

Construction and evaluation of a *gfp*-tagged *Salmonella* Typhimurium strain for environmental applications[#]

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Abstract

Salmonella enterica ser. Typhimurium was isolated from freshwater sediments and chromosomally labelled with a stable variant of the green fluorescent protein (GFP). The pUT mini-Tn5 Km transposon was used to introduce the *gfp* gene onto the chromosome of the *S. Typhimurium* strain by tri-parental mating. Southern Blot hybridisation confirmed that the gene had integrated into the chromosome. The *gfp* gene was stably maintained and the labelled strain was not growth-rate impaired. The incorporation of the *gfp* gene did not convey any significant loss of phenotype which would affect the survival and behaviour of the tagged strains. The tagged *S. Typhimurium* strain was used to spike an established drinking water biofilm and was able to colonise and persist within the biofilm. The tagged strain was also successfully used to study the survival of *S. Typhimurium* in natural sediments under different temperatures. These tagged strains can therefore be used to study the fate and survival of different *Salmonella* strains in water environments.

Keywords: biofilm, green fluorescent protein, *Salmonella* Typhimurium, survival

Introduction

Salmonella enterica is a primary agent of gastroenteritis in both humans and animals and each year an estimated 12.5 million people are affected (Hunter, 1997). Although often associated with food-borne transmission, *Salmonella* is also frequently isolated from water sources, which may serve as a reservoir for infection. *Salmonella* have been detected in sewage, freshwater, marine, coastal and groundwater (Baudart et al., 2000; Ho and Tam, 2000; Gannon et al., 2004). The presence of this bacterium in the aquatic environment is often linked to direct or indirect contamination by human or animal excretion (Baudart et al., 2000; O'Shea and Field, 1992; Moganedi et al., 2007).

Compared to *E. coli*, *Salmonella* appears to withstand a wider variety of environmental fluctuations and may persist in various water environments for extended periods. (Winfield and Groisman, 2003). Biofilms of potable water distribution systems have the potential to harbour *Salmonella*. This is problematic, as these pathogens could be released back into the flow following detachment (Camper et al., 1999; Szewyk et al., 2000). Sediments may protect enteric bacteria from certain stressors associated with aquatic environments and they are known to act as nutrient sources that may support bacterial growth (Lim and Flint, 1989). *Salmonella enterica* serovar Typhimurium mr-DT-104 was found to survive for several months in aquatic environments, with enhanced survival in sediments relative to overlying water (Moore et al., 2003). In order to protect and manage the quality of water sources, a clear understanding of the fate, survival and transport of this bacterium in various aqueous environments is necessary.

Detailed studies on the fate and persistence of non-typhoidal *Salmonella* in water environments have been limited due to the lack of a stably tagged strain readily distinguishable *in vivo* from the rest of the bacterial community. A range of specific detection techniques has been described in the past decades, including use of fluorescent antibodies (Cloete and De Bruyn, 2001) and fluorescent *in situ* hybridisation (FISH) (Amann et al., 1995). Genetic tags described include the luciferase (*lux*) operon (Meikle et al., 1994), the expression of which exerts significant energy drainage on the cell. A more recent approach to study the behaviour of a specific strain in natural environments is by tagging the bacterial cell with the gene encoding the green fluorescent protein (GFP) (Möller et al., 1998). GFP can be visualised by epi-fluorescence microscopy, fluorescing green during excitation with UV or blue light. The *gfp* gene obtained from the jellyfish *Aequorea victoria* (Chalfie et al., 1994) overcomes many of the limitations posed by systems such as luciferase and end-point approaches such as FISH. Cells tagged with *gfp* therefore do not require prior fixing, staining or addition of substrate for visualisation (Chalfie et al., 1994; Valdivia et al., 1996). GFP is a very stable protein (Andersen et al., 1998) and its detection is independent of cellular energy reserves at the time of viewing. Green fluorescence phenotypes are detectable in all growth phases even under starved conditions (Tombolini et al., 1997). Frana and Carlson (2001) developed and used a plasmid encoding *gfp* in *Salmonella* Typhimurium phage type DT104. It was found that fluorescence was maintained for up to 50 propagations under non-selective conditions. For strains that are destined for use in an environmental study, it is imperative that the desired traits are encoded and maintained on the chromosome, ensuring stability of the engineered phenotype (Eberl et al., 1997). The chromosomal tagging of bacterial strains using GFP allows for the possibility of performing survival studies under mixed culture conditions including temporal observations over extended periods of time. In this way, a more realistic indication of the growth potential of *Salmonella* in aquatic environments can be obtained due to the interaction with competing indigenous micro-organisms.

