pSTM6-275, a conjugative IncHI2 plasmid of Salmonella that confers antibiotic and heavy metal resistance under changing physiological conditions

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Running Head: IncHI2 plasmid structure, transfer and resistance

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Abstract. Detailed annotation of an IncHI2 plasmid, pSTM6-275, from *Salmonella enterica* serotype 1,4,5,12:i:- TW-Stm6 revealed a composite structure including antimicrobial resistance genes on mobile genetic elements. The plasmid was thermosensitive for transfer to *E.coli* and conferred reduced susceptibility to antibiotics, copper sulphate and silver nitrate. Metal ion susceptibility was dependent on physiological conditions, giving an insight into the environments where this trait might confer a fitness advantage.
Salmonella enterica is a common enteric pathogen of humans and animals, and is found in many environmental and animal reservoirs with zoonotic potential. Distinct clones of multi-drug resistant S. enterica serovar Typhimurium have emerged and dominated in succession (1, 2). A recent clone of S. Typhimurium (SO4698-09) carries a genomic island which contributes to enhanced resistance to copper sulphate, a common animal feed additive (3). We recently reported the genome sequence of S. Typhimurium (SO4698-09), which has the same antigenic formula, phage type and sequence type as strain SO4698-09, and also carries the genomic island, SGI-4 (3-5). The assembled genome of TW-Stm6 comprised a 4,999,862 bp chromosome, a 4 kb MOB0 plasmid (pSTM6-4); and a 275.8 kb IncHI2 plasmid (pSTM6-275). Here we report the detailed annotation of pSTM6-275, its genetic structure, function and transmission of antibiotic and heavy metal resistance genes to other bacteria.

Annotations were revised using EcoGene v3 (www.ecogene.org), UniProt (www.uniprot.org), RFAM (rfam.xfam.org), BLAST (blast.ncbi.nlm.nih.gov) and literature searches. Plasmid typing was performed using PlasmidFinder (cge.cbs.dtu.dk/services/PlasmidFinder-1.3), Plasmid MLST database (pubmlst.org/plasmid), and local searches of custom database sequences and IncHI typing (7). IS elements were typed using ISFinder (https://www-is.biotoul.fr). A diagram of the 275,801 bp plasmid pSTM6-275 (CP019647.1) is shown in Fig. 1A. Many genes were clustered in functional units, such as the ter operon (tellurium resistance); the sil locus (silver efflux), the pco locus (copper efflux) and the transfer regions Tra1/Tra2. Tra2 has an origin of plasmid transfer, oriT; a potential replication terminus (ter) site and a tus gene encoding the replication-termination protein Tus. The plasmid copy number, determined by normalising sequence depth relative to the chromosome was 1.4. In silico typing of plasmid pSTM6-275 indicated it belonged to the IncHI2 (subtype 3), and was...
similar to plasmid IncHI2 reference plasmid R478 (NP_941280.1) (Fig. 1B). BLASTN searches of the GenBank database (Oct 4, 2017) failed to find any other plasmid that matched (≥99% nt identity) more than about 70% of pSTM6-275. Figure 2A depicts regions that are rich in mobile genetic elements, including IS elements, transposons and integrons, and containing multiple resistance genes (blaTEM, strA, strB, sul3, aadA1, aadA2, cmlA, aphA2 and two copies of tetA) encoding resistance to ampicillin, streptomycin, spectinomycin, sulphonamide, trimethoprim, chloramphenicol, kanamycin and tetracycline. Copies of blaTEM, strA, strB, tetB and sul2 occur on the chromosome in SGI-4 (5). The class I integron In27 (dfrA12_gcuF_aadA2_AqacE), encoding resistance to trimethoprim and streptomycin/spectinomycin, also contains a truncated sul1 gene, disrupted by IS26. A second integron, In1412, classified as a novel class I integron (8), was 9240 bp and contained the array estX3_psp_aadA2_cmlA1_aadA1_qacHD4::IS1203 conferring resistance to streptomycin/spectinomycin and chloramphenicol (Fig. 2). A fragment of the macrolide efflux MFS gene mefB (9) and a sul3-element/domain (10) lie proximal to qacH and are flanked by divergent IS26 elements.

