Exploitation of a β -lactamase reporter gene fusion in the carbapenem antibiotic production operon to study adaptive evolution in *Erwinia carotovora*

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Erwinia carotovora subsp. *carotovora* strain ATTN10 produces the β -lactam antibiotic 1-carbapen-2-em-3-carboxylic acid (carbapenem) by expressing the *carABCDEFHG* operon. Mutants exhibiting increased carbapenem gene transcription were positively selected using an engineered strain with a functional β -lactamase translational fusion in *carH*, the last gene of the operon. However, spontaneous ampicillin-resistant mutants were isolated even when transcription of *carH::blaM* was blocked by a strongly polar mutation in *carE*. The mechanism of resistance was shown to be due to cryptic IS10 elements transposing upstream of *carH::blaM*, thereby providing new promoters enabling *carH::blaM* transcription. Southern blots showed that IS10 was present in multicopy in ATTN10. In addition, a Tn10 genetic remnant was discovered. The results offer insights into the genetic archaeology of strain ATTN10 and highlight the powerful impacts of cryptic IS elements in bacterial adaptive evolution.

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INTRODUCTION

Erwinia carotovora subsp. *carotovora* (Ecc) strain ATCC 39048 is a Gram-negative phytopathogenic member of the Enterobacteriaceae and a causal agent of potato soft rot. In our earlier development of genetic analysis tools for this strain, Tn10 was introduced by λ ::Tn10 mutagenesis into the genetically 'virgin' version of ATCC 39048, in the selection of a mutant defective in the endogenous DNA restriction system (McGowan *et al.*, 1996). This restrictionless mutant strain was then subjected to fusaric acid eduction (Bochner *et al.*, 1980) to yield the tetracycline (Tc)-sensitive derivative strain ATTN10, which was still restrictionless (McGowan *et al.*, 1996). ATTN10 has subsequently been studied as a model genetically tractable phytopathogen with which to understand the regulation of bacterial virulence factors involved in plant disease.

ATTN10 is known to express the *carABCDEFHG* gene operon, which enables production of the β -lactam antibiotic 1-carbapen-2-em-3-carboxylic acid (carbapenem, Car) (McGowan *et al.*, 1996; reviewed by Coulthurst *et al.*, 2005). The first three genes, *carABC*, are essential for Car biosynthesis (McGowan *et al.*, 1997). *carDE* are also involved in Car biosynthesis, but are not essential, because when they are disrupted by in-frame deletions, Car is still produced, though at a reduced level. *carFG* are involved in Car intrinsic resistance via an uncharacterized, novel

mechanism (McGowan *et al.*, 1997). The presence of *carFG* does not confer cross-resistance to related β -lactam antibiotics such as imipenem (McGowan *et al.*, 1997). CarF and CarG both contain predicted signal peptides that direct their export to the periplasm (McGowan *et al.*, 1997). The *carH* gene product has no known function, but is also exported to the periplasm (McGowan *et al.*, 1997).

It has been shown recently that the ATTN10 *carABCDEFHG* gene cluster contains two promoters (McGowan *et al.*, 2005). The first promoter (P1) is located immediately upstream of *carA*. Transcription from P1 is increased by the presence of the quorum-sensing signal molecule, *N*-(3-oxohexanoyl)-L-homoserine lactone (OHHL), produced by the CarI enzyme (McGowan *et al.*, 1996). The OHHL signal molecule interacts with CarR with a dissociation constant of 1.8 μ M (Welch *et al.*, 2000). It is thought that the CarR:OHHL complex activates transcription of the *carABCDEFHG* operon, and that this leads to production of Car (McGowan *et al.*, 1997). The second promoter (P2) is located within *carD* and is not regulated by quorum sensing (McGowan *et al.*, 2005). P2 is a weak promoter that allows low levels of expression of a *carG::lacZ* transcriptional fusion (McGowan *et al.*, 2005). P2 is presumed to maintain auto-resistance to leaky production of Car by an individual cell or by its neighbours.

