[Frontiers in Bioscience, Elite, 7, 423-433, June 1, 2015]

Induction of crossover by introduction of aro554:Tn10 into *Salmonella* chromosome

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1. ABSTRACT

One of the ancient methods for the generation of bacterial mutants was to insert the composite transposable elements (e.g. Tn*10*) flanked by desired gene sequences, into the bacterial chromosome. This mechanism of DNA integrating into a chromosome can sometimes not only lead to the creation of desired mutants but also induced other recombination event within the chromosome. Several studies have reported alterations such as deletion, insertion, inversion and both deletion/inversion in the bacterial chromosome due to the insertion of composite transposable elements. In this study it has been found that a Tn*10* mutagenesis event not only leads to the inactivation of desired gene (Δ*aorA*), and consequential deletion of other genes upstream of *aroA* and insertion of IS*10*, also has resulted in a large-scale chromosomal rearrangement in the *Salmonella* Typhimurium chromosome. This rearrangement consists of exchange of genetic material between the 10 minute and the 19 minute on a circular chromosomal map (approximately 440 kbps), possibly due to crossover between the two regions. Results from this study are the first evidence of such a large scale rearrangement in the bacterial genome due to the insertion of transposable elements.

2. INTRODUCTION

Salmonella Typhimurium mutants harbouring mutations in *aroA, aroC, aroD, htrA, ompR* or *phoPQ* have been developed and evaluated as potential vaccines. Some of the mutants have also been tested for their ability to deliver heterologous antigens of bacterial, viral and parasitic origin (1-3). The induction of strong humoral and cellular responses makes mutant *Salmonella* spp. ideal candidates for attenuated bacterial vaccines or for vectored antigen delivery (4-6) because they induce both TH1 and TH2 immunity.

STM-1 is a derivative of *S.* Typhimurium harbouring an *aroA* mutation, which causes a complete, non-reverting aromatic biosynthesis (aro) defect. This strain has been used as a vaccine to protect poultry, calves and adult cattle against *Salmonella* challenge (7), and has also been used experimentally for the delivery of heterologous antigen (8).

However, to date the genetic basis of attenuation of STM-1 has not been fully characterised. Therefore to enable specific site directed insertion of foreign gene (s) into a defined site of the STM-1 chromosome, it was necessary to determine the exact nature of the attenuation.

The *aroA* attenuation in STM-1 was created according to the method described by Hoiseth and Stocker (9). The Polymerase Chain Reaction (PCR), Southern blot analysis, and Pulse Field Gel Electrophoresis (PFGE) were applied to identify the changes in the chromosome of STM-1 with respect to the parent strain (*S.* Typhimurium 82/6915).

3. MATERIALS AND METHODS

3.1. Growth media and supplements

All *E. coli* and *Salmonella* strains were grown on solid microbiological media under aerobic conditions at 37°C for 16h. In instances where broth cultures were used, the strains were grown aerobically at 37°C for 16h on a Ratek orbital shaker at 120-150 rpm. Antibiotic supplements were added to an appropriate concentration when required.

3.2. PCR amplification

It was assumed in this study that the parent strain of STM-1, *S.* Typhimurium 82/6915 has a similar architecture to LT2 (as shown in Figure 1). Subsequent experiments have shown this assumption to be correct. All primers used were designed according to the LT2 sequence. The PCR amplifications were carried out in 25 to 50 µL reactions containing: 1-2 U Taq DNA polymerase (Perkin Elmer, USA), 1.5 to 2.0 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-Cl (pH 9.0) (Invitrogen, USA), 0.2 mM dNTPs (Roche Diagnostics, Germany), 50 - 100 ng genomic DNA and 0.2 mM of each primer. The primers used in this investigation are listed in Table 2. The PCR cycling conditions were as follows: denaturation at 94°C 1-2 min, denaturation at 94°C 30 secs, annealing at 50-65°C 30 sec and extension at 72°C at 30 s/kb for 30-35 cycles. The Expand Long Template DNA polymerase (Promega) was either used to amplify DNA segments >4kb in size or where proof reading activity was required. The PCR amplifications were carried out in 25 to 50 microliter reactions containing: 1-2 U Expand Long Template DNA polymerase, Promega buffer 1 or buffer 3 and 0.2 mM dNTPs, 50-100ng genomic DNA.

3.3. DNA sequence analysis

All DNA sequencing was carried out using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (supplied by Monash University, Australia) according to the manufacturer's instructions.

3.4. Southern blotting

The *aroA, pflB* and *rfbL* gene products were labelled to be used as probes. The labelling was performed using the DIG DNA Labelling and Detection kit (Roche Diagnostics, Germany) based on the random priming system. All procedures were carried out according to the manufacturer's instructions using digoxigenin (DIG)-dUTP as the label. The concentration of the probe was determined by a spot test with DIG-labelled control DNA. Approximately 3-5 microgram genomic DNA of STM-1 and *S.* Typhimuirum parent strain was digested with *Sac*I (10 U) (Promega USA) restriction enzyme. After electrophoresis in a 1percent agarose gel for 2h at 80 V, the gel was treated with depurination solution (HCl (0.25 M) diluted in dH_2O) for 10 min and twice in denaturing solution (NaOH (0.5 M), NaCl (1.5 M) dissolved in dH_2O) for 10 min each, followed by two 15 min washes in neutralisation solution (Tris-HCl (0.5 M pH 7.5), NaCl (3M). The DNA was transferred to the nylon membrane via capillary transfer. The DNA was hybridised overnight with denatured probes (20 ng/mL) at 65°C in standard hybridisation buffer (SSC (5X), N-lauroylsarcosine (0.1%), SDS (0.02%) and Blocking Reagent (1%) diluted in dH_2O). Following hybridisation the membrane was washed twice with 2 x SSC wash buffer for 5 min at room temperature, twice in 0.5 x SSC wash buffer for 15 min at 65°C and equilibrated in maleic acid wash buffer (Tween® 20 (0.3 percent w/v) in Maleic Acid Buffer) and blocked for 1h at room temperature in blocking solution. The blocking solution was replaced with anti-DIG Antibody Solution (Anti-DIG antibody 1:5000 dilution in blocking solution) and incubated for 1h at room temperature. Unbound antibody was removed by two washes in maleic acid wash buffer for 10 min at room temperature. Substrate solution (200 µL NBT/BCIP substrate solution diluted in 10 mL of detection buffer) was then added and the membrane placed in the dark until a colour precipitate had formed.

