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Development of a novel inducible bioluminescent and antibiotic resistance tagging system and its use to investigate the role of antibiotic production by *Pectobacterium carotovorum* ssp. *carotovorum* during potato tuber infection

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Abstract We report the construction of a novel Tn7 vector for the tagging and enumeration of target bacteria from complex microbial communities. The system utilises a cassette for inducible bioluminescence and tetracycline resistance that integrates at a defined neutral position present in most Gram-negative species. We used this approach to chromosomally tag *Pectobacterium* such that it could be enumerated in mixed consortia without placing a significant bioburden on the tagged strain. Two *Pectobacterium* strains, a carbapenem antibiotic producer and an isogenic knock-out strain were tagged using this system. The modified *Pectobacterium* strains were used to compare the extent to which potato tuber-associated and endophytic bacteria can gain advantage and multiply *in planta*, utilising the nutrients released by a *Pectobacterium* infection, when the infecting *Pectobacterium* is either an antibiotic producer (Car+) or a carbapenem knock-out (Car−) strain. We show that the ability to synthesise carbapenem has a significant effect upon *Pectobacterium* numbers throughout the course of the infection. Whilst limiting the number of other bacterial species, carbepenem production allows the *Pectobacterium* to replicate to higher titres in the rotting tuber. We anticipate that the Tn7 tagging vector will be of use to other researchers studying ecological interactions in complex environments.

Keywords *Erwinia carotovora* · Tn7 vector · Soft rot

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Introduction

Tagging of bacteria with marker genes is a commonly used approach to follow target species in a mixed population. However, in complex ecosystems the constitutive expression of marker genes may have a negative impact on the fitness of the tagged organisms (Sayler et al. 2001; Jansson 2003; Dandie et al. 2005). A system which would allow routine site-specific insertion and tightly controlled expression of marker genes would provide an optimal tool for such studies. The Tn7 transposon inserts into most bacterial chromosomes at a unique neutral site (*att*Tn7),

downstream of the *glmS* gene (Peters and Craig 2001). It inserts with high frequency, as a single copy in a fixed orientation. The inserted sequences are stably maintained during growth and it is believed that the insertion within this site does not affect its host (Peters and Craig 2001). Taking advantage of these characteristics, we developed a novel Tn7 system that allows the facile insertion of genes for tetracycline resistance and tetracycline-inducible bioluminescence into most bacterial species in which the *att*Tn7 site is present. Plating of mixed bacterial populations on tetracycline-containing media provides efficient selection of the tagged strain, which is confirmed by the induced bioluminescence. This system allows the facile identification and enumeration of the tagged strain, even when present as a minor constituent of the bacterial population. We have used this vector for the first time to tag two strains of *Pectobacterium* and we show that in both cases the marker cassette inserted at the predicted single site (downstream of the *glmS* gene). The tagged *Pectobacterium* strains were then used in an established potato-infection model (Llama-Palacios et al. 2002) to investigate the potential role of antibiotic production during infection.

Species of *Pectobacterium carotovorum* ssp. *carotovorum* (*Pcc*) and *Pectobacterium atrosepticum* (formerly known as *Erwinia carotovora* ssp. *carotovora* and *Erwinia carotovora* ssp. *atroseptica*) are important pathogens of potato, causing soft rot of tubers and blackleg of stems (Pérombelon 2002). Some *Pcc* strains produce a broad-spectrum β-lactam antibiotic, 1-carbapen-2-em-3-acid carboxylic acid (carbapenem), thought to have a role in niche defence by eliminating competing bacteria from sites of infection (Whitehead et al. 2002). In *Pcc*, production of carbapenems is controlled in a cell density-dependent manner, requiring the accumulation of AHL - N-(3-oxohexanoyl)-L-homoserine lactone (3-oxo-C6 HSL) in the growth medium and *in planta* during infections (Bainton et al. 1992; Pirhonen et al. 1993; Swift et al. 1993; Liu et al. 2008). *Pcc* produces 3-oxo-C6 HSL, which binds to an activator protein (CarR), initiating the transcription of the carbapenem biosynthetic and resistance gene cluster (*car A-H*) (Jones et al. 1993; McGowan et al. 1995, 1997; Welch et al. 2000). This gene cluster contains two promoters (McGowan et al. 2005), the first (P1) is located upstream of *carA* and is transcribed in the presence of 3-oxo-C6 HSL, and the second promoter (P2) is located within *carD* and directs the transcription of *Pcc*'s carbapenem resistance genes (*carE-H*), providing auto-resistance to carbapenem. The regulation of carbapenem production has been studied in liquid cultures (reviewed by Coulthurst et al. 2005) and it has been shown that carbapenem is produced during the transition between late log and stationary phases of growth (Whitehead et al. 2002). Whilst the genes involved in the regulation of carbapenem synthesis have been extensively studied in liquid monocultures, less is known of the ecological importance of antibiotic production during plant infection. The impact of carbapenem on potato-associated bacterial communities during *Pectobacterium* infections has not been tested and no advantage to the *Pectobacterium* has been demonstrated.