[#] Journal series publication 3595 from the South Dakota Agricultural Experiment Station.

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Received 10 September 2007; accepted in revised form 11 December 2007.

The aim of this investigation was to chromosomally tag a clinically relevant *Salmonella* strain isolated from an aqueous ecosystem with the *gfp* gene. The stability of maintenance, expression of *gfp*, and its suitability for application in studies of biofilms and freshwater sediments is demonstrated.

Experimental

Isolation and identification of *Salmonella* strains

Sediment samples were collected from rivers in the Vhembe region of the Limpopo Province of South Africa. After pre-enrichment in buffered peptone water (BPW) (Oxoid) for 24 h at 37°C, 1 ml of the pre-enrichment was inoculated into 9 ml of Rappaport-Vassiliadis (RV) broth (Oxoid) for selective enrichment and incubated for 24 h at 42°C. A loop-full of broth from each positive tube was plated onto XLD (xylose lysine deoxycholate) agar (Oxoid) and incubated at 37°C for 24 h. Black colonies were identified using the API 20E biochemical test strips (bioMérieux). Serological typing of selected isolates was performed at the ARC-Onderstepoort Veterinary Institute, Pretoria, South Africa. Care was taken to select for environmental strains that belonged to *Salmonella* serotype Typhimurium, as it is the most commonly isolated serotype from patients in this area. Isolates were maintained on Microbank beads (Pro-lab diagnostics) at -80°C.

Chromosomal tagging

S. ser. Typhimurium isolates were confirmed susceptible to ampicillin (100 µg/ml) and kanamycin (100 µg/ml) using Luria Bertani (LB) agar. The pUT mini-Tn5 Km transposon (De Lorenzo et al., 1990) was used to insert the *gfp*mut3b* gene into the chromosome. *Salmonella* was transformed by tri-parental mating using *E. coli* HB101 (pRK2013) (Figurski and Helinski, 1979) as the helper strain, *E. coli* CC118λpir (pSM1695) (Sternberg et al., 1999) as the donor strain and a *Salmonella* isolate as the recipient (Table 1). A loop-full of each of the donor, helper and recipient strains was mixed together on LB agar and incubated for 24 h at 37°C. The mixed culture was then streaked onto XLD agar supplemented with 100 µg/ml of kanamycin and incubated at 37°C for 24 h. Black colonies were selected and confirmed as tri-transconjugants by their ability to fluoresce green when exposed to blue light. Confirmed tri-transconjugant colonies were inoculated into 10 ml of LB broth and incubated at 37°C. Chromosomal integrants were selected by replica-plating kanamycin-resistant colonies onto ampicillin agar and selecting sensitive

colonies. The presence of *gfp* was confirmed by fluorescence microscopy using an inverted Zeiss Axiovert 200 fluorescent microscope (Excitation – 490 nm and Emission – 510 nm), fitted with a 100x/1.4 Zeiss Neofluor objective. The images were captured using a Nikon charge-coupled device (CCD) camera.

