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Cryptic carbapenem antibiotic production genes are widespread in *Erwinia carotovora*: facile *trans* activation by the *carR* transcriptional regulator

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Few strains of *Erwinia carotovora* subsp. *carotovora* (*Ecc*) make carbapenem antibiotics. Strain GS101 makes the basic carbapenem molecule, 1-carbapen-2-em-3-carboxylic acid (Car). The production of this antibiotic has been shown to be cell density dependent, requiring the accumulation of the small diffusible molecule *N*-(3-oxohexanoyl)-L-homoserine lactone (OHHL) in the growth medium. When the concentration of this inducer rises above a threshold level, OHHL is proposed to interact with the transcriptional activator of the carbapenem cluster (CarR) and induce carbapenem biosynthesis. The introduction of the GS101 *carR* gene into an *Ecc* strain (SCRI 193) which is naturally carbapenem-negative resulted in the production of Car. This suggested that strain SCRI 193 contained functional cryptic carbapenem biosynthetic genes, but lacked a functional *carR* homologue. The distribution of *trans*-activatable antibiotic genes was assayed in *Erwinia* strains from a culture collection and was found to be common in a large proportion of *Ecc* strains. Significantly, amongst the *Ecc* strains identified, a larger proportion contained *trans*-activatable cryptic genes than produced antibiotics constitutively. Southern hybridization of the chromosomal DNA of cryptic *Ecc* strains confirmed the presence of both the *car* biosynthetic cluster and the regulatory genes. Identification of homologues of the transcriptional activator *carR* suggests that the cause of the silencing of the carbapenem biosynthetic cluster in these strains is not the deletion of *carR*. In an attempt to identify the cause of the silencing in the *Ecc* strain SCRI 193 the *carR* homologue from this strain was cloned and sequenced. The SCRI 193 CarR homologue was 94% identical to the GS101 CarR and contained 14 amino acid substitutions. Both homologues could be expressed from their native promoters and ribosome-binding sites using an *in vitro* prokaryotic transcription and translation assay, and when the SCRI 193 *carR* homologue was cloned in multicopy plasmids and reintroduced into SCRI 193, antibiotic production was observed. This suggested that the mutation causing the silencing of the biosynthetic cluster in SCRI 193 was leaky and the cryptic Car phenotype could be suppressed by multiple copies of the apparently mutant transcriptional activator.

Keywords: cryptic genes, carbapenem, *Erwinia*, LuxR homologue

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Abbreviations: *Eca*, *Erwinia carotovora* subsp. *atroseptica*; *Ecc*, *Erwinia carotovora* subsp. *carotovora*; OHHL, *N*-(3-oxohexanoyl)-L-homoserine lactone; RLU, relative light units.

The GenBank accession number for the sequence reported in this paper is AF041840.

INTRODUCTION

Erwinia carotovora subsp. *carotovora* (*Ecc*) is a phytopathogen, causing soft-rot diseases in a range of economically important crops, including potatoes and carrots (Pérombelon & Kelman, 1980). Some strains of this Gram-negative enteric bacterium make the simple carbapenem antibiotic 1-carbapen-2-em-3-carboxylic acid (Car) (Parker *et al*., 1982). Carbapenems are among the most recently discovered β-lactams and they exhibit a broad spectrum of activity against both aerobes and anaerobes, including nosocomial pathogens (Bycroft *et al*., 1988; Neu *et al*., 1994). This class of antibiotics has been of particular interest due to their relative resistance to most clinically encountered β-lactamases (Ubukata *et al*., 1990).

The first carbapenem to be discovered was thienamycin, which is produced by *Streptomyces cattleya* (Kahan *et al*., 1979). Other structurally complex carbapenems have been found to be produced by a range of Gram-positive *Streptomyces* spp. (Williamson *et al*., 1985). The results of studies with *Streptomyces* and *Serratia marcescens* suggested that the carbapenems are synthesized by a biochemical route distinct from that of penicillins and cephalosporins (Williamson *et al*., 1985; Bycroft *et al*., 1988). However, problems with slow growth and poor genetic tractability of *Streptomyces* producers have made analysis of carbapenem production in these strains problematic. Such problems with the *Streptomyces* strains encouraged investigation of the biosynthesis of 1-carbapen-2-em-3-carboxylic acid in the genetically tractable, and fast growing, *Ecc* GS101. A molecular genetic study has now led to the identification of a novel cluster of eight genes (*carA–H*) responsible for the production of Car (Fig. 1; McGowan *et al*., 1996). Additional regulatory genes (*carI/R*) have been identified which are homologous to the regulatory genes (*luxI/R*) of the luminescence (*lux*) operon in the marine bacterium *Photobacterium* (*Vibrio*) *fischeri* (Swift *et al*., 1993; McGowan *et al*., 1995). The two carbapenem regulatory genes lie within a quorum-sensing regulon that links transcriptional activation of the biosynthetic cluster to cell density via the small diffusible molecule *N*-(3-oxohexanoyl)-L-homoserine lactone (OHHL; Bainton *et al*., 1992). The *carI* gene product is a putative autoinducer synthase that mediates the synthesis of the OHHL inducer (Swift *et al*., 1993; Pirhonen *et al*., 1993). The genetic organization of the *car* regulon is unlike the equivalent regulon in *P. fischeri*, as *carI* is unlinked to the *carRA–H* cluster.