3.5. Pulsed field gel electrophoresis

The STM-1 and the parent strain were grown in 5 mL LB at 37°C for 3-4h. The bacterial cells were collected after centrifugation. The cells were washed in PBS, re-suspended in 250 microliter of PIV buffer (Tris (10 mM, pH 8.0), NaCl (1M) and kept on ice.

The cell suspension was mixed with an equal volume of 2 percent low melting point agarose (dissolved in PIV buffer) and poured into sterile, pre-chilled moulds. The plugs were allowed to set, the cells were lysed in 0.5 mL PFGE lysis buffer (Tris-HCl (6 mM), NaCl (1 M) and EDTA (0.1M). Following lysis, the plugs were allowed to harden at 4°C for 20 min and incubated in 0.5 mL of ESP solution (EDTA (0.5 M), N-lauryl sarcosine (1 percent w/v) and Proteinase K (1 mg/mL), at 50°C overnight. After overnight incubation, the plugs were hardened at 4°C for 20 min, and then incubated in 0.5 mL of Fluoride/TE solution (Phenyl methyl sulfonyl fluoride (18.6 mg/mL) in of isopropanol (1 mL). The plugs were then washed 6 times in TE buffer over 6 h at 37°C following fluoride treatment.

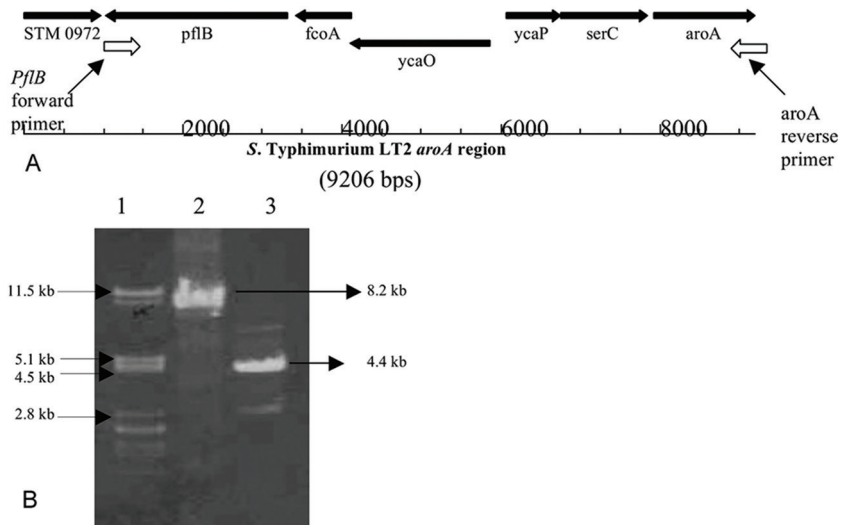

Figure 1. a. Map of the aroA region in *S.* Typhimurium LT2 at 19 minutes at location 48. The parent *S.* Typhimurium 82/6915 is assumed to have similar genomic architecture as the LT2 sequence. b. Amplification of the Pflb-aroA region. The product in the wild type is 8.2 kb whereas in STM-1 the product is 4.4 kb, a difference of approximately 3.8 kb. Lanes: 1, lambda x *Pst*1 marker; 2, Parent Strain; 3, STM-1.

Table 1. Bacterial strains used in this study

Bacterial strain/plasmids	Genotype or description	Reference/source
Escherichia coli		
DH5a	*supE ΔlacU169 (ϕ80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Hanahan, 1983 Cite by Reference number
TOP10F'	*F' lacIq, Tn10 (*TetR*)), mcrA, D (mrr-hsdRMS-mcrBC), f80lacZDM15, DlacX74, deoR, recA1, araD139 D (ara-leu) 7697, galK, rpsL (*StrR*), endA1, nupG*	Invitrogen
Salmonella Typhimurium		
LT2-9121	*leu hsdL trpD2 rpsL120 ilv452 metE551 metA22 hsdA hsdB*	Prof P. Reeves, Department of Microbiology, University of Sydney
82/6915	Wild type parent strain of STM-1	RMIT Biotechnology
STM-1	*ΔaroA⁻ Δserc⁻*	RMIT Biotechnology

A portion of the DNA plug was cut and digested with 10-15U of restriction enzyme for 6-8h at 37°C. Following restriction enzyme digestion, the DNA plugs were inserted into the wells of 1% agarose gel in TBE buffer and the wells were sealed with 2% low melting molten agarose. The agarose gel was then subjected to PFGE at 220 V for 22h (PFGE CHEF-DR II unit, Biorad) with the pulse time ramped from 2.2- 63.8 s. The gel was stained in ethidium bromide and examined by UV transillumination.

4. RESULTS

4.1. Amplification of PflB-aroA region

To characterise the STM-1 sequence upstream of *aroA*, the PflB-aroA region was amplified using the pflB forward primer and aroA reverse primer as shown in Figure 1a. The wild type *S.* Typhimurium parent strain was used as a positive control for all PCR's. In the parent strain the amplified product was 8.2 kb as expected from the LT2 sequence, but the product amplified in STM-1 was only 4.4 kb, Figure 1b. The amplified PCR product was ligated into pCR2.1 (Invitrogen), to characterise the unknown region upstream of *aroA* located at 19 minutes on the *S.* Typhimurium circular map.