The aim of this research was to create and exploit a strain with a β -lactamase translational fusion in the *carH* gene to enable the positive selection of mutants exhibiting increased transcription of the *carABCDEFHG* operon. We presumed that these spontaneous mutants would have point mutations

Abbreviations: Ap, ampicillin; Tc, tetracycline; Car, 1-carbapen-2-em-3-carboxylic acid; Ecc, *Erwinia carotovora* subspecies *carotovora*.

within the P1 and P2 promoters that would lead to increased expression of the *carABCDEFGH* genes. In addition, we predicted that we might isolate a class of mutants with extra-operonic mutations defining new genes involved in carbapenem operon regulation. However, our results were surprisingly unexpected, and led to the discovery that Tn10 had not been entirely lost from the ATTN10 genome, highlighting the powerful role of mobile genetic elements in the adaptive evolution of bacteria that are challenged by a potentially lethal chemical selection pressure.

METHODS

Bacterial strains, plasmids and growth conditions. All *Ecc* and *Escherichia coli* strains, bacteriophage and plasmids used in this study are listed in Table 1. Strains were routinely grown on

Luria–Bertani broth agar (LBA) (Miller, 1972), at 30 °C for *Ecc* strains and 37 °C for *E. coli* strains, supplemented with antibiotics, as necessary. Liquid cultures of *Ecc* were grown in sterile conical flasks containing one-tenth volume Luria–Bertani broth (LB) at 30 °C at 300 r.p.m. in shaking water baths.

DNA manipulations and sequencing. All molecular biological techniques, unless stated otherwise, were performed by standard procedures (Sambrook *et al.*, 1989). Restriction enzymes and T4 DNA ligase were purchased from NEB and were used according to the manufacturer's instructions. Oligonucleotide primers were obtained from Sigma Genosys and are listed in Table 2. DNA sequencing was performed by the DNA sequencing facility, University of Cambridge. Nucleotide sequence data were analysed using the GCG package (Genetics Computer Group, University of Wisconsin); sequences were compared to protein and nucleotide databases using the BLAST suite of programs (Altschul *et al.*, 1990) at GenBank (<http://www.ncbi.nlm.nih.gov/BLAST/>).

Table 1. Strains, plasmids and phages used in this study

Strain	Relevant genotype or description	Reference or source
<i>E. coli</i>		
CC118 λ pir	<i>araD139</i> Δ (<i>ara leu</i>)7697 Δ <i>lacX74</i> <i>phoA20</i> <i>galE</i> <i>galK</i> <i>thi</i> <i>rpsE</i> <i>rpoB</i> <i>araEam</i> <i>recA1</i> (λ pir)	Herrero <i>et al.</i> (1990)
HH26 (pNJ5000)	<i>E. coli</i> helper strain used in marker exchange	Grinter (1983)
ESS	β -Lactam super-sensitive strain used in Car bioassays	Bainton <i>et al.</i> (1992)
<i>Ecc</i>		
ATCC 39048	Original 'virgin' Car ⁺ strain	Laboratory stock
ATTN10	Restrictionless Tc ^S derivative of a Tn10-containing ATCC 39048 (made by fusaric acid curing)	McGowan <i>et al.</i> (1996)
SBE1	<i>carH::blaM</i> derivative of ATTN10	This study
SM11	Δ <i>carB</i> derivative of ATTN10; Car [−]	McGowan <i>et al.</i> (1997)
SBE5	<i>carH::blaM</i> derivative of SM11	This study
SBE7	Δ <i>carB</i> , <i>carE::</i> Ω , <i>carH::blaM</i> derivative of SBE5	This study
SBE9	Δ <i>carB</i> , <i>carE::</i> Ω , <i>carH::blaM</i> derivative of ATCC 39048	This study
SBE7 5.1	Spontaneous ampicillin-resistant mutant derived from SBE7	This study
SBE7 5.2	Spontaneous ampicillin-resistant mutant derived from SBE7	This study
SBE7 5.3	Spontaneous ampicillin-resistant mutant derived from SBE7	This study
SBE7 5.4	Spontaneous ampicillin-resistant mutant derived from SBE7	This study
SBE7 5.5	Spontaneous ampicillin-resistant mutant derived from SBE7	This study
SBE7 5.6	Spontaneous ampicillin-resistant mutant derived from SBE7	This study
SBE7 5.7	Spontaneous ampicillin-resistant mutant derived from SBE7	This study
SBE7 5.8	Spontaneous ampicillin-resistant mutant derived from SBE7	This study
SBE7 5.9	Spontaneous ampicillin-resistant mutant derived from SBE7	This study
SBE7 5.10	Spontaneous ampicillin-resistant mutant derived from SBE7	This study
Plasmid		
pKNG101	<i>sacB</i> , <i>oriR6K</i> , Sm ^R	Kaniga <i>et al.</i> (1991)
pSMG45	<i>lacZ'</i> / <i>carE</i> fusion, <i>carFGH</i> , ' <i>kduI</i>	McGowan <i>et al.</i> (1997)
pSMG45HBlaMCAA +	pSMG45 plasmid with EZ TnBlaM <i>carH</i> translational fusion (see Methods)	This study
pSMG45HBlaMTAA +	pSMG45HBlaMCAA + with stop codon corrected to TAA (see Methods)	This study
pSMG45HBlaMTAA −	<i>SmaI</i> / <i>SnaBI</i> -digested pSMG45HBlaMTAA + 6.2 kb band self-ligated to remove <i>oriR6K</i>	This study
pKNG101HBlaMTAA −	<i>BglII</i> – <i>SpeI</i> 2.4 kb pSMG45HBlaMTAA − band ligated into <i>BamHI</i> – <i>XbaI</i> 6.5 kb pKNG101 band	This study
pSMG112	<i>carD</i> , <i>carE::</i> Ω , <i>carF</i> , <i>carG'</i> cloned into pKNG101	McGowan <i>et al.</i> (2005)
Bacteriophage		
ϕ KP	Generalized <i>Ecc</i> transducing phage	Toth <i>et al.</i> (1993)