A Tn7-family transposon (32.4 kb) carrying silver and copper resistance loci, silESRCFBAGP and pcoGE/ABCDRSE2 (Fig. 2A), occurs between the integrons and is delimited by inverted terminal repeats and flanking 5bp direct repeats (Fig. 1.) Similar elements have been detected in other IncHI2 plasmids from animal-associated bacteria (11). Sil and Pco systems are comprised of metal ion-binding proteins and transporters (12-14). A sil-pco locus with the same gene arrangement also occurs on the TW-Stm6 chromosome in SGI-4, but unlike the plasmid version it lacks Tn7-like msABCD genes and inverted terminal repeats, suggesting a different evolutionary history.
Many IncHI2 plasmids are thermosensitive for transfer, with 27-33°C being the permissive temperature and >37°C non-permissive (15). Since pSTM6-275 has all the genes for proteins required for self-transmission, this function and its thermosensitive character were examined. Plasmid stability and conjugal transfer of pSTM6-275 from Salmonella to E. coli DH5α were tested according to published methods (15) and the results are from two experiments performed in duplicate. Transfer occurred at 1.3 x 10^5 transconjugants per donor at 27°C but no transfer was detected at 37°C. Transconjugants coinherited resistance to ampicillin, sulphonamide, streptomycin, spectinomycin, kanamycin, tetracycline, trimethoprim and chloramphenicol consistent with the plasmid structure. Colonies (n=112) derived from a culture of the transconjugants, grown at 44 °C for 24 hr without antibiotic selection retained all resistance markers, indicating the plasmid was not thermosensitive for maintenance in E. coli. In this respect, pSTM6-275 differs from the reported phenotype of plasmid R478 (15).

Minimum inhibitory concentrations of CuSO₄ and AgNO₃ were determined by agar dilution assays using LB agar (pH 7.2, 25mM HEPES) (11, 13) using Oxoid AnaeroGen™ sachets if required. The sensitivity to of Salmonella TW-Stm6; E. coli DH5α and two E. coli pSTM-275 transconjugants are shown in Table 1. Both transconjugants had the same MICs. The Salmonella donor had a higher AgNO₃ MIC at 27°C (800µM) than 37°C (50µM) and the MIC was not influenced by oxygen availability. The plasmid increased the MIC of AgNO₃ for E. coli from 200 to 800µM at 27°C however this effect was not seen at the higher temperature where the MIC for all strains was 50µM. The MIC to CuSO₄ for E. coli was affected by oxygen. E. coli was most sensitive to CuSO₄ under anaerobic conditions but plasmid-bearing transconjugants were less sensitive possibly due to more efficient efflux.
ST3 IncHI2 plasmids are widely spread in food producing animals (11) and despite their potential to disseminate antimicrobial resistance genes, few complete sequences have been characterised in detail. Our results suggest that transmission of pSTM6-275 is probably restricted to outside a mammalian host given the thermosensitive nature of transfer, suggesting it is well adapted for persistence in the environment. Furthermore, expression of at least some of the metal resistance traits were influenced by physiological conditions. Copper metabolism in enterobacteria is complex, as several genes can be involved, including those involved in transport, oxidation and regulation. In the present study, *E. coli* DH5α was sensitive to 1.56mM CuSO₄ without oxygen and acquisition of pSTM6-275 decreased the sensitivity to 6.25mM under anaerobic conditions. *E. coli* can regulate copper levels by expressing chromosomal genes encoding a periplasmic copper oxidase, CueO, a cytoplasmic copper transporter, CopA and the Cus efflux system (16). In the presence of oxygen and amino acids, copper homeostasis is achieved by CueO oxidation and CopA-mediated efflux. Cus is induced under anaerobic conditions or nutrient limitation and *E. coli* CueO and Cus are not sufficient to confer Cu(I) resistance under anaerobic conditions where nutrients are plentiful (17). *Salmonella* does not have a *cus*-encoded copper efflux pump and relies on CueO and CopA for copper homeostasis (16). CueO is sufficient for low level Cu(I) tolerance and is required for virulence in mice (18). The high level (800µM) and temperature dependence of silver sensitivity shown by TW-Stm6 and *E. coli* pSTM6-275 transconjugants was unexpected. To our knowledge, this has not been previously reported and the mechanism underlying this phenotype is unclear. It may be due to differences in thermoregulation of *sil* gene expression, the effect of temperature on the secondary structure of SilE/PcoE that alter the amount of ion binding or changes in the outer membrane composition (19, 20).
pSTM6-275 carries a novel class I integron, In1412, that is most similar to the sul3-integron type IIIc region of the *E. coli* plasmid pRYC306 (HQ875016.1). To evolve from pRYC306 to pSTM-275, one could hypothesise that (i), an IS440 element inserted into qacH/I; (ii), an IS26 element inserted in *mefB*, in the opposite orientation to the IS26 element near sul3, and (iii), an inversion occurred via the outward facing IS26 elements, splitting the 8bp direct repeats originally on the IS26 near the sul3 gene so they end up on two separate IS26 copies.