To investigate the adaptive value of carbapenem production in the presence of potentially competing or antagonistic bacterial species *in planta*, we used our newly developed inducible marker system which allowed us to selectively recover and enumerate the infecting *Pectobacterium* strains. Our results indicate that when the soft-rot causing *Pectobacterium* is unable to produce antibiotics, competing bacteria are able to grow to higher cell densities and this is at the expense of the *Pectobacterium* itself.

Materials and methods

Bacterial strains used in this study

The wild-type bacterial strain *Pcc* ATCC39048 was obtained from the Scottish Crop Research Institute (SCRI) culture collection from stocks stored at −80°C. Tn7 site-specific insertion was used to create a carbapenem-producing *tetR-luxABCDE-tetA* labelled strain (*Pcc*-*tetR*-luxABCDE-*tetA*) and an isogenic carbapenem knock-out strain (*Pcc*-carC^{-} *tetR*-luxABCDE-*tetA*). All *Pectobacterium* strains used in the present study were routinely grown on Luria-Bertani (LB, Sambrook et al. 1989) medium at 29°C.

Insertional mutagenesis of *carC*

Based on the known nucleotide sequence of the wild-type *Pcc* ATCC39048, two primers leftcarB (5′ TGCCGGAACTTAAGCATGGT3′) and rightcarD (5′AGAACCAAACGATCCACTGC) were designed to generate a 1,824 bp fragment following PCR

amplification. This fragment incorporates 368 bp of *carB*, 485 bp of *carD* and the whole *carC* (821 bp) of the carbapenem biosynthetic gene cluster. The *carBCD* PCR product was gel-purified using Wizard column (Promega) and cloned into pGEMT Easy vector (Promega). The resulting plasmid (pGEMT-carBCD) was digested with *Eco*RV in order to remove a 648 bp fragment from within the *carC* coding sequence, then religated. The whole *carBC-carCD* fragment was then released by *Not*I (Promega) digestion and further cloned into the *sacB*-based suicide vector pDM4 (Milton et al. 1996). The resulting plasmid was conjugated from *E. coli* S17-1λ pir into *Pcc* (ATCC39048) as described by Milton et al. (1996). Chloramphenicol-resistant transconjugants were selected on minimal M9 medium (Sambrook et al. 1989) containing 25 μg ml^{-1} chloramphenicol (Cm). Colonies in which excision of the integrated vector sequences occurred through homologous recombination were selected by growth on LB plates containing 5% sucrose and assaying for chloramphenicol sensitivity and lack of carbapenem production. Selected colonies were checked for the absence of the excised *carC* fragment by colony PCR, using the same leftcarB and rightcarD primers which in the case of *carC*$^-$ mutants amplified a ~1.2 bp fragment.

Construction of pGRG25-*tetR-luxABCDE-tetA* site-specific tagging vector

To generate the tetracycline inducible *luxABCDE* reporter vector we used the multisite Gateway cloning (Invitrogen). First the 5′ end entry vector was created by amplifying the tetracycline repressor (*tetR*) and the promoter of the tetracycline resistance gene (*PtetA*) from Tn10 (Bertrand et al. 1983) using the TetRattB4f-5′GGGGACAACTTTGTATAGAAAAGTTGGCGGCCGCACGGAAAAAGGTTATGCTGCTTTTAAGACCC 3′ and PtetAattB1r-5′GGGGACTGCTTTTTTGTACAAACTTGTTCACTTTTCTCTATCACTGATAGGG 3′ primers and the amplified fragment was cloned into the pDONRP4-P1R entry vector (Invitrogen) using the BP recombination reaction according to the supplier's protocol. The forward primer contains a *Not*I restriction site (underlined) in order to facilitate the release of the whole marker gene cassette after the LR recombination reaction. The generated entry clone (pDONRattL4R1-*tetR*) was characterised by sequence analysis. Secondly, a 3′ end entry clone was generated by amplifying the tetracycline resistance gene (*tetA*) from pBR322 (Bolivar et al. 1977) using the TetAf 5′ - AATGCGGCCGCTTCTCATGTTTGACAGCTTAT - 3′ and TetAr 5′ - CATTCAGGTCGAGGTGGCCCGGCTCCATGC 3′ primers, and cloning of the PCR product into the *Pme*I site of a previously described entry vector (Perehinec et al. 2007), downstream of the *rrnBT1T2* bi-directional transcriptional terminator and within the *attR2* and *attL3* recombination sites. The generated entry vector (pDONRattR2L3-T1T2-*tetA*) was characterised by restriction digest using *Eco*RV restriction enzyme (Promega). The *luxABCDE* reporter entry clone (pDONR221-*luxABCDE* attL1L2) was kindly donated by Tania Perehinec. The Multisite Gateway LR reaction between the entry clones and the pCR2.1-DESTR4-R3 (Perehinec et al. 2007) destination vector was performed according to the supplier's protocol in order to generate the pCR2.1-*tetR-luxABCDE-tetA* expression clone. The whole *tetR-luxABCDE-tetA* reporter gene cassette was released by *Not*I digestion and subcloned into the multiple cloning site of the pGRG25 site-specific Tn7 transposition vector (McKenzie and Craig 2006). The orientation of the insert was checked by PCR using the Tn7Rf 5′ - TGTGGGCGGACAATAAAGTCTTAAACTGAAC - 3′ and PtetAattB1r, or the Tn7Lr 5′ - CTGTGGGCGGACAAAATAGTTGGGAACTGGG - 3′ and PtetAattB1r pimer pairs.