Table 2. Primers used in this study

Primer	Sequence (5'–3')
CarF5'	CGCTAATTGTTGGACTGTGCTG
BlaRP1	CCAGCGTTTCTGGGTGATCGATGAT
CarHF1	CATGAATTCGGTAACACAAGATACCTACC
CarHB1	CATGGATCCTTAGTAATCCAGCTCTACCG
INBlaM3'	CATGGTACCTTACCAATGCTTAATCAGTGA
IS105'2	ACAAGATGTGCGAACTCGAT
IS103'	TCCCCAAAGCGTAACCATGT
jemC1	AGCATAACCTTTTCCGTGATGGT
jemC2	GATCTATGATTCCCTTTGTCAACAGCA
Sp2	GCCTTGATGTTACCCGAGAGC

Engineering of *carH::blaM* translational fusion plasmids.

The plasmid pSMG45 was mutagenized *in vitro* using the EZ TnBlaM kit (Epicentre) as described in the manufacturer's manual. Briefly, 1 µl reaction mixture was used to transform electrocompetent CC118λ*pir* cells, which were plated onto LBA supplemented with 5 µg ampicillin (Ap) ml⁻¹ and incubated overnight at 37 °C. Resistant transformant colonies were screened by colony PCR using the primers CarF5' and BlaRP1. One colony was identified as carrying an insertion in *carH*. Sequencing of the *carH* gene using primers CarHF1 and CarHB1 confirmed that the EZ TnBlaM had inserted into the *carH* gene at bp 7713 as defined by the *carA–H* gene cluster accession number U17224. This insertion was predicted to yield a functional translational fusion of the *carH* and *blaM* genes.

Unfortunately, sequencing revealed that bp 823 of the original EZ TnBlaM transposon DNA was a cytosine residue rather than the documented thymine residue listed in the manufacturer's manual. This single base pair alteration had the effect of altering the *blaM* TAA stop codon to a CAA codon encoding glutamine. Furthermore, this base pair alteration extended the ORF of the *blaM* gene by an additional 23 codons.

To correct the T-C base pair alteration, a PCR strategy was devised. Briefly, primers CarF5' and INBlaM3' were used to amplify *carFG*, *carH'::blaM* from pSMG45HBlaMCAA+ using the Hi Fi PCR kit supplied by Roche. The PCR product was digested with *SpeI* and *KpnI* and cloned into the 5.0 kb pSMG45HBlaMCAA+ *SpeI/KpnI* band to generate the plasmid pSMG45HBlaMTAA+. CC118λ*pir* (pSMG45HBlaMTAA+) grown on LBA had a MIC > 500 µg Ap ml⁻¹, whilst CC118λ*pir* (pSMG45HBlaMCAA+) had an MIC of < 50 µg Ap ml⁻¹, confirming that the altered stop codon was dramatically influencing CarHφBlaM β-lactamase activity in *E. coli* (data not shown).