IS26 is a frequently occurring and highly active insertion element in the genomes and plasmids of *Salmonella*, commonly mediating recombination events that generate new types or combinations of virulence determinants (21). Recently documented examples include novel plasmids and chromosomal loci (22-24).

The evolution of pSTM6-275 appears to be complex, and the function and regulation of many of its genes remain to be fully characterized, particularly accessory genes such as those involved in resistance, regulatory cross-talk and those specifying uncharacterized proteins with unknown function. Identification of other ST3 IncHI2 plasmids from human, veterinary and environmental sources may provide further insights into the evolution of these plasmids and their role in dissemination of resistance. This work adds to our understanding of the organisation and function of a ST3 IncHI2 plasmid which may confer a fitness advantage for persistence in agricultural effluent.

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FIG 1 A. Diagram of plasmid pSTM6-275 (275.8kb). Tracks show (from outermost to centre); a, scale in kbp; b, predicted coding sequences (CDS) of the top (blue) and bottom (black) strands; c, transposons and IS elements (red); d, integrons (green) and Tra regions (grey); e, GC%; f, GC-skew; and g, cumulative GC-skew.

B. Alignment of pSTM6-275 and the IncHI2 reference plasmid R478 (BX664015.1). Regions of nucleotide sequence similarity ≥85% are indicated in red. Backbone regions and some of the more important proteins and loci of pSTM6-275 are indicated at the top.

FIG 2. A, Gene map of the resistance regions of pSTM6-275. Antibiotic-, metal- and disinfectant-resistance genes are clustered into two, nearby regions, 87.1-151.4 kb (upper panel) and 162.1-173.7 kb (lower panel). In each panel, genes and operons are shown above the horizontal line, and IS elements, transposons and integrons are displayed under the line. Integron attC sites are shown as triangles within integron borders. The scale at the top indicates kbp.

B. Comparison of sul3-integron type IIIc region of the E.coli plasmid pRYC306 (HQ875016) with the corresponding region of pSTM6-275. Near identical sequences are shaded red, or grey (for IS26 elements). As indicated in the diagram, the flanking 8bp direct repeats (CTTAGGTC) of pRYC306 IS element IS26 (nt 7321 - 6502) are found split between the two leftmost copies of IS26 in pSTM6-275. A 49 bp sequence, depicted as solid blue arrows close to the horizontal lines, occurs twice in pRYC306 (near IS26 and near sul3) and once in pSTM6-275. Size scales, in kbp, are shown at the top and bottom.
Table 1. Minimal inhibitory concentrations of CuSO₄ (mM) and AgNO₃ (µM)

| temp | 37°C | 37°C | 27°C | 27°C |
|------|------|------|------|------|
| oxygen | + | - | + | - |
| Donorᵃ | 50 | 50 | 800 | 800 |
| AgNO₃ Recipientᵇ | 50 | 50 | 50 | 50 |
| Transconjugantsᶜ | 50 | 50 | 800 | 800 |
| Donorᵃ | 12.5 | 12.5 | 12.5 | 12.5 |
| CuSO₄ Recipientᵇ | 6.25 | 1.56 | 6.26 | 1.56 |
| Transconjugantsᶜ | 6.25 | 6.25 | 6.25 | 6.25 |

ᵃ Salmonella Typhimurium TW-Stm6,ᵇ E. coli DH5α,ᶜ 2 transconjugants