Tagging the wild-type *Pcc* ATCC39048 and the carC$^-$ knockout strains using the site-specific pGRG25-*tetR-luxABCDE-tetA* Tn7 transposition vector

The pGRG25-*tetR-luxABCDE-tetA* construct was delivered to the wild-type *Pcc*ATCC39048 strain and to the *carC*$^-$ knock-out strain by conjugation using the donor *E. coli* S17-1 λ-pir strain. Prior to conjugation, both strains were grown overnight in LB broth cultures, washed twice with 10 mM $MgSO_4$, mixed in 1:2 donor:recipient ratio and incubated on LB agar plates for 6 h at 29°C. After incubation, the mating mixture was scraped off the LB plates, resuspended in 10 mM $MgSO_4$ and plated onto M9 plates containing 10 μg ml^{-1} of tetracycline. To select for *tetR-luxABCDE-tetA* tagged transconjugants, plates were incubated for 48 h at 29°C. Tetracycline-resistant luminescent colonies were identified using a

NightOWL Imaging System (EG&G Berthold). To facilitate transgene insertion and loss of the temperature-sensitive plasmid, a few luminescent colonies from both conjugations were selected and grown overnight at 37°C, non-selectively in LB containing 2% arabinose. Cells from this culture were plated on LB plates and incubated overnight at 37°C. Colonies were selected and replica plated on LB plates containing 10 μg ml^{-1} tetracycline and on LB plates containing 50 μg ml^{-1} ampicillin. Colonies which grew only on tetracycline-containing plates were selected and insertion into the target site was verified by PCR using the glmSEcaF (5′-CCGCTTGCGCTGATTGATGCAGACATG -3′) and TetAr (5′ CATTCAGGTCGAGGTGGCCCGGCT CCATGC 3′) primer pairs.

DNA blot analysis

Genomic DNA from selected tetracycline-inducible bioluminescent colonies (*Pcc-tetR-luxABCDE-tetA* and *Pcc-carC*$^{-}$*-tetR-luxABCDE-tetA*) and of the wild-type *Pcc* ATCC39048 strain was extracted using a CTAB method described by Ausubel et al. (1994). Genomic DNA, along with the pGRG25-*tetR->luxABCDE-tetA* plasmid was digested with either *Eco*RI or *Bam*HI (Promega), electrophoresed on 1% agarose gels and transferred to Hybond N + (Amersham, UK) membranes using a standard neutral transfer procedure (Sambrook et al. 1989). The DNA probe (full length *tetA*) was random prime-labelled with [α-32P] dCTP using a RediprimeTM II (Amersham, UK) kit. The probe was hybridised to the filter overnight in: 10× Denhardt's, 1% SDS, 5× SSC, 1 mg ml^{-1} sonicated Salmon sperm DNA at 65°C. Membrane washing was carried out with 2×, 1× and 0.1× SSC, 0.1% SDS solutions at 65°C, 10 min each. Signals were detected using auto-radiographic film (KODAK X-OMAT AR).

Growth and bioluminescence measurements

Pectobacterium cultures were grown overnight in LB broth at 29°C with shaking (150 rpm). Cells were recovered by centrifugation, washed with the same volume of 10 mM $MgSO_4$ and diluted 1/100 into LB broth or LB broth supplemented with 10 μg ml^{-1} tetracycline. Replicate samples (200 μl) were placed into the wells of a 96-clear-bottom microtiter plate (Porvair) and incubated at 28°C in a Tecan Genesis Pro microplate reader. Absorbance (600 nm) and bioluminescence (RLU) readings were taken at 30 min periods over 24 h.