4.2. Sequence analysis of amplified PCR products

Sequence analysis of the aroA-pflB region was performed using the chromosomal walking method to sequence the entire 4.4 kb product. This sequence analysis indicated an insertion and deletion in STM-1, with respect to the parent strain. The IS*10* left hand side (IS*10* LH) of the Tn*10* element (approximately 1.4 kb) is inserted adjacent to *aroA*. Several genes including *serC*, *ycaP*, *ycoA*, *fcoA* and part of the *pflB* have been deleted. Also deleted is the first 90 bp of the *aroA*

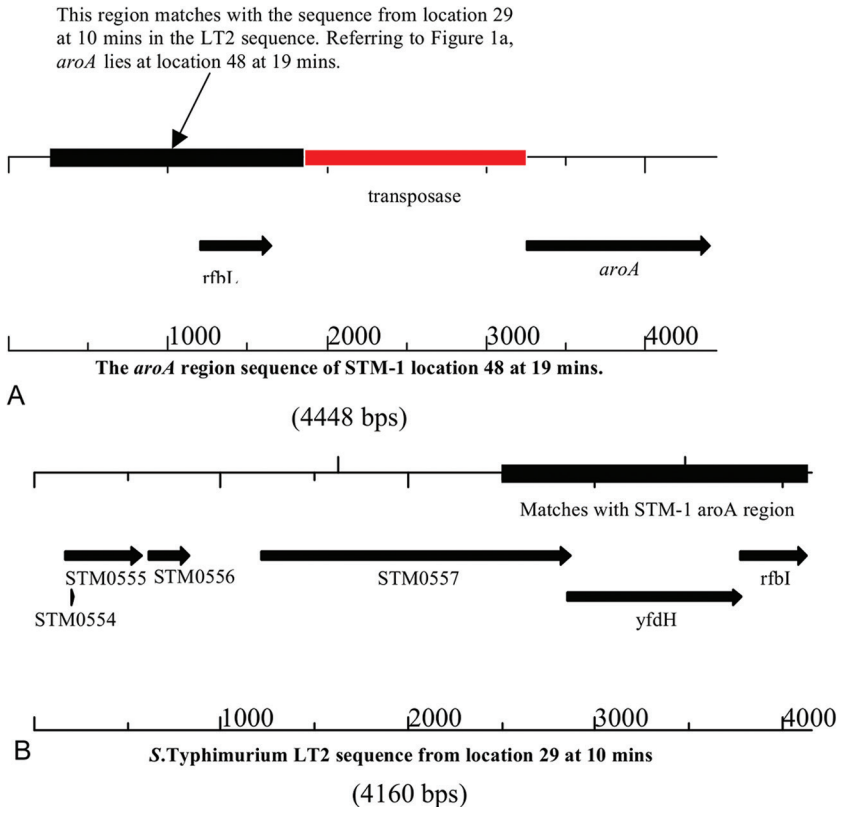

Figure 2. a. Sequence analysis of STM-1 around the *aroA* region. The sequence region depicted in the red bar matches with the IS*10* LH of the Tn*10* element. The sequence depicted in blue is the *S.* Typhimurium sequence that matches with the sequence from location 29 approximately at 10 mins in the circular map of *S.* Typhimurium LT2. The *aroA* gene is at 19 mins, location 48, a difference of 440 kb. b. *S.* Typhimurium sequence from location 29 at 10 minutes. The sequence marked in blue matches with the STM-1 sequence from location 48 at 19 mins..

gene (Figure 2a). The *serC* gene, which encodes an enzyme involved in serine biosynthesis is of metabolic importance for the growth of the bacterial cell, the *pfl*B gene encodes pyruvate formate lyase, which is a central enzyme in bacterial anaerobic metabolism catalysing the reversible reaction of pyruvate and coenzyme A into acetyl-CoA and formate (10), *ycaP* encodes for a putative inner membrane protein, *ycaO* encodes for a putative cytoplasmic protein, and *fcoA* encodes for a putative formate transporter. Each of these genes are appeared to be missing in STM-1.

The sequence analysis also indicates the possibility of a chromosomal rearrangement, as the sequence adjacent to the IS*10* LH completely aligns with *S.* Typhimurium LT2 sequence from location 29, at 10 minutes (Figure 2b), whereas *aroA* lies in the location 48 at 19 minutes on the LT2 map, a difference of approximately 440 kb. This sequence at location 29 of LT2 at 10 minutes matches with the *rfbL* gene of phage P22.

4.3. Amplification of the *serC, ycaO, ycaP, fcoA, pflB* and *sopD* genes

Attempts were made to amplify *ycaO*, *ycaP, fcoA, pflB* and *sopD* genes to confirm the sequence analysis results (Figure 3.a, 3.b and 3.c). The negative PCR results confirmed the deletion of genes *serC, ycaP ycaO and focA* from the *aroA* region in STM-1, a deletion of approximately 5 kb. However, the *pflB* and *sopD* genes initially thought to be deleted, as determined by sequence analysis, were found to be present after using the PCR amplification to identify individual genes (Figure 3.d).

4.4. Amplification of the pflB-aroA region with pflB and aroA170 bp primers

The pflB-aroA region was amplified using a pflB forward primer, but a different reverse primer designed at 170 bp downstream of the *aroA* gene (Table-2), to confirm the presence of the pflB gene in the aroA region of STM-1 chromosome.

The result in the parent strain was as expected but there was no amplification in STM-1 (Figure 4). The PCR further confirms that although the *pflB* gene is present in the STM-1 chromosome it does not exist in the same region as in the parent strain, which indicates the possibility of chromosomal rearrangement, and suggested that this rearrangement may be more complex than a simple inversion.

