened, as an outbreak of multidrug

resistant *K. pneumonia* was reported in Rotterdam hospital and several other places²⁷ in 2011 and it followed the spread of drug-resistant *A. baumannii*¹⁴. The analysis of previous fluxes of GIs also implicates a possible transfer of new virulence genes to *Brucella* (Fig. 1, cell a4), *Mycobacterium* and *Nocardia* (cells d2-3), which may pose a serious impact on human health. There are many GIs in *Mycobacterium*, which most likely originated from β -Proteobacteria as discussed above (Fig. 1). All these are quite old and void of heavy metal resistance operons, but this does not rule out the possibility for new PAIs to be acquired by mycobacteria in future. An unexpected high frequency of mercury-resistant strains showing also an increased tolerance to gentamicin, streptomycin and D-cycloserine has been reported among the clinical nontuberculous mycobacteria isolates of the species *Mycobacterium avium*, *M. intracellulare*, and *M. scrofulaceum*²⁸. The genome of a fish pathogen *Mycobacterium marinum*, which sometimes causes opportunistic infections in humans, comprises a 23-kb mercury-resistance plasmid pMM23²⁹. Blastn and oligonucleotide composition analysis showed that this plasmid together with the mercury resistance operon which it possesses originate from either *Nocardia* or *Pseudonocardia* (Fig. 1). It is very likely that this plasmid has been acquired by *M. marinum* quite recently as it shows very strong sequence and pattern similarities to *Nocardia*. These newly acquired genes may be behind the reported increased drug resistance of *M. marinum* isolates. A mercury resistance plasmid similar to that of *M. marinum* together with multiple GIs of *Pseudomonas* and *Actinobacteria* origin were identified in *Mycobacterium abscessus*, a pseudotuberculous lung disease causing microbe³⁰.

Drug and heavy metal resistance genes are abundant in the bacterial world. It is likely that these genes started being abundant in microorganisms soon after life began, when the world was heavily polluted with volcanic activity¹⁹. A recent study has revealed that at least 30,000 years ago the major antibiotic resistance genes were already present, as these were identified in a permafrost sediment DNA sample³¹. In spite of the abundance of potential virulence factors in nature, there is a clear periodicity in the occurrence of outbreaks. In our previous publication⁸ we hypothesised that this periodicity may reflect the oscillation of genetic vector activity. Bacteria develop resistance against foreign invasions initiated by existing vectors (plasmids and phages) and the efficiency of horizontal gene exchange gradually decreases. Thereafter, new vectors evolve by undergoing several mutations which allow them to evade host resistance mechanisms³² and get incorporated into host chromosomes. These bring new virulence genes or simply activate the gene exchange between potentially pathogenic micro-organisms that eventually lead to the achievement of effective combination of pathogenicity determinants in new virulence GIs⁸. Mutations which arise from these vectors in order to gain access to host organisms allow the interchange of various genomic fragments between bacteria. The interchanges may include fragments from both plasmids and phages. The latter could be the result of hybrid genomic islands which have been reported to possess elements of both: temperate phages and conjugative plasmids^{33, 34}.

There is a general belief that the infection outbreaks may possibly be caused by former commensal strains after their acquisitions of PAIs from pathogenic bacteria or by the change of a host niche³⁵. Analysis of the distribution of GIs showed that commensal *E. coli* and pathogenic *Shigella* share mobile genetic elements⁸, but this did not turn either commensal *E. coli* to pathogenicity or pathogenic *Shigella* to commensalism. These microorganisms are highly specialized and saturated with horizontally acquired elements. One more acquisition will not be sufficient to change their lifestyles. Acquisition of a GI may have more profound effects on genetically naïve micro-organisms which were initially not exposed to the horizontal exchange events due to geographic isolation. This was the case with the latest outbreak caused by a naïve *E. coli* TY2482 after it acquired a number of GIs from a general pool of PAIs of enterobacteria, and from environmental micro-flora. Climate change and general pollution may mix bacterial populations and activate previously dormant vectors for which all current pathogens will be “genetically naïve”, i.e. not exposed to these types of genetic

vectors and thus become more vulnerable. Again this probably was the case with *E. coli* TY2482, after it acquired mercury resistance operons from micro-organisms which are not known to be the usual sources of PAIs for enterobacteria⁸. Our further work will be aimed at monitoring unusual recent GI inserts in potentially pathogenic micro-organism and tracking down organisms which are likely to be probable donors and recipients of such entities. Potentially, this monitoring system will allow the likelihood estimate of new deadly outbreaks in future.

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Fig. 1. The figure illustrates the grouping of GIs which share similarities in both composition and sequence. Each node corresponds to one GI. Nodes sharing compositional similarity of 75% and above are linked by edges. As it was shown previously, this level of compositional similarity implies common ancestry between mobile genomic elements⁸. Plasmid genomes are depicted by ovals. More details about these GIs are available on an interactive map at <http://www.bi.up.ac.za/SeqWord/maps/map.html>. A) Nodes are coloured according to the taxonomic belonging of the host micro-organisms. B) Grey gradient colours depict divergence of GI pattern from the tetranucleotide usage pattern of the host chromosome.