Tuber infections and bacterial enumeration

Potato tubers (cv. King Edward) were washed and surface-sterilised by spraying with 70% ethanol, followed by rinsing with sterile demineralised water and drying in a flow cabinet. Slices of 0.7 cm were cut transversely and slices of similar weight (10–12 g) were chosen and placed in sterile Petri dishes (Sterilin, UK) prior to inoculation. For the infections 10 ml cultures of the *Pectobacterium* strains (OD_{600}= 0.450) were centrifuged (3,000 rpm for 5 min), washed with the same volume of 10 mM $MgSO_4$, and diluted to 10^8cells ml^{-1}. Tuber slices were inoculated with 100 μl of bacterial suspension (10^7 *Pectobacterium* cells/tuber slice) mixed with an equal volume of tuber wash (TW, ~10^6 culturable bacteria/ tuber slice). Tuber wash was prepared from bacteria recovered from the surface of potato tubers (1 g of tuber wash resuspended in 9 ml of 10 mM $MgSO_4$). TW was diluted and plated on LB agar in order to establish the total culturable bacteria. Four tuber slices for each time-point were inoculated with this bacterial suspension. Control tuber slices were also inoculated, for each time-point, with 100 μl tuber wash mixed with the same volume of 10 mM $MgSO_4$. All inoculated samples were placed in plastic boxes and sealed to maintain humidity. For bacterial cultivation and enumeration from infected tuber slices Luria-Bertani medium (Sambrook et al. 1989) was used. After each incubation time-point (0–9 days) tuber slices were weighed and homogenised with 1:10 volume (weight:volume) of 10 mM $MgSO_4$ in filter stomacher bags to enable collection of the ten-fold diluted liquid fraction. A serial dilution of this suspension was performed and 100 μl of appropriate dilutions were plated in duplicate on LB plates and LB plates containing 5 μg ml^{-1} tetracycline. All plates were incubated at 25°C for 48 h.

Statistical data analysis

Design-Expert 7.1.5. for Windows (Stat-Ease Inc, Minneapolis, MN, USA) was used for all statistical analysis of the data (ANOVA; $P<0.05$).

Results

Creation of an isogenic carbapenem knock-out strain by homologous recombination

A carbapenem-negative strain (*carC*$^{-}$) was created from the *Pcc* ATCC39048 parent by two-step homologous recombination to delete a 648 bp nucleotide fragment of *carC*. This deletion left the downstream carbapenem resistance genes intact and fully functional. Gene deletion was confirmed by PCR analysis and loss of antibiotic production was demonstrated by the inability of the knock-out strain to inhibit growth of a carbapenem super-sensitive *E. coli* tester strain (ESS, Bainton et al. 1992; Fig. 1). Similar T-streaks with the wild-type (*Pcc* ATCC39048) and the knock-out strain (*carC*$^{-}$) indicated that the expression of the downstream genes, which have an independent promoter and confer carbapenem resistance, had not been compromised.

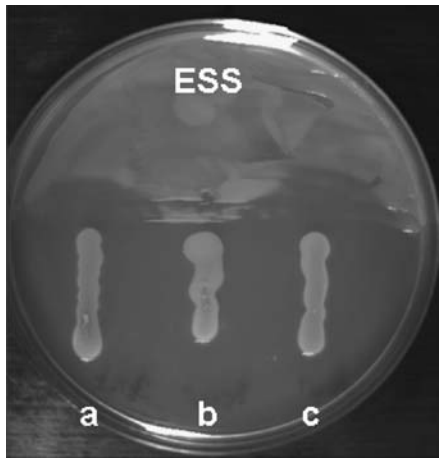

Fig. 1 Assay for carbapenem production by wild-type (*Pcc* ATCC39048) and tagged strains. Tagged *Pcc- tetR-luxABCDE-tetA* (**a**), *Pcc*-carC^{-}- *tetR-luxABCDE-tetA* knock-out strain (**b**) and the wild-type *Pcc* ATCC39048 (**c**) strain were cross-streaked against the *E. coli* sensor strain (ESS). Both the tagged *Pcc- tetR-luxABCDE-tetA* (**a**) and the wild-type *Pcc* ATCC39048 (**c**) strain produce carbapenem which inhibits the growth of the *E. coli* sensor strain (ESS). The *Pcc*-carC^{-}-*tetR-luxABCDE-tetA* knock-out strain (**b**) has lost its ability to inhibit ESS growth