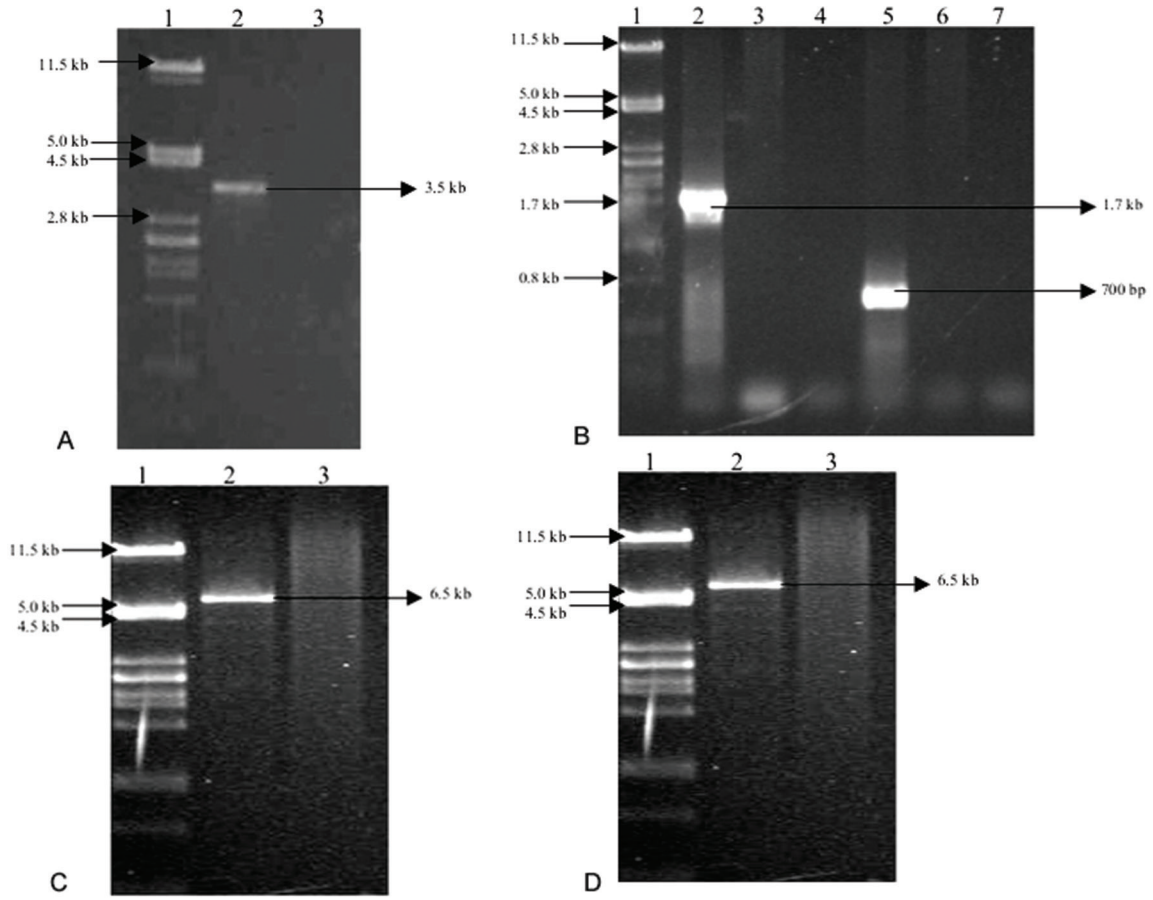

Figure 3. a. Amplification of the *serC* gene. The PCR confirms the deletion of the *serC* gene from STM-1. Lanes: 1, lambda x *Pst*I marker; 2, strain; 3, STM-1. 3b. Amplification of the *ycaP* and *ycaO* genes. The PCR confirms the absence of the *ycaP* and *ycaO* genes in STM-1. Lanes: 1, lambda x *Pst*I marker; 2, parent strain (*ycaO* gene); 3, STM-1 (ycaO gene); 4, No DNA control; 5, parent strain (*ycaP* gene); 6, STM-1 (*ycaP* gene); 7, No DNA control. 3c. Amplification of the *fcoA* gene. The PCR confirms the absence of the *fcoA* gene. Lanes: 1, lambda x *Pst*I marker; 2, parent strain; 3, STM-1. 3d. Amplification of the *sopD* and *pflb* genes. The PCR result illustrates the presence of the *pflB* and *sopD* genes initially thought to be missing after sequence analysis of the STM-1 chromosome. Lanes: 1, parent strain; 2, STM-1 (*sopD* gene); 3, no DNA control; 4, lambda x *Pst*I marker; 5, parent strain; 6, STM-1 (*pflB* gene).

4.5. Amplification of the *rfbL* gene

The sequence analysis of STM-1 also showed that the sequence which exists at location 29 in the parent strain, now exists adjacent to the *aroA* gene in STM-1 which is at location 48 (Figure 2b).

The amplification of this gene by PCR confirms the presence of this sequence in both the parent strain and in STM-1 (data not shown). As mentioned earlier the sequence of the parent strain *S.* Typhimurium 82/6915 is assumed to align with the *S.* Typhimurium LT2 sequence, therefore this PCR was performed to confirm the presence of the rfbL sequence in both the parent strain and STM-1. This means that the presence of phage P22 DNA is a normal occurrence in strains of *S*. Typhimurium and is not simply the result of the delivery of exogenous DNA driving the creation of STM-1.

4.6. Amplification of pflb-aroA region using rfbL and aroA 170 bp primers and pflB and rfbL primers

After confirmation that the rfbL sequence is a part of the parent strain it was essential to confirm the position of that *rfbL* gene in both the parent strain and STM-1. The *aroA* region was amplified using the rfbL forward primer and aroA 170bp reverse primer. While there was no amplification in the parent strain, in STM-1 the size of the amplified product was 2.3 kb (data not shown), which was expected from the sequence analysis and further confirms the presence of *rfbL* gene in the aroA location in STM-1.