Marker exchange. Engineering of *carE::Ω* and *carH::blaM* was carried out using the pKNG101-derived plasmids pSMG112 and pKNG101HBlaMTAA–, respectively, as described previously (Kaniga *et al.*, 1991). To avoid selecting for unwanted mutations during the marker exchange process that could lead to enhanced β-lactamase activity from the *carH::blaM* mutation, Ap was not used to select for colonies containing the *carH::blaM* marker. Instead, sucrose-resistant, streptomycin (Sm)-sensitive colonies were screened by patching onto LBA and LBA plus 5 µg Ap ml⁻¹. Any colonies that were able to grow on the LBA plus 5 µg Ap ml⁻¹ plates were then picked from the LBA-only plate, and the presence of the *carH::blaM* marker was confirmed by PCR analysis.

Carbapenem plate assays. Carbapenem production assays on overnight cultures of *Ecc* samples were carried out as described previously (McGowan *et al.*, 1996), using the β-lactam sensor strain

ESS. The only alteration was that, in some experiments, freshly prepared stock solutions of clavulanate were added to the LBA overlay to the desired final concentration to inhibit *carH::blaM* β-lactamase activity (Reading & Cole, 1977).

Nitrocefin-based β-lactamase assays. Expression of *carH::blaM* throughout growth was assayed using the chromogenic substrate nitrocefin (GlaxoSmithKline). Aliquots (1 ml) were extracted from liquid cultures of *Ecc* and resuspended in phosphate buffer, pH 7.0. The samples were then sonicated on ice for a total time of 3 min and 45 s, composed of eight 15 s periods of sonication with 15 s gaps between them. Cell debris was pelleted using a benchtop centrifuge at 13 000 r.p.m. for 5 min. To 800 µl of the sample incubated at 30 °C, 20 µl of 4 mg nitrocefin ml⁻¹ stock solution was added, and the change in OD₅₅₀ was recorded in a spectrophotometer over time and recorded relative to the OD₆₀₀ of the culture at each time-point.

Southern blotting using DIG-labelled probes. Southern blotting experiments were carried out using standard procedures (Sambrook *et al.*, 1989). DIG-labelled probes hybridizing to IS10 and *jemC* were created by PCR using the DIG-labelled dNTP mix supplied by Roche. The IS10 probe was PCR-amplified using primers IS105'2 and IS103'. DIG-labelled probes were detected using FAb fragments supplied by Roche. Peroxidase activity was detected using the CDP-star kit supplied by Roche.

RESULTS AND DISCUSSION**CarHφBlaM is functionally expressed in *Ecc* throughout growth**

A *carH::blaM* translational fusion was constructed in the ATTn10 *carA–H* operon by marker exchange to generate

Fig. 1. (a) Diagrammatic representation of the *carA–H* cluster in ATTn10. The P1 promoter enables transcription of *carA–H* and is regulated by the CarIR quorum-sensing system (McGowan *et al.* 1996). The P2 promoter is located within *carD* and enables weak expression of CarEFGH. (b) The *carA–H* cluster in SBE1 has a β-lactamase translational fusion in the *carH* gene. (c) SBE7 has an identical *carA–H* gene cluster to that of SBE1, except that an Ω interposon in *carE* prevents expression of CarF, CarG and CarHφBlaM from the P1 and P2 promoters.

Fig. 2. Clavulanate inhibits carbapenem degradation by CarH ϕ BlaM β -lactamase activity. Spots of ATTn10 (left hand side of each plate) and SBE1 (right hand side of each plate) were added to 0.7% Luria broth top agar, which had been seeded with the β -lactam sensor strain ESS, containing increasing concentrations of clavulanate: (a) 0 μ g clavulanate ml⁻¹, (b) 5 μ g clavulanate ml⁻¹, (c) 10 μ g clavulanate ml⁻¹, (d) 25 μ g clavulanate ml⁻¹ and (e) 50 μ g clavulanate ml⁻¹, and incubated overnight at 30 °C. The zones of clearing are proportional to the levels of carbapenem secreted by ATTn10 and SBE1.

strain SBE1 (Fig. 1b). To test whether the CarH ϕ BlaM protein was functional *in vivo*, ATTn10 and SBE1 Car production was assessed by spotting cultures onto ESS overlays supplemented with clavulanate (Fig. 2). Clavulanate was used to inhibit the β -lactamase activity (Reading & Cole, 1977) conferred by CarH ϕ BlaM. In the absence of clavulanate, the zone of clearing on the ESS lawn around SBE1 was reduced compared to that of ATTn10 (Fig. 2a). However, addition of clavulanate to the ESS overlays restored the size of the Car haloes around SBE1 to the same as that of ATTn10 (Fig. 2b–e). These results confirmed that i) SBE1 was producing the same levels of Car as that of ATTn10, and ii) the CarH ϕ BlaM β -lactamase was functional, as it could hydrolyse Car unless excess clavulanate was present as a competitive inhibitor.