tetR-luxABCDE-tetA transgene insertion into the *Pcc* chromosome

To facilitate the isolation and enumeration of the *Pcc* strains from a mixed population, we created a novel Tn7-based vector that allows the facile insertion of genes for inducible tetracycline resistance and bioluminescence at a unique, neutral site within the *Pcc* genome. To do so, a tetracycline (Tc)-inducible bioluminescent *luxABCDE* reporter cassette (pCR2.1-*tetR-luxABCDE-tetA*) was first created by multisite Gateway cloning (Invitrogen). In this construct, the expression of both the *luxABCDE* and *tetA* marker genes are under *tetR* repression in the absence of inducer (Corbel and Rossi 2002). The entire *tetR-luxABCDE-tetA* reporter cassette was then introduced into the pGRG25 site-specific transposition vector (McKenzie and Craig 2006), between its Tn7 left and right borders (Fig. 2a) to give the pGRG25- *tetR-luxABCDE-tetA* vector. This vector carries all four transposase genes (*TnsABCD*) flanked by the left and right ends of Tn7 to promote transposition into the *att*Tn7site of the bacterial chromosome. The *att*Tn7 site is found at the 3′ end of the *glmS* gene in many Gram-negative bacteria. The pGRG25-*tetR-luxABCDE-tetA* plasmid was delivered to *Pectobacterium* strains by conjugation as described in the Materials and methods section. In most organisms Tn7 inserts in a single-site downstream of *glmS,* therefore no genes are disrupted and no fitness costs to the host are encountered (McKenzie and Craig 2006). The pGRG25-*tetR-luxABCDE-tetA* vector also carries an *araC* promoter to mediate arabinose-inducible expression of the transposase genes, and a temperature-sensitive origin of replication which allows the host to be cured of the plasmid after transposition and marker insertion.

After plasmid curing, a few colonies for each of the labelled *Pectobacterium* strains were selected and checked by PCR for the insertion of the transgenes in the *att*Tn7 site using oligonucleotides glmSEca and TetAr. The forward primer (glmSEca) was designed according to the published sequence for the *glmS* terminator region of the *P. atrosepticum* SCRI1043 strain, as no sequence data was available for the *Pcc glmS* gene. Tagged strains gave a positive amplification product of the expected size (~1.7 kb) but no amplicon was obtained from control wild-type strains as there is no binding site for the TetAr primer. DNA sequencing confirmed that the fragment contained the *glmS* target sequence for the *Pcc* strain as well as the Tn7 insertion site (not shown). Absence of additional

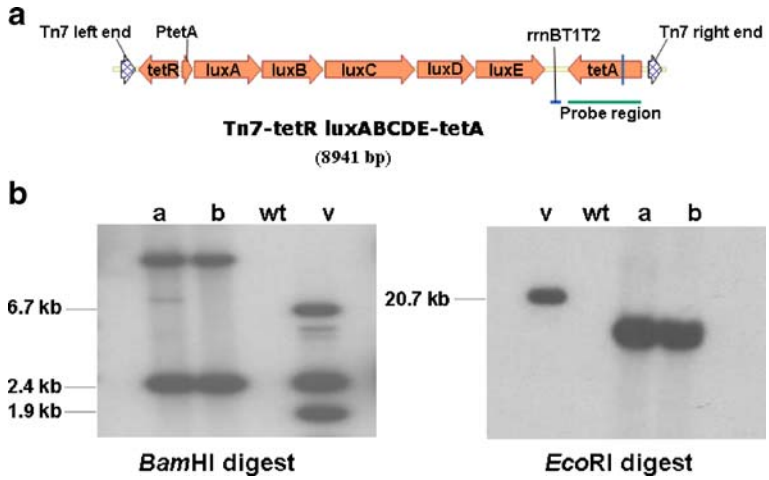

Fig. 2 Site-specific tagging of *Pectobacterium*. **a** Schematic representation of the *tetR-luxABCDE-tetA* reporter cassette introduced into the *Pectobacterium*'s chromosome. **b** Southern analysis to confirm identical unique insertion of the *tetR-luxABCDE-tetA* transgenes and the curing of the Tn7 vector. **a** *Pcc- tetR-luxABCDE-tetA*, **b** *Pcc*-carC^{-}-*tetR-luxABCDE-tetA*, (**wt**) wild-type (*Pcc* ATCC39048; negative control), (**v**) pGRG25- *tetR-luxABCDE-tetA* construct

off-target insertions of the marker genes into the chromosome and loss of the original vector was confirmed by Southern blot analysis (Fig. 2b).

Both *tetR-luxABCDE-tetA*-labelled *Pectobacterium* strains perform equally in liquid culture and maintain their inducible luminescent properties

Growth and bioluminescent properties of both labelled *Pectobacterium* strains (*Pcc- tetR-luxABCDE-tetA* and *Pcc*-carC^{-}-*tetR-luxABCDE-tetA*) were compared in liquid LB media in the presence and absence of tetracycline (Tc). Three single colonies for each strain were selected and grown overnight. From the overnight-grown cultures, replicate samples (five replicates for each culture) were tested. The results shown in Fig. 3a represent the average growth rate in LB broth of the three biological replicates for each strain. Both the carbapenem-producing strain (*Pcc-tetR- luxABCDE-tetA*) and the carbapenem-negative strain (*Pcc*-carC^{-}-*tetR-luxABCDE-tetA*) grew equally well and the luminescent signals could only be detected in the presence of tetracycline (Tc) (Fig. 3b). No significant differences between the growth rate and bioluminescent properties of the two strains were observed. These results demonstrated that both strains maintain the ability to express the luminescence genes and that they perform equally in liquid cultures.