These results confirm the existence of a chromosomal segment from location 29 at 10 mins in the parent strain, in location 48 at 19 mins in STM-1. The result also confirms the possibility of a chromosomal

Table 2. PCR primers used in this study

Primer Name	Sequence 5'® 3'	Amplifies/Purpose	Tm°C*
AROA3.5KB FWD	TGCTTCTCACTGGTCGCACTGTCCG	*aroA 3.5kb* downstrean	72
AROAEND	AGGCGTACTCATTCGCGCCAGTTG	*aroA end*	54.7
AROA170BP REV	CAGGGCATTGAGCATATGGCG	*aroA* 170bp downstream rev	66
SERCFWD	TGACAGACGCTCGAAGTATTCGCC	*serC* gene	68
YCAP FWD	TTGCAACGAATGGCGCTGGATAACG	*ycaP* gene	70
YCAP REV	TTAGCCCGGCTTGCCTTCGTCCA	*ycaP* gene	72
YCAO FWD	AAGACGCCGCGCTGGAAGACTCCAT	*ycaO* gene *fwd*	74
YCAO REV	TTTGACCAGTATGCCGCTTTCGC	*ycaO* gene *rev*	68
FCAO FWD	ATAGACGCCTGCTTCTTCAGCC	*fcaO* gene	64
PFLB FWD	GCGTGAAGGTACGAGTAATAACGTCC	*pflB* gene	67
PFLB REV	CACTCCGTATGAGGGTGACGAGTCC	*pflB* gene	69
SOPD FWD	GCGTCGCATTCAACGCGCAATCAGG	*sopD* gene	73
SOPD REV	AGGCTCCATATCAGTGGGGC	*sopD* gene	65
RFBL FWD	GAGCGCCGTTGTTGGCTGGATGG	*rfbL* gene	72
RFBL REV	TAGCAGCATTCGCGCAGCCTGCC	*rfbL* gene	74
RPSA REV	TCCAGGTGCAGAGTATCACGCACCG	*rpsA* gene	72
M13 REV	CAGGAAACAGCTATGAC	Sequencing primer	44.5
M13 –20	GTAAAACGACGGCCAG	Sequencing primer	45.8

rearrangement of the genomic DNA of STM-1 other than an inversion, as PCRs with the pflB forward primer and aroA reverse primer only amplifies the region in the parent strain, and not in STM-1. This shows that the *pflB* gene does not exist in the same region as *aroA* in STM-1. Also the PCR with the rfbL forward primer and aroA reverse primer gives the expected product only in STM-1, (data not shown).

4.7. Southern Blot analysis

The sequence analysis and PCR results indicate that there is a deletion of an approximately 5 kb DNA segment and insertion of approximately 1.4 kb in STM-1. The PCR of individual genes also confirms the presence of the *pflB* gene, but not in the same region as in the parent strain. The sequence analysis and the PCR results indicate that the sequence of DNA which was originally present at location 29 at 10 minutes in the parent strain, now exists adjacent to the *aroA* gene in STM-1 which is at location 48 at 19 minutes in STM-1. However, the possibility of the existence of sequence from location 29 at location 48 required further evidence. It also requires additional investigation to determine if this change in the STM-1 genome is due to a chromosomal rearrangement rather than a simple inversion, as the inversion of such a large segment of DNA has never been reported in *Salmonella* spp. or *E coli*.

To further analyse the events leading to the STM-1 chromosomal architecture, Southern blots, using three different probes, (aroA, pflB and rfbL) were performed. The genomic DNA of both the parent strain and STM-1 was digested with *Sac*I after analysing the DNA sequence information from *S*. Typhimurium LT2 strain from the database. The digestion with *Sac*I yields a 15 kb fragment in the *aroA* region of the LT2 genome. Southern blot analysis with the pflB probe yields a 15 kb product in both the parent strain and STM-1 (Figure 5; lanes 1 & 2). The size of the fragment when probed with aroA in the parent strain was 15 kb but in STM-1 the fragment size is only 12 kb (Figure 5; lanes 4 & 5). The existence of a 15 kb fragment in the parent strain probed with both aroA and pflB further confirms that the architecture of the parent strain is similar to LT2 as previously indicated by PCR, and also confirms that the *pflB* gene and the *aroA* gene exists in the same region in the parent strain. However, the difference in the fragment size amplified in STM-1 between the pflB probe and aroA probe indicates a probable genomic rearrangement.

The Southern blot with the rfbL probe yields an approximately 4 kb fragment in the parent strain whereas the size of the fragment in STM-1 is 12 kb (Figure 5; Lanes 5 & 6). This indicates the presence of the *rfbL* gene adjacent to the aroA gene in STM-1, as both the probes yield a 12 kb fragment in STM-1. These Southern blots

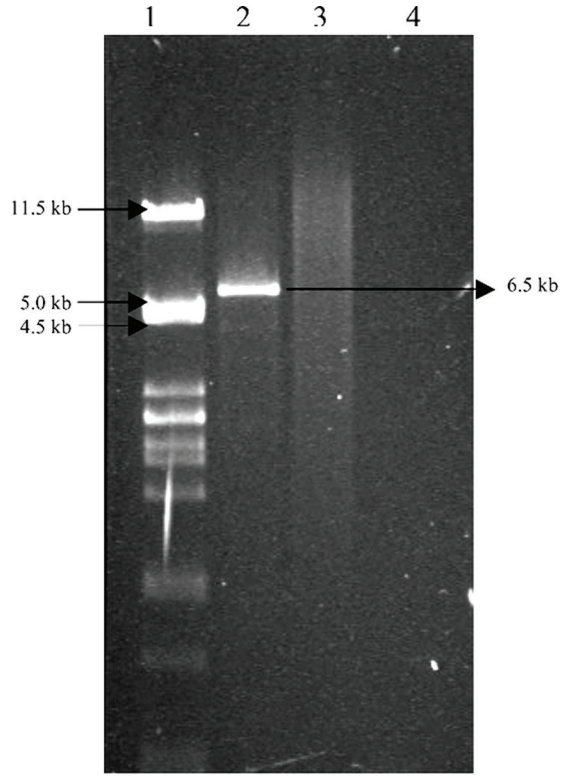

Figure 4. Amplification of the pflB-aroA region with pflB forward and 170 bp aroA rev primers. The PCR shows that the *pflB* gene, shown to be present in the STM-1 chromosome, does not exist at the same location as in the parent strain. Lanes: 1 lambda x PstI marker; 2, parent strain; 3, STM-1; 4, no DNA control.