CarH ϕ BlaM expression was detected throughout growth by measuring β -lactamase activity (Fig. 3). The level of β -lactamase activity in SBE1 increased dramatically during mid-exponential growth, as predicted, due to *carA*–*H* transcription via quorum sensing. The level of β -lactamase activity from SBE1 began to decline again once the cells were in stationary phase, suggesting that the CarH ϕ BlaM hybrid was turned over *in vivo*, presumably because of proteolysis. Previous studies have shown that β -galactosidase also declines during stationary phase (McGowan *et al.* 2005). It seems likely that both CarH ϕ BlaM and β -galactosidase are inactivated by proteases that are induced, or are most active, during the stationary phase of growth.

A third strain (SBE7, Fig. 1c) was constructed, in which transcription of *carH::blaM* was disrupted. SBE7 *carH::blaM* transcription from both of the known *carA*–*H* promoters was blocked by the presence of the strongly polar omega interposon sequence (Prentki & Krisch, 1984) in *carE* (Figs 1c and 3). Consistent with our previous data on transcription of the operon (McGowan *et al.*, 2005), transcription of *carH::blaM* was switched off in strain SBE7.

The *carH::blaM* fusion strain can give rise to ampicillin-resistant mutants, despite the interposon insertion

The survival frequency of ATTn10 and SBE1 in response to increasing concentrations of Ap is recorded in Fig. 4. As a negative control we also included *Ecc* strain SBE7, which cannot transcribe the *carH::blaM* gene because of the *carE::Ω* mutation, as described above. Surprisingly, SBE7 had an e.o.p. on LBA supplemented with 5 μ g Ap ml⁻¹ of 1×10^{-5} , which was significant, because ATTn10 could not grow on this medium. *Ecc* growth on LBA supplemented with 25 μ g Ap ml⁻¹ was undetectable after 24 h at 30 °C, with the exception of SBE1, which had an e.o.p. of 1×10^{-7} relative to that in the absence of Ap.

The e.o.p. values of SBE1 and SBE7 when grown on LBA supplemented with 5 μ g Ap ml⁻¹ were 1×10^{-4} and 1×10^{-5} , respectively. Because transcription of *carH::blaM* in SBE7 cannot occur from either of the *carA*–*H* promoters because of the polar *carE::Ω* mutation, SBE7

Fig. 3. Detection of β -lactamase activity throughout growth using the chromogenic cephalosporin nitrocefin. The following are shown: log OD₆₀₀ of SBE1 (diamonds, solid line) and SBE7 (triangles, solid line) grown at 30 °C in Luria broth at 300 r.p.m. in shaking water baths; β -lactamase activity of SBE1 (diamonds, broken line) and SBE7 (triangles, broken line) throughout growth.

Fig. 4. Survival efficiency of SBE1 (black bars), SBE7 (grey bars) and ATTn10 (white bars) in response to different Ap concentrations.

Ap^R colonies could only arise if the *carH::blaM* fusion was transcribed from a new promoter.

Expression of CarH ϕ BlaM can occur spontaneously in SBE7 because of the action of mobile DNA providing functional promoters