Carbapenem production provides a competitive advantage in the presence of the competitors

Labelling the *Pectobacterium* strains with tetracycline-inducible *luxABCDE* marker genes allowed the growth and relative number of the two strains to be monitored throughout the nine-day infection. To do so, we used a tuber slice system in which washed and surface-sterilised tubers were sliced and inoculated with one of the labelled *Pectobacterium* strains (~10^7 cells added per tuber slice) along with bacteria recovered from the surface of potato tubers (tuber wash; ~10^6 culturable bacteria added per tuber slice). In order to monitor the population dynamics during the course of *Pectobacterium* infection, enumeration of bacteria at different time-points was carried out. Colony-forming unit (CFU) counts of culturable bacteria recovered from the infected tuber slices were determined per whole tuber slice by plating on LB agar plates. To establish the proportion of *tetR-luxABCDE-tetA*-labelled *Pectobacterium* from the total number of culturable bacteria, enumeration of luminescent bacteria grown on LB plates supplemented with tetracycline was carried out using a NightOWL Imaging System (EG&G Berthold).

Immediately after inoculation (time point 0), the total number of bacteria/tuber slice (including *Pectobacterium*) recovered was between $1.5–1.7\times10^7$ CFU/tuber slice for both treatments and ~2×10^5/tuber

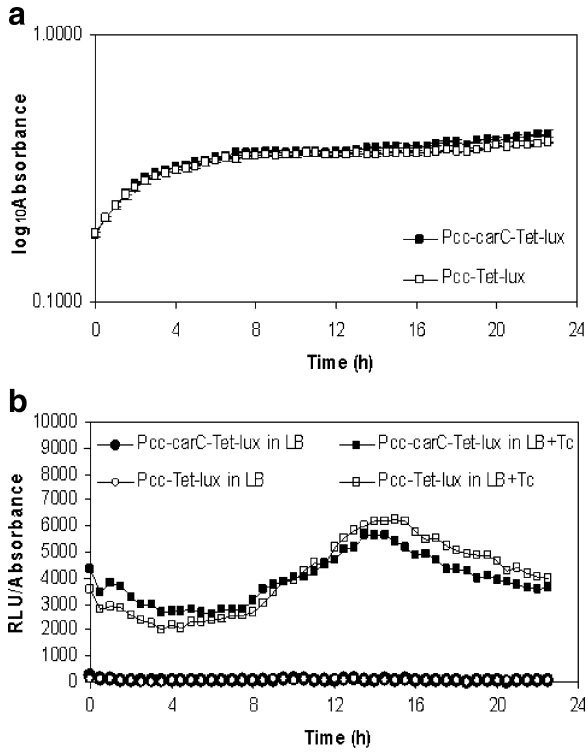

Fig. 3 Growth rate and bioluminescence of the tagged strains. **a** The carbapenem-producing - *Pcc- tetR-luxABCDE-tetA* (□) and the carbapenem-negative - *Pcc*-carC⁻- *tetR-luxABCDE-tetA* (■) strains were grown in LB broth at 28°C, and absorbance (at 600 nm) was measured at 30 min intervals. Both strains grew at comparable levels and reached similar population densities. **b** Bioluminescence of the two tagged strains was measured in LB broth in the presence and in the absence of tetracycline using a Tecan Genesis Pro microplate reader. The *Y*-axis values show the ratio of relative light units/population density (RLU/OD_{600nm}) measured in the presence (*Pcc- tetR-luxABCDE-tetA* (□), *Pcc*-carC⁻- *tetR-luxABCDE-tetA* (■)) and in the absence *Pcc- tetR-luxABCDE-tetA* (○), *Pcc*-carC⁻- *tetR-luxABCDE-tetA* (●) of tetracycline. Induction following addition of tetracycline (Tc) was similar in both bacterial strains and no bioluminescent signals were detected in the absence of the inducer (Tc). The results are means ± SE of three biological replicates (five replicates each)

slice for the control tuber slices inoculated with tuber wash (TW) only. The proportion of inoculated *Pectobacterium* from the total number of cultivable bacteria was around 85% for both infections. No significant differences between the treatments were recorded at this time-point (ANOVA, $P=0.3270$) (Fig. 4a). Twenty-four hours (day 1) after inoculation the total number of bacteria increased ca. 20-fold in both infections and the initiation of rotting was evident. No significant differences between the total number of bacteria was observed at this time-point (ANOVA, $P=0.0905$), but the proportion that the *Pectobacterium* contributed to this total varied dramatically in the two infections.