indicate the possibility of a chromosomal rearrangement other than an inversion in the STM-1 genomic DNA, as the rfbL probe yields a 12 kb fragment in STM-1, the same fragment size that is observed with the aroA probe, whereas the rfbL probe in the parent strain yields only a 4 kb fragment as shown in Figure 5. This indicates that the *rfbL* fragment now exists in a different chromosomal location, presumed to be in the *aroA* region at location 48 at 19 minutes. The *plfB* gene does not exist in the *aroA* region in STM-1, as the pflB probe yields a 15 kb fragment in the STM-1, whereas the aroA probe yields a 12 kb fragment. These southern blots therefore confirm a chromosomal rearrangement in the STM-1 genomic DNA.

4.8. PFGE analysis of STM-1 genomic DNA

The PCR and Southern blot analysis indicates the possibility of a chromosomal rearrangement. PFGE was used to further analyse the nature of the rearrangement in STM-1 genomic DNA. The rationale behind this experiment was to study the pattern of digestion of the genomic DNA of both parent strain and STM-1, considering the fact that if such a large rearrangement (approximately 440 kb) has occurred, as the sequence analysis, PCR and Southern blot analysis indicate, there should be an observable difference after pulse field electrophoresis. Chromosomal DNA was digested with *Xba*I to study the pattern of digestion by PFGE.

The pattern of genomic DNA digestion is similar in both the parent strain and STM-1 as shown in Figure 6, which was not expected considering the fact that all the above sequence analysis, PCR and Southern blot results indicates the possibility of large chromosomal rearrangement. Such a large rearrangement could have produced an observable change in the pattern of digestion.

4.9. PFGE-Southern blot analysis of STM-1 genomic DNA

For further confirmation, two different restriction enzymes *Spe*I and *Not*I were used for further PFGE analysis and PFGE followed by southern blot. This experiment was planned keeping in mind two possibilities, either (1) the rearrangement in the STM-1 genome is of a nature that cannot be visualised from the parent strain after *Xba*I digestion, but may be after digestion with *Spe*I and *Not*I, or (2) even if the pattern of digestion with other enzymes does not reveal changes between the parent strain and the STM-1, PFGE followed by the Southern blotting might be able to show the difference in the pattern of digestion, if any exists between the parent strain and the STM-1.

The digestion pattern after PFGE did not show any observable differences between the parent and STM-1 genomic DNA as shown in Figure 7a, but the PFGE followed by Southern blot with the pflB probe, yields different size products as shown in Figure 7b. This confirms that there is a rearrangement in the STM-1 chromosome due to the insertion of the transposon Tn*10*.

4.10. Amplification of the aroA-rpsA region

PCR was used to determine the sequence organisation downstream of the *aroA* gene. The forward primer was designed from the *aroA* gene at 400 bp and the reverse primer was designed at the end of the *rpsA* gene. As shown in Figure 8, there was no difference in the size of the amplified product in either parent strain or STM-1, suggesting that there is no rearrangement in the segment downstream of the *aroA* gene.

5. DISCUSSION

An aromatic vitamin dependent strain of *Salmonella* Typhimurium STM-1 was created by transposon insertion/deletion. Sequence and PCR analysis of the *aroA* region in this strain STM-1 indicates not only a deletion and insertion, but also a large-scale rearrangement in the STM-1 chromosome. Initially it was thought that this significant change in the gene rearrangement within the chromosome could have been

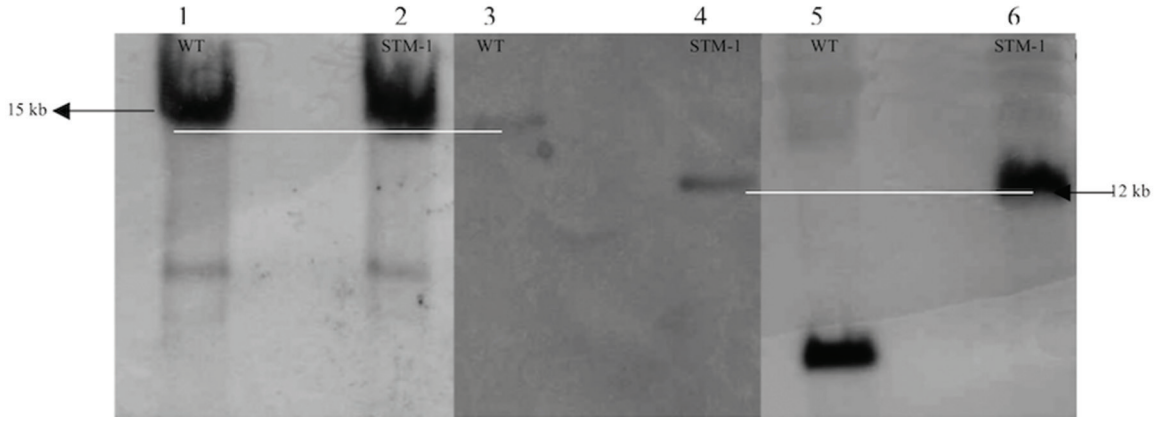

Figure 5. Southern blot analysis with the pflB, aroA and rfbL probes. The Southern blot with the pflB probe yields a 15 kb fragment in both the parent strain and STM-1 (Lane 1 & 2), the aroA probe yields a 15 kb fragment in the parent strain however, only a 12 kb fragment in STM-1 (Lane 3 & 4). The rfbL probe yields a 4 kb fragment in the parent strain and a 12 kb fragment in STM-1 (Lane 5 & 6), which is similar to the size of fragment yielded with the aroA probe. This confirms the existence of the *rfbL* gene adjacent to the *aroA* gene,and indicates a possible genomic rearrangement in STM-1 which are at different locations in the parent strain. Lanes: 1, parent strain probed with pflB; 2, STM-1 probed with pflB; 3, parent strain probed with aroA; 4, STM-1 probed with aroA; 5, parent strain probed with rfbL; 6, STM-1 probed with rfbL.