To determine the nature of the SBE7 Ap^R mutants, 10 of the colonies (SBE7 5.1–5.10) that grew on 5 µg Ap ml⁻¹ were selected at random for further investigation. The most likely explanation for Ap^R in SBE7 5.1–5.10 was that a genetic alteration (presumably insertions or deletions) had occurred immediately upstream of *carH::blaM*. Therefore, the region between *carH::blaM* and *carE::Ω* in these mutants was amplified by PCR analysis using primers Sp2 and BlaRP1 (Fig. 5a). It was clear that, in the Ap^R SBE7 mutants, the size of the 1871 bp region (Fig. 5a, lane F) between *carH::blaM* and *carE::Ω* had increased. In most cases, the increase in size was approximately 1.3 kb. In the case of SBE7 5.5, the increase in size was approximately 1.8 kb (Fig. 5a, lane E). Sequencing of four of the PCR products from SBE7 5.1 to 5.4 revealed that IS10R from the transposon Tn10 had inserted in the region between *carH::blaM* and *carE::Ω* (Fig. 5b). The site of the IS10 insertion in strains SBE7 5.1, 5.3 and 5.4 was identical in each case, suggesting that they could be siblings. In all cases, the IS10R P_{out} promoter was orientated in the correct direction to enable transcription of the downstream *carH::blaM* gene (Fig. 5c). Previous data has shown that both IS10 elements possess a fairly strong P_{out} promoter (Simons *et al.*, 1983) that is capable of allowing expression of adjacent ORFs (Ciampi *et al.*, 1982). It seems that, due to these random insertions, the P_{out} promoter from IS10 has enabled SBE7 5.1–5.10 to drive transcription of the otherwise cryptic *carH::blaM* fusion.

Our results suggest that ATTn10 provides a useful model to study the adaptive evolution processes seen in natural isolates that possess IS10R. As an example, earlier research

by other groups has shown that IS elements can induce antibiotic resistance in *E. coli* (Jellen-Ritter & Kern, 2001; Kobayashi *et al.*, 2001) and *Salmonella enterica* (Olliver *et al.*, 2005). In all three of these studies, IS elements were shown to provide mechanisms of broad-spectrum antibiotic resistance by providing promoters that are capable of expressing the multidrug resistance pump AcrEF. Such experiments show that IS elements can play a crucial role in the evolution of antibiotic resistance in clinical isolates.

Transposon Tn10 has been extensively degraded in the ATTn10 genome

Mutant SBE7 5.5 was particularly interesting, because the PCR product from this mutant was enlarged by approximately 1.8 kb instead of 1.3 kb (Fig. 5a, lane E). Sequencing of the enlarged PCR product showed that this mutant contained genetic remnants of Tn10 orientated such that the IS10L end was closest to *carH::blaM*. In SBE7 5.5, transcription of *carH::blaM* appears to be from the P_{out} promoter of IS10L, as this promoter is in the correct orientation. The Tn10 sequence in this IS element was severely degraded, however, by the presence of two large deletions in its sequence (Fig. 6). Both deletions are defined by the Tn10 sequence accession number AF162223 (Chalmers *et al.*, 2000), and are as follows: deletion 1 removes bp 340–4426, which includes most of IS10L and ends in the 5' end of *jemC*, removing *jemAB* in the process; deletion 2 removes bp 4698–7818. Deletion 2 removes the 3' end of the *jemC* gene and the entire *tetRACD* gene cluster, and ends precisely at the point at which the IS10R inverted repeat sequence begins. This 1939 bp Tn10 genetic remnant will from now on be referred to as Tn10Δ.

The process leading to the generation of deletion 1 is not clear, but may involve strand slippage mechanisms (Farabaugh *et al.*, 1978; Albertini *et al.*, 1982; Singer & Westlye, 1988; Marvo *et al.*, 1983; Uematsu *et al.*, 1999) during DNA replication or repair, because it is flanked at the

Fig. 5. (a) Colony PCR analysis of the following strains using primers BlaRP1 and SP2: A, SBE7 5.1; B, SBE7 5.2; C, SBE7 5.3; D, SBE7 5.4; E, SBE7 5.5; F, SBE7; G, SBE7 5.6; H, SBE7 5.7; I, SBE7 5.8; J, SBE7 5.9; K, SBE7 5.10. The lanes designated M are GibcoBRL Life Sciences 1 kb ladders. (b) Diagrammatic representation of IS10R transposition upstream of *carH::blaM* but downstream of *carE::Ω*, leading to the IS10R P_{out} promoter becoming correctly orientated to enable transcription of *carH::blaM*. (c) The DNA sequence of *carF* and *carG* from GenBank accession number U17224. Boxed 'atg' sequences indicate the start codons of *carF* and *carG*. The stop codons for *carF* and *carG* are underlined and in italics. The bold sequences in upper-case type represent the sites of the IS10 insertions in SBE7 5.1–5.5. Three of the mutants, SBE7 5.1, 5.3 and 5.4, had IS10 insertions that duplicated bp 7074–7082. One of the mutants, SBE7 5.2, had an insertion that duplicated bp 6985–6993. The last mutant, SBE7 5.5, had an enlarged IS10 insertion that duplicated bp 6832–6840.

termini by an imperfect 7 bp repeat sequence G(C/A)T(G/C)TAT. Alternatively, processes other than strand slippage (Balbinder, 2001) may be responsible for deletion 1.