Verification of chromosomal tagging

Chromosomal DNA was extracted from two tagged isolates, the wild-type strain and the donor strain using the Dneasy tissue kit (Qiagen). The *gfp* gene of 720 bp was amplified by PCR using the primers P_{gfp(up)} (5'-ATATAGCATGCGTAAAGGA-GAAGAACTTTTCA-3') and P_{gfp(down)} (5'-CTCTCAAGCT-TATTTGTATAGTTCATCCATGC-3') (Andersen et al., 1998). The 50 µl PCR reaction mixture contained 50 pmol/µl of each primer, 2.5 mM deoxynucleotide triphosphate (dNTP), 5 µl of 10 x PCR buffer (containing 15 mM MgCl₂), 1U of Taq polymerase and 50 to 100 ng of genomic template DNA. The reaction mixture was subjected to 30 amplification cycles of 92°C for 1 min, 60°C for 1 min, and 72°C for 1 min on a GeneAmp® 2700 PCR System (Applied Biosystems). An initial start of 92°C for 5 min and a final elongation step at 72°C for 5 min was also included. Southern Blot hybridisation was performed using the *gfp* gene as amplified by PCR from pSM1695 as a probe. The amplicon was excised from an agarose gel using the QIAquick® PCR purification kit (Qiagen) and random primed with digoxigenin using the DIG High Prime DNA Labelling and Detection Starter Kit I (Roche). Southern blotting was performed using the protocol as supplied by Roche, transferring agarose-resolved DNA extracts to Hybond™-N+ nylon membrane (Amersham) by vacuum and hybridising at 64°C using 20 ng of probe DNA. Hybridised probe was detected chromogenically using NBT/BCIP (Roche).

Survival studies

Stability and maintenance of the *gfp* gene

Tagged strains and the wild-type strain were inoculated separately into 10 ml of LB or 1/10 strength R2A broth and incubated at 37°C while shaking at 180 r/min. Aliquots of 100 µl were transferred axenically to fresh broth daily for 30 d. The expression and stability of the *gfp* gene in cells were verified by fluorescence microscopy

Fitness of the *gfp*-tagged strain

The wild-type and tagged strains were cultured overnight in LB broth. One ml of the overnight culture was inoculated into

TABLE 1
Strains and plasmids used for tri-parental mating

Strains and plasmids	Relevant characteristics	Reference or source
<i>E. coli</i> K-12 strains		
HB101	<i>E. coli</i> K-12/B hybrid	JB Andersen (personal gift)
CC118λpir	λpir lysogen	JB Andersen (personal gift)
<i>Salmonella</i> strains		
<i>S. Typhimurium</i>	Recipient strain; environmental isolate	This study
Tagged <i>S. Typhimurium</i>	<i>S. Typhimurium</i> X HB101 (pRK2013) X CC118λpir (pSM1695); <i>S. Typhimurium</i> with mini Tn5-Km- <i>rrnB</i> P1-RBSII- <i>gfp</i> mut3b*-To-T1 cassette randomly inserted into chromosome; Km ^R	This study
Plasmids		
pRK2013	RK2 transfer function; helper plasmid in tri-parental matings	Figurski and Helinski, 1979
pSM1695	Amp ^R Km ^R ; delivery plasmid for mini Tn5-Km- <i>rrnB</i> P1-RBSII- <i>gfp</i> mut3b*-To-T1	Sternberg et al., 1999

250 ml side-arm flasks containing 100 ml LB or $1/_{10}$ strength R2A broth and incubated while shaking at 37°C. The OD at 600 nm was determined spectrophotometrically. Phenotype Microarrays (Biolog) were used to compare the phenotypic profiles of the wild type and tagged Strain 1. The absorbance at 590 nm was determined after 24h using an EL_x800 Universal Microplate Reader (Bio-Tek Instruments Inc.).

Fate of *Salmonella* in an established biofilm

A flow cell was used to simulate biofilm formation in a water distribution system. The cell, having 6 channels with an individual dimension of 2 x 2 x 15 mm was covered with a glass cover-slip applied with silicone sealant (Bostik®) and connected to a Watson Marlow 205S peristaltic pump. The system was sterilised by perfusing with bleach (2%) and rinsed with sterile dH₂O. A simulated drinking water biofilm was established in sterile tap water supplemented with 1 mg/l acetate by inoculating the flow cells with a mixture of 5 non-fluorescing isolates obtained from biofilms of drinking water systems. Flow was paused for 1 h to allow bacteria to attach to the glass slide and then set at a flow rate of 0.4 mm/s through the flow cell. After 72 h, flow cells were spiked with *gfp*-tagged *Salmonella* pre-grown in $1/_{10}$ strength R2A medium and diluted to 10⁶ and 10² CFU/ml respectively. The flow was maintained for a further 144 h and the biofilms were viewed periodically by phase contrast and epifluorescence microscopy.