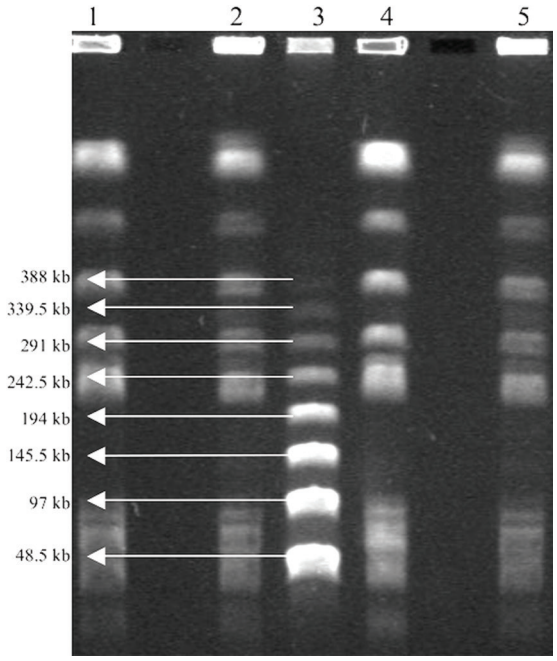

Figure 6. PFGE after digestion with *Xba*I. The pattern of digestion in both the parent strain and STM-1 is similar, which contradicts the results of sequence analysis and PCR and Southern blot. Lanes: 1, parent strain; 2, STM-1; 3, PFGE lambda marker; 4, parent strain; 5, STM-1.

caused by an inversion, but extensive PCR analysis ruled this out. Consequently pulse field gel separation of large regions of the chromosome and southern blot analysis was undertaken to determine if exchange of DNA within the chromosome had occurred. Southern blotting analysis results also indicate exchange of genetic material between 10 minutes and 19 minutes on circular map, indicating changes in the STM-1 chromosomal DNA. Introduction of Tn*10* into the bacterial chromosome and its deletion can promote several types of recombination events such as deletion, inversion and deletion/inversion. However, approximately 10 percent changes in the chromosome due to Tn*10* element are intricate or/ unidentified (10-12).

Initially PFGE analysis performed after digesting both the parent strain and STM-1 genomic DNA with *Xba*I and further PFGE analysis performed after digesting with the *Spe*I, and *Not*I, reveal only minor change in the PFGE pattern between the parent strain and STM-1. This is likely to be the case because the enzymes *Xba*I, *Spe*I and *Not*I cut very infrequently in the chromosome and since the amount of DNA that “crossed over” during rearrangement was relatively small (440 kb) it is unlikely that rearrangement will lead to multiple changes in the restriction sites and so the visible changes in the PFGE pattern may be minimal. Chromosomal rearrangement such as an inversion of 440 kbps could have created a major change in the pattern of digestion of STM-1 chromosomal DNA in comparison to the parent strain chromosomal DNA. Therefore large-scale rearrangement in the STM-1chromosomal DNA can be best explained by the phenomenon of a genomic rearrangement such as cross over of DNA segment from one location to another, this would have preserved the integrity of the restriction sites and should lead to only minor changes, in the restriction pattern. That said it appears that there is a difference between the parent and the STM-1 PFGE genomic pattern with an obvious band at 97 kb that is missing in STM-1 and this region aligns with the changed sites in the southern blot. Overall, what initially appeared to be a simple deletion of the *aroA* gene rendering the mutant aromatic vitamin dependent has also induced a significant genetic rearrangement in the bacterial chromosome. Fortunately the consequences of these