Deletion 2 terminates with a 5 bp imperfect direct repeat, ATGC(T/A), and therefore may have formed by a strand misalignment mechanism (Farabaugh *et al.*, 1978; Albertini *et al.*, 1982; Singer & Westlye, 1988; Marvo *et al.*, 1983; Uematsu *et al.*, 1999). However, the direct transition of the IS10R inverted repeat sequence that defines the boundaries of the mobile genetic element to the site of a deletion is characteristic of an IS10 adjacent deletion, as described elsewhere (Roberts *et al.*, 1991; Chalmers & Kleckner, 1996). It seems very likely that deletion 2 has been generated by an IS10-mediated adjacent deletion. Two lines of evidence support this hypothesis: i) deletion 2 is immediately flanked at the 3' end by the IS10 inside end, and ii) deletion 2 is immediately flanked at the 5' end by a 5'-GGTTATGCT-3' sequence which closely matches the 5'-NGCTNAGCN-3'

IS10 transposition consensus sequence (Halling & Kleckner, 1982).

The Tc^S phenotype of the ATTn10 strain can be explained by the presence of deletion 2. This is because deletion 2 has led to the complete loss of the *tetRACD* gene cluster from Tn10. Furthermore, a Southern blot to probe for *jemC* in ATTn10 genomic DNA cut with *Bsi*IEI, which cuts either end of Tn10, generated only one hybridizing band that corresponded to the size expected for Tn10Δ, confirming that the *tetRACD* and *jemAB* genes are absent in the ATTn10 genome (data not shown). Deletion 2 would offer a selective advantage when Tn10-containing strains were exposed to fusaric acid because it removes the *tetA* gene that encodes the TetA efflux pump which confers fusaric acid sensitivity. An example of how fusaric acid treatment can lead to the generation of hybrid IS elements in strains containing Tn10 has been documented in *E. coli* (Bogosian *et al.*, 1993). The IS10R fusion in the strain under study generated a 1329 bp element

Fig. 6. (a) Diagrammatic representation of the regions of Tn10 that have been deleted in Tn10Δ. Genes and IS10 elements are represented as grey rectangles; deletions are represented as black rectangles. (b) Representation of Tn10Δ insertion upstream of *carH::blaM* in Ecc strain SBE7 5.5. Transcription of *carH::blaM* is likely to occur from the P_{out} promoter of the IS10L fragment within Tn10Δ which is represented by a black bent arrow. All genes are drawn approximately to scale, with the exception of *carH::blaM*.

(designated IS10L/R1) that contained sequence from IS10R as well as sequence from IS10L. The Tn10Δ element isolated in strain SBE7 5.5 is a new example of how the strong selection pressure exacted by fusaric acid treatment of Tn10-containing strains can encourage the evolution of novel, hybrid IS elements.

ATTn10 contains multiple copies of IS10

Genomic DNA from SBE7, SBE9 and SBE7 5.1–5.10 was analysed by Southern blot experiments using DIG-labelled probes to the transposase gene of IS10 (Fig. 7). No hybridizing bands were detected in SBE9 DNA. SBE9 is not derived from ATTn10, and therefore has never been exposed to Tn10. However, it was clear from the SBE7 sample, which does derive from ATTn10, that there were at least 15 independent hybridizing bands. A band approximately 6.8 kb in size, corresponding to an IS10 insertion in *carFG*, was present in all the Ap^R SBE7 mutants except SBE7 5.5 (Fig. 7), supporting the PCR and DNA sequencing data (Fig. 5). A band at the predicted 7.4 kb was present in the SBE7 5.5 genomic DNA sample, but this co-migrated with at least one other band that was present in SBE7 and all the derived mutant strains. The 7.4 kb band was more distinguishable in SBE7 5.5 than in the other strains when probing for the presence of *jemC* (data not shown).