Survival of *Salmonella* in natural freshwater sediments

Sediment samples of 500 ml were collected from a small stream near Thabazimbi, Limpopo Province, South Africa, which forms part of the Limpopo basin. The sediment was confirmed free of *Salmonella* by the MPN method using BPW followed by RV broth and streaking onto XLD agar (detection limit 0.3 CFU/ml). The sediment samples were mixed with 24 h LB-grown recombinant 1 *Salmonella*, washed and resuspended in PBS to a final density of 10⁹ cells/g of sediment. The spiked sediment microcosms were incubated at 8, 16 and 28°C, and the number of recoverable tagged isolate was determined every 7 d for 23 weeks. *Salmonella* was enumerated by re-suspending 10 g of sediment in 90 ml of extraction buffer (0.04 g EGTA, 0.12 g TRIS, 0.1 g peptone and 0.04 mg N-dodecyl-N, N dimethyl-3-ammonio-1-propanesulfonate (Zwittergent) (Camper et al., 1985), followed by sonication for 5 min to assist the release of the bacteria from the sediment. Dilutions were prepared in triplicate series of $1/_{4}$ strength Ringers solution, followed by pre-enrichment in BPW, enrichment in RV broth and confirmation on XLD agar as described above. The MPN table (Beliaeff and Mary, 1993) was used to estimate the level of bacteria in each sample by scoring all dilutions yielding black colonies as presumptive *Salmonella* positive. Single black colonies were selected and examined by fluorescence microscopy to verify the presence of the tagged isolate after enumeration.

Results

Chromosomal tagging of a *Salmonella* isolate

Several *Salmonella* were obtained from the river sediment and one *S. ser. Typhimurium* isolate sensitive to both ampicillin and kanamycin was selected for tagging with *gfp*. After tri-parental mating pSM1695 was successfully introduced into *S. ser. Typhimurium* as confirmed by initial resistance to both ampicillin and

kanamycin. Two fluorescing tri-transconjugants were obtained, confirmed sensitive to ampicillin by replica plating, and were assumed to harbour genomic integrations of the pUT mini-Tn5 Km transposon harbouring *gfp*. The *gfp* gene was amplified successfully by PCR from chromosomal extracts. Southern Blot hybridisation confirmed that the *gfp* gene had integrated into the chromosome of the tagged isolates. Neither recombinant harboured plasmid pSM1695 as determined by sensitivity to ampicillin and by absence of detectable plasmid determined by agarose gel electrophoresis of plasmid extracts. The *gfp* gene could also not be detected by PCR using plasmid extract.

Maintenance of the *gfp* gene

The stability of the GFP labelling was tested by continuous batch culturing for 30 d under non-selecting conditions. The GFP was expressed in all cells cultured in both LB and $1/_{10}$ strength R2A as determined by fluorescence microscopy. This showed that the *gfpmut3b** gene was maintained stably under both nutrient-rich and nutrient-poor conditions in all progeny.

Fitness of the recombinant strains

Fitness under nutrient-rich and nutrient-poor conditions

The tagged isolates did not display discernable growth rate or yield deficiencies when compared to the wild-type strain, both under nutrient-rich (LB broth) (Fig. 1a) and nutrient-poor ($1/_{10}$ strength R2A medium) conditions (Fig. 1b). This indicated that the integration did not affect growth of the recombinant strains. GFP fluorescence was clearly visible by fluorescence microscopy in cells grown in $1/_{10}$ strength R2A, showing that *gfp* was still expressed constitutively during growth in nutrient-limiting conditions.