An increase in the total number of bacteria was observed in the control tuber slices, possibly due to release of nutrients following tissue damage caused by slicing and incubation at 24ºC. However, no significant additional bacterial growth was observed after this 24 h time-point in the control tuber slices and none of the control slices showed any signs of rotting or tissue maceration throughout the course of the experiment. The number of carbapenem-producing *Pectobacterium* (*Pcc-tetR-luxABCDE-tetA*), one day after inoculation was around 10-fold higher than the carbapenem-negative strain (*Pcc*-carC⁻- *tetR-luxABCDE-tetA;* Fig. 4b). As a percentage of total culturable bacteria, the proportion of *Pectobacteria* at this time-point was therefore much higher (~80%) for the carbapenem-producing (*Pcc- tetR-luxABCDE-tetA*) strain than for the carbapenem-negative strain (~15%) (Fig. 4c). Both the number of tagged *Pectobacterium* and their proportion of the total cultivable bacteria were significantly different in both cases (ANOVA, $P=0.0002$) and (ANOVA, $P<0.0001$) respectively. This also implied a significant difference (ANOVA, $P=0.0005$) between the numbers of other competing bacteria present in the two infections. Two days after inoculation, there had been a slight rise in *Pectobacterium* numbers from the day 1 value and the carbapenem-producing *Pectobacterium* (*Pcc- tetR-luxABCDE-tetA*) reached their population peak at this time.

The carbapenem-negative strain (*Pcc*-carC⁻- *tetR-luxABCDE-tetA*) cell number continued to rise slightly over the following 24 h, but did not reach the levels of the carbapenem-producing strains (Fig. 4b). The total number of bacteria present in both infections were very similar at this time point, although the number of non-carbapenem-producing *Pectobacterium* was still significantly lower (ANOVA, $P<0.0001$) than the number of the carbapenem-producing strain. In both infections, *Pectobacterium* proportions decreased from 85% to < 40% in the case of the carbapenem producer whilst in the case of the non-carbapenem producer strain this decreased from

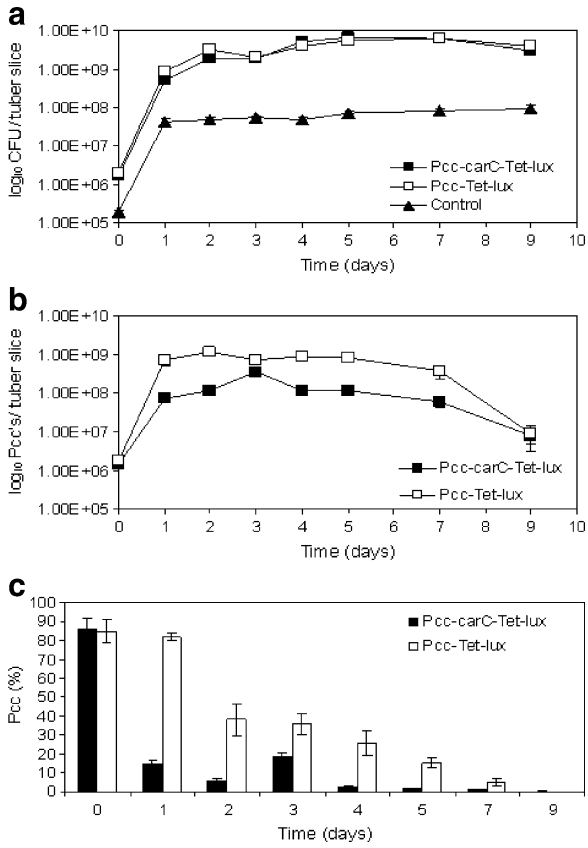

Fig. 4 Potato infection assay. **a** Total number of culturable bacteria (CFU) isolated from tuber slices infected with tuber wash (TW) plus either *Pcc*-carC^{-}- *tetR-luxABCDE-tetA* (■) or *Pcc- tetR-luxABCDE-tetA* (□) strains or TW alone (○) inoculated with tuber wash (TW) only. No significant differences were observed between the total number of bacteria present in the two infections throughout the nine-day infection. Significant differences were observed between the *Pectobacterium* infected and control tuber slices at each time-point. **b** Total number of luminescent *Pectobacteria* - *Pcc*-carC^{-}-*tetR-luxABCDE-tetA* (■) and *Pcc- tetR-luxABCDE-tetA* (□) isolated from tuber slices at eight time-points. Significant differences between the numbers of the two *Pectobacteria* were seen one day after infection and persisted until the later stages (7–9 days), when both strains declined to similar low levels. **c** Proportion of the *Pectobacterium*s from the total CFU of cultivated bacteria over the nine-day infection. Significant differences between the proportion of the two strains (*Pcc*-carC^{-}-*tetR-luxABCDE-tetA* (*full bars*) and *Pcc-tetR-luxABCDE-tetA* (*open bars*) were observed one day after infection and persisted over the course of the infection. The results are means ± SE of four replicates for each time-point