There are other examples in the literature of strains that contain multiple copies of IS10: a strain of *Enterobacter cloacae* has been found to contain at least 15 copies of IS10 (Matsutani, 1991), and a strain of *Salmonella enterica* has also been shown to possess at least 15 chromosomal copies (Olliver *et al.*, 2005). IS10 copy number would normally be

Fig. 7. Southern blot to detect the IS10 transposase gene from chromosomal DNA of several Ecc strains digested with AatII. The lanes are as follows: A, SBE7 5.1; B, SBE7 5.2; C, SBE7 5.3; D, SBE7 5.4; E, SBE7 5.5; F, SBE7; G, SBE9; H, SBE7 5.6; I, SBE7 5.7; J, SBE7 5.8; K, SBE7 5.9; L, SBE7 5.10. All marker band sizes are approximate values. The dashed arrow shows the predicted 6.8 kb band that corresponds to an IS10 insertion in *carFG* in SBE7, which is also indicated with a circle in lane H. Examples of unique IS10 hybridizing bands present in some of the SBE7 ampicillin-resistant mutants are boxed within rectangles.

kept low by several mechanisms. Firstly the transcription and translation levels of the IS10R promoter are fairly weak (Simons *et al.*, 1983). Secondly, transcription of the transposase gene is limited to a small fraction of the cell cycle because of methylation of the two *dam* sites located in IS10R (Roberts *et al.*, 1985). Thirdly, IS10R has been shown to possess mechanisms to limit its copy number in the cell by a process known as 'multicopy inhibition' (Simons & Kleckner, 1983). This effect is believed to be due to RNA produced by transcription from the P_{out} promoter inhibiting translation of the transposase gene by hybridizing to the first 36 bp of the mRNA message and occluding ribosome binding. This effect has been shown to function *in trans*, so that as the number of IS10 copies in the cell increases, multicopy inhibition becomes more effective at preventing IS10 transposition. It may be that multicopy inhibition is less effective in some genetic backgrounds, perhaps because the P_{out} RNA transcript is turned over rapidly in some strains.

It is possible to differentiate SBE7 5.1–5.10 from each other by the number and size of their IS10R hybridizing bands, which in many cases were different from each other. As an example, SBE7 5.10 contains an easily distinguishable hybridizing band at around 2.2 kb that is absent in SBE7 and the rest of the Ap^R SBE7 mutants. Whether these IS10 hybridizing bands were generated before or after selection on Ap is not known. It has been shown that growth in the presence of β -lactams can induce the SOS response (Miller *et al.*, 2004). The SOS response in turn has been shown to

induce *IS10* transposition (Eichenbaum & Livneh, 1998). Therefore, any colonies that are initially able to grow in the presence of Ap may undergo increased rates of *IS10R* transposition that may explain the high level of heterogeneity among SBE7 5.1–5.10.

One observation from Fig. 7 is that none of the *IS10* hybridizing bands present in the SBE7 parental strain is absent in SBE7 5.1–5.10. As *IS10R* has been shown previously to transpose by a non-replicative mechanism (Bender & Kleckner, 1986; Kleckner *et al.*, 1995), it seems clear that during transposition, *IS10R* is maintained in our strains at the donor molecule site. Mechanisms for how this can occur have been described elsewhere (Bender *et al.*, 1991), and our results agree with a model in which intramolecular rejoining to lose *IS10R* from the donor molecule site is an uncommon event.

Final summary

This research has generated a functionally expressed β -lactamase translational fusion in *carH*, and shown that in the absence of a functional promoter, this construct can serve as an effective mobile promoter trap. This has led to the identification of cryptic *IS10* elements in the ATtn10 chromosome. This finding was initially surprising, as it was assumed that *IS10* might have been lost from the chromosome during fusaric acid treatment to cure Tn10-dependent Tc^R in the progenitor strain. This promoter trap offered insights into the molecular events that may have led to ATtn10 Tc^S with the discovery of the Tn10 genetic remnant Tn10 Δ in the SBE7 Ap^R mutant SBE7 5.5. Southern blot experiments subsequently demonstrated that ATtn10 contains at least 15 copies of *IS10*. These data show that ATtn10 may be a useful model strain in which to study adaptive evolution, and our recent observations that ATtn10 may be hypermutable are consistent with this notion (S. D. Bowden and G. P. C. Salmond, unpublished data).

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