Phenotypic comparison

The Biolog Phenotype Microarrays indicated 21 out of a total of 379 phenotypic changes in tagged Strain 1 in comparison to the wild-type strain, only 5 of which demonstrated impaired growth (D-aspartic acid as a carbon source and D-asparagine, N-acetyl-D-mannosamine and inositol hexaphosphate as a nitrogen source). The recombinant was curiously able to grow on 16 additional nutrients, including inositol, bromo succinic acid, tricarballic acid, dihydroxy fumaric acid, 2-hydroxy benzoic acid, D-tartaric acid, D,L- α -amino caprylic acid, L-arginine, L-asparagine, xanthine, alanine-glutamine, D-mannose-1-phosphate, D-mannose-6-phosphate, cysteamine-5-phosphate, O-phospho-D-serine and D-methionine). None of these sources appear essential for growth of *Salmonella*, nor would they be expected to be major nutrients in the environment. The results indicated that the incorporation of the *gfp*-bearing transposon was not detrimental to the fitness of the tagged strain or its ability to utilise a range of prevalent carbon, nitrogen and sulphur sources, yet the observed phenotypic changes are intriguing.

Visual evaluation of *Salmonella* in an established mixed culture biofilm

After 72 h a 5-strain biofilm with developed micro-colonies had formed in the acetate-containing drinking water. No fluorescence could be detected microscopically. *Salmonella* was found to attach to and survive in the mixed culture biofilm. Individual fluorescing cells could be visualised 24 h after inoculation among the mixed culture biofilm at both spiking concen-

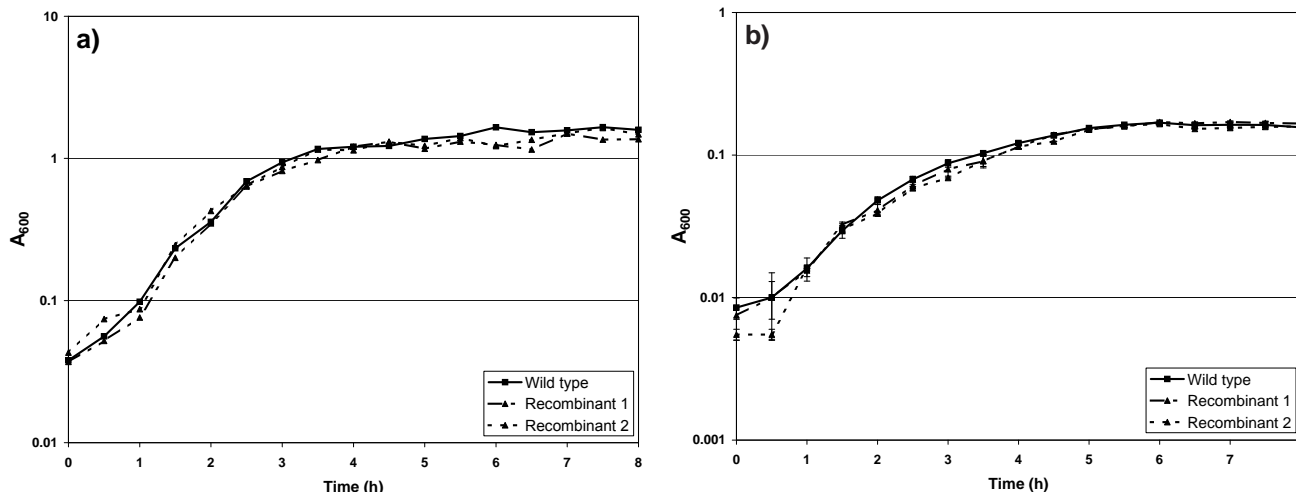


Figure 1
Growth of *Salmonella Typhimurium* wild-type vs. tagged strains in LB broth (a) and $1/10$ strength R2A medium (b) at 37°C. The error bars denote the standard error of the mean.

tration of 10^6 CFU/ml and 10^2 CFU/ml. *Salmonella* persisted in the established biofilm, forming small micro-colonies after 48 h, and continued to persist in the established biofilm at 72 h (Fig. 2). The *gfp*-tagged strain, therefore, facilitated the study of the interaction of *Salmonella* with a consortium of other bacteria in a biofilm. All fluorescing colonies obtained following plating of serial dilutions of biofilm onto LB agar were confirmed as kanamycin-resistant *Salmonella* following streaking onto XLD agar, indicating a lack of interspecies transfer of *gfp* in the biofilm.