85% to < 10% (Fig. 4c). The number of carbapenem producing *Pectobacterium* showed a slight decrease at the 3 day time-point, but remained at similar population density levels for the next three time-points (4, 5 and 7 days) of the infection. Similarly, the number of non-carbapenem producers started to decline after reaching the maximum population density (3 days after inoculation), and remained 10-fold lower than the carbapenem-producing strain for the next three time-points (4, 5 and 7 days) during the infection (Fig. 4b). Nine days after inoculation, both the number and proportion of *Pectobacterium* declined dramatically (10–20 fold) in both infections, while the total number of bacteria present in the rotted tuber remained at high levels, similar to the earlier time-points (Fig. 4a). This observation suggests that the *Pectobacterium* was displaced by the replication of other bacterial species. This also implies that carbapenem production provides a competitive advantage at the beginning of the infection, but may not assist *Pcc* persistence in fully rotted tubers.

Discussion

It has been suggested (Whitehead et al. 2002) that carbapenem production serves to prevent or limit competing bacteria from utilising the nutrients released following the infection-derived maceration of the plant tissues. Our results support this model and indicate that in *Pectobacterium* species, carbapenem antibiotic production contributes to the ecological fitness of the producing strain. We have shown that carbapenem production indeed affects the competitiveness of the *Pectobacterium* throughout the course of the infection by ensuring a higher *Pectobacterium* population density and by reducing competitors. This is particularly pronounced at the beginning of the infection.

Mazzola et al. (1992), studying the role of phenazine (Phz) antibiotic production by fluorescent pseudomonads in soil, showed that for *Pseudomonas fluorescens* 2–79 and *Pseudomonas aureofaciens* 30–84 Phz can contribute to the ecological competence of these strains in soil and in the rhizosphere of wheat. These authors found that population sizes of Phz^{-} mutants declined more rapidly than did population sizes of the corresponding parental Phz^{+} strains and they concluded that the reduced survival of the Phz^{-} strains is due to a diminished ability to compete with the resident microflora.

The rotting tuber is a somewhat different environment, as a mass of nutrients are rapidly released and

potentially made available to both *Pectobacterium* and competing bacteria; and the release of these nutrients is dependent upon the presence of the *Pectobacterium* itself. Our results demonstrate the importance of antibiotic production to the *Pectobacterium* for competitiveness in the presence of other bacterial species. They imply that the carbapenem antibiotic production by *Pcc* has a significant effect on the total number of competing bacteria, but does not ensure *Pcc* persistence in the fully rotted tuber. The *carC*⁻ strain reached a population size approximately 10-fold lower than the carbapenem-producing strain, however tuber slices infected with the carbapenem non-producer (*carC*⁻) were still fully rotted. These findings imply that the smaller *carC*⁻ population still produces sufficient plant cell wall-degrading exoenzymes to achieve normal levels of plant tissue maceration. Similarly, the same total number of bacteria was seen in both infections, suggesting that the maximum bacterial community size was reached given the available nutrients. When the infection was caused by the non-carbapenem producing strain (*carC*⁻), a greater percentage of the total bacteria supported by the rotted tubers were non-*Pectobacterium*.

Given the apparent competitive advantage of antibiotic production, it is curious that some *Pcc* isolates do not appear to produce carbapenem. Interestingly, Holden et al. (1998) reported that seven out of 43 *Pcc* isolates and two out of 39 *P. atrosepticum* strains tested carry cryptic genes for carbapenem synthesis. They were able to functionally re-activate these genes by the expression of an introduced *CarR* transcriptional activator on a high-copy number plasmid.

It will be interesting to test which bacterial species are specifically excluded by carbapenem. It is likely that many of the bacteria present will not be directly competing with the *Pectobacterium*, and some may even play a beneficial role. For example, it is possible that some bacterial species may assist the complete breakdown and release of nutrients from starch grains as *Pectobacterium* cultures are not able to use this major released nutrient as a sole carbon source.

The use of Tn7 tagging system allowed us to monitor the potential role of carbapenem production by *Pectobacterium* strains in a complex environment, such as during potato tuber infection. This was possible as the chromosomal insertion and controlled expression of the marker genes themselves had no negative impact on growth rate or competitiveness of the strains. Therefore we anticipate that this tagging system could be of a great use in complex competition trials to compare a variety of virulence determinants in different infection models given that Tn7 was shown to transpose in at least 20 bacterial species (Choi et al. 2005).

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