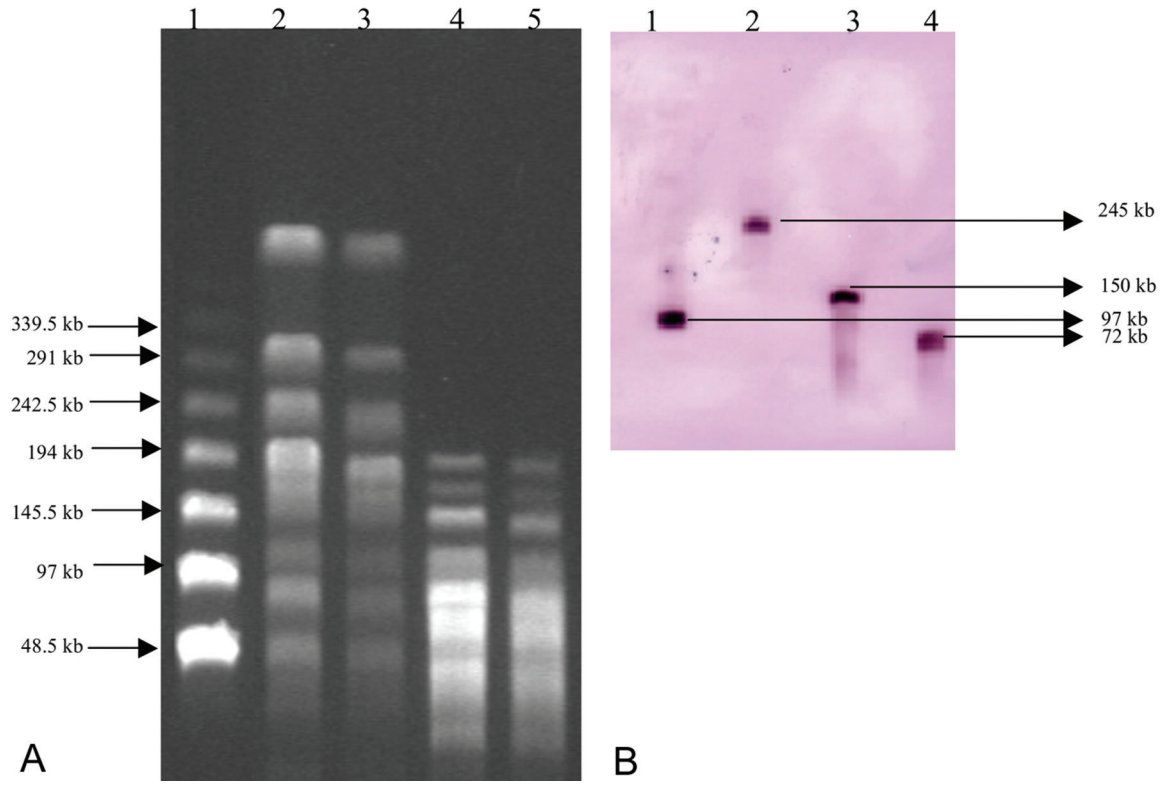

Figure 7. a. PFGE with *Spe*I and *Not*I. Digestion with the three different restriction enzymes does not reveal any difference in the pattern of digestion between the parent strain and STM-1. Lanes: 1, PFGE lambda marker; 2, parent strain; 3, STM-1digested with *Spe*I; 4, parent strain; 5, STM-1 digested with *Not*I. b. Southern blot analysis with the pflB probe after PFGE with *Spe*I and *Not*I. The observation of different sized fragments detected confirms a rearrangement in the STM-1 genome. Lanes: 1, parent strain; 2, STM-1 digested with *Spe*I; 3, parent strain; 4, STM-1 digested with *Not*I.

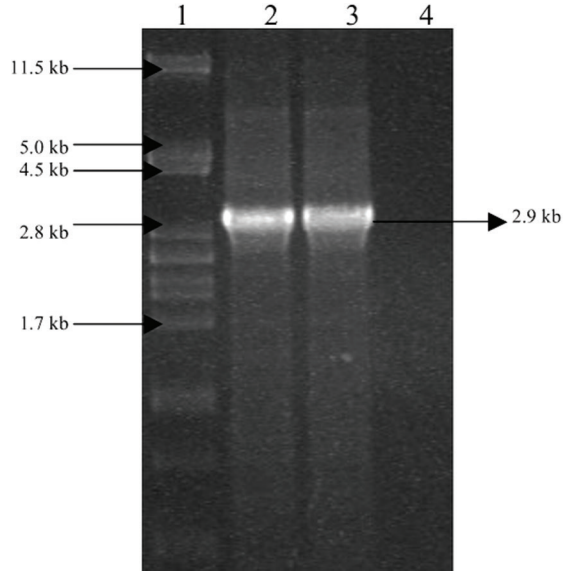

Figure 8. Amplification of the aroA-rpsA region with aroA 400 bp fwd and rpsA rev primers. There is no rearrangement downstream of aroA in the STM-1 chromosome. Lanes: 1, lambda x *Pst*I marker; 2, parent strain; 3, STM-1; 4, no DNA control.

changes have not been detrimental to the features of the STM-1 except for the aromatic vitamin dependence. This is likely to be because the crossover did not inactivate any other essential gene function.

A study by Chalmers and Kleckner, (13) also indicates that transposition events mediated by Tn*10* transposons and IS*10* insertion sequence promote an array of DNA rearrangements. Transposition is a complex phenomenon in which several factors are responsible for insertion and release of Tn*10* transposable element. Architectural heterodimer protein IHF (integration host factor) which regulates recombination of bacteriophage and was first discovered during the integration of bacteriophage l in *E. coli (*14) and negative supercoiling are two such factors which play important roles in Tn*10* transposition events (15-18), although only one factor is involved. Both IHF and negative supercoiling imparts topological changes in the chromosome during inter and intra transposition events such as deletion, inversion and deletion/inversion. However, the presence or absence of IHF and different concentration of IHF can direct the recombination events in a different ways. At lower IHF concentration an equivalent levels of deletion and

inversion with reduced topological complexity has been observed, this also induces the generation of attached deletion circles and inversion knot circles and noticeable level of free deletion circles (15).

In *S.* Typhimurium, inversions from 5 kb up to >60 kb have been reported (19) but the inversion of a very large fragment has never been reported. A likely explanation of the chromosomal rearrangement in STM-1 is a crossover between two distant points on the chromosome, not due to a specific target sequence but due to the presence of segment of the Tn*10* transposon and facilitated by the conserved three-dimensional structure of the bacterial chromosome which allowed contact and cross over between segments of the chromosome from widely divergent regions, possibly intiated by the transposition insertion sequences. This phenomenon has been observed in *E. coli* genomic DNA caused by the insertion of the Tn*10* transposable element (20). In the natural state the chromosome exists in a three dimensional form and is in a highly strained conformation. A transposition event with recombination induces complex structural changes further straining the already stressed conformation of bacterial chromosome. These changes may situate location 29 and 48 in a close proximity, and may be responsible for the crossover between two unspecific target sequences as has occurred in this case. There are clear shifts in the position of pflB in the parent and STM-1 mutant as shown in the southern blotting (figure 7b). This information, plus the PCR results show that the STM-1 has rearranged some of its chromosomal DNA. Furthermore the sequence analysis result also supports the above findings.

From these results it appears that the introduction and deletion of the composite transposable element Tn*10* in the bacterial chromosome can induce chromosomal rearrangement which can be other then a deletion, inversion or simultaneous deletion/inversion in nature. This can also lead to the insertion of residual Tn*10* or IS*10* but can also lead to a more complex process where the large segments of DNA can exchange position, in an unpredictable fashion.

6. ACKNOWLEDGMENTS

M.S. thanks RMIT University for a postgraduate scholarship.

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Key Words: *Salmonella*, aroA554::Tn*10*, chromosome

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