Survival of *Salmonella* in natural freshwater sediments

The *Salmonella* recombinant introduced into a river sediment sample declined exponentially over several weeks (Fig. 3). Initially most of the inoculated CFU could be recovered using BPW followed by RV broth and XLD culturing. All *Salmonella* obtained displayed green fluorescence, indicating stable maintenance of the *gfp* gene under sediment conditions. The initial rate of decline was independent of the incubation temperature. After 5 weeks the recombinants surviving at 28°C died, while survivors at both 8 and 16°C respectively persisted for a further 16 weeks before none could be detected in the sediment samples. A repeat of this experiment using fresh sediment produced similar results.

Discussion

S. Typhimurium is often endemic in developing countries and was commonly isolated from patients in a rural area in South Africa (Venter, 2005). *Salmonella enterica* serotype Typhimurium is also increasingly found to persist in a range of environments, pointing to its ability to endure in various habitats (Islam et al., 2004; Natvig et al., 2002). In this study the construction and evaluation of a *S. Typhimurium* strain tagged genomically with the *gfp* gene is described.

In aquatic systems bacteria may encounter low nutrient conditions, limiting the ability to express non-essential genes. The inserted gene under control of the promoter of the *rrnB* operon (Sternberg et al., 1999) was expressed as determined by epifluorescence microscopy during growth in a variety of culture media, ranging from LB broth to $1/10$ strength R2A and drinking water containing 1 mg/l acetate. This indicated that the gene was expressed even when cells were subjected to severe nutrient limitation and even after extended periods of growth. Fluorescence could be viewed for both cells in suspension as well as for biofilm-associated cells.

Recombinant bacteria are known to lose foreign genes in the absence of selective pressure or under stress conditions (Sobecky et al., 1992). Autecological studies or studies dealing with the biological relationship between an individual species and its environment should accurately reflect the prevailing

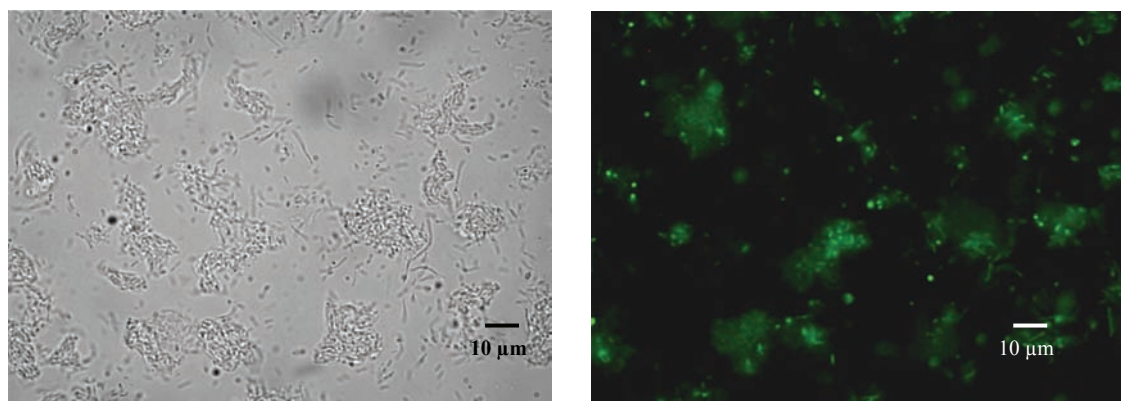


Figure 2
Mixed culture biofilm spiked with *Salmonella Typhimurium* of 10^6 CFU/ml after 72 h viewed by phase contrast (A) and fluorescence microscopy (B)

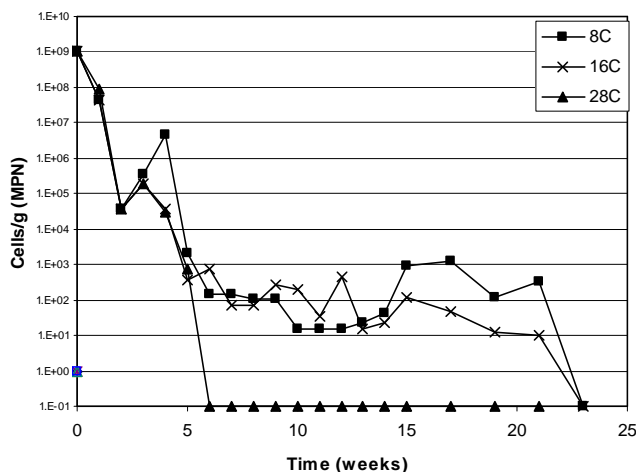


Figure 3

The effect of temperature on the survival of *Salmonella Typhimurium* in freshwater sediments

environmental conditions and should exclude factors foreign to the system, such as supplementation with antibiotics for selective plasmid maintenance. The recombinant *S. Typhimurium* maintained the *gfp* gene in the absence of selective pressure in axenic broth batch culture, in competitive conditions such as biofilm and in sediment microcosms. It is therefore demonstrated to be suitable for autecological studies.

The insertion point of the mini-Tn5 transposon is selected at random, and may therefore involve the disruption of a chromosomal sequence. Environmentally important functions can thus be affected (De Lorenzo et al., 1998). The *gfp*-labelled recombinant was not found to be growth rate impaired in nutrient-rich or nutrient-poor medium, as growth rates of recombinant and parental strains were indistinguishable. A thorough phenotypic interrogation using the Biolog Phenotypic Microarrays indicated loss of growth on 5 nutrient sources, which are not anticipated as significant nutrient sources in aqueous systems. The gain of function for 16 nutrients cannot be explained, but may point to inactivation of a repressor. The recombinant strain was also able to form biofilms as efficiently as the wild type under pure culture conditions in drinking water supplemented with acetate (data not shown).

The *gfp*-tagged *S. Typhimurium* was evaluated for applications in the study of both drinking water biofilms and sediments. *Salmonella* was able to colonise a pre-formed drinking water biofilm, and was able to persist and grow while expressing green fluorescence. The tagged strain could also be used to study the survival of *Salmonella* in freshwater sediments. The GFP label could be used to verify that *Salmonella* isolated from the sediments during the entire study period was indeed the same clone used during the initial spiking of the sample. The results indicated that *Salmonella* survived for extended periods in sediments at temperatures typically associated with freshwater streams and rivers in South Africa. A sub-population of the *Salmonella* survived for extended periods at 8 and 16°C respectively, but not at 28°C. This is in agreement with studies on the survival of various pathogenic bacteria and indicator organisms in the environment (Barcina et al., 1997; Anderson et al., 2005). While no net increase in the *Salmonella* culturable count was observed, the strain could still have grown in specific niches while decreasing in others due to either grazing or amensalistic challenges.

Conclusions

The *gfp* tagged strain of *S. Typhimurium* could therefore be used *in situ* to study the growth, survival and dissemination of *Salmonella* in water environments such as sediments or drinking water distribution systems. The tagged strain will facilitate long-term monitoring of *Salmonella* survival, growth and dissemination in nutrient-limited conditions and microbial communities, improving our knowledge and understanding of the ecology of this devastating pathogen in aqueous environments.

Acknowledgements

We thank JB Anderson for his kind gift of pSM1695, Raynard MacDonald for assistance with microscopy and Mulde Willems for assistance with sediment work. This research was supported by a grant from the Water Research Commission of South Africa (WRC K5/1276) to VSB and SNV, National Research Foundation (NRF) grant 2046811 to VSB, and by the South Dakota Agricultural Experiment Station. LMB was supported by a scholarship from the NRF of South Africa.

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