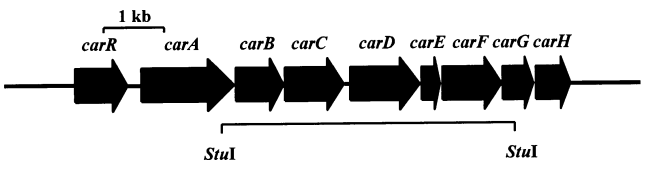

Fig. 1. Genetic organization of the *Ecc* GS101 *car* locus. *Stu*I sites are marked on the bottom of the schematic diagram to indicate the DNA used as *car* biosynthetic probe in the Southern hybridizations.

The *carR* gene product has homology to the growing family of LuxR-like bacterial transcriptional effectors and so is proposed to be a transcriptional activator (Fuqua *et al*., 1996; Swift *et al*., 1996; Salmond *et al*., 1995). CarR is thought to act in concert with OHHL, stimulating the transcription of the *car* biosynthetic cluster when a threshold concentration of the inducer molecule has accumulated in the growth medium (McGowan *et al*., 1995). *carR* is located upstream of the biosynthetic cluster, separated by an intergenic region of approximately 150 bp. It has been proposed that this region may contain a biosynthetic cluster promoter and a putative CarR binding site, to which the activated CarR–OHHL complex might bind, stimulating transcription of the carbapenem cluster (McGowan *et al*., 1996).

In contrast to the above, little is known about the regulation of carbapenem production by actinomycetes. *Streptomyces* spp. produce a spectrum of carbapenem molecules containing distinct chemical substitutions (Moellering *et al*., 1989). This diversity in antibiotic structure and widespread distribution of producing strains has not been seen in other bacterial genera. The number of Gram-negative bacteria isolated from the environment, in mass screening programmes, which produce β-lactam antibiotics has been found to be low (Sykes *et al*., 1981). This contrasts sharply with myxobacteria, filamentous fungi and actinomycetes, which are prolific producers of antibiotics (Foster *et al*., 1992). Over 40% of filamentous fungi and actinomycetes produce antibiotics when freshly isolated from soil (Demain, 1995). However, there are species-specific exceptions to the assertion that the frequency of antibiotic production is low in Gram-negative bacteria. Classically, certain species of the genus *Pseudomonas* have been associated with the biological control of plant pathogens via the production of antibiotics. Fluorescent *Pseudomonas* strains which produce 2,4-diacetylphloroglucinol were frequently identified in soils which suppressed take-all of wheat caused by the fungal pathogen *Gaeumannomyces graminis* var. *tritici* (Keel *et al*., 1992).

Here we report that the distribution of carbapenem production genes in *Erwinia carotovora* strains is far more widespread than previously realized. However, the majority of strains investigated here carry carbapenem genes that are cryptic and can be activated by the introduction of the GS101 *carR* carbapenem transcriptional activator *in trans*. Our results suggest that a significant proportion of the cryptic genes are due to defects in the carbapenem-specific, pheromone-dependent, CarR regulator.

METHODS

Bacterial strains and growth conditions. Bacterial strains other than the *Erwinia carotovora* strains surveyed for their Car phenotype are listed in Table 1; the latter strains are listed in Tables 3 and 4 (see Results). The bacteriophage and vectors

Table 1. Bacterial strains

Strain	Relevant characteristics	Source or reference
Escherichia coli		
DH1	F⁻ *recA1 endA1 thi1 hsdR17* (r_K^- m_K^+) *gyrA96 supE44 relA1*	Hanahan (1983)
ESS	β-Lactam supersensitive indicator strain	Bainton *et al.* (1992); from SmithKline Beecham Pharmaceuticals
CC118(*λpir*)	As CC118, lysogenized with *λpir*	Herrero *et al.* (1990)
Serratia marcescens		
SBON1	Wild-type, Car⁻	G. S. A. B. Stewart
ATCC 39006	Wild-type, Car⁺	Bycroft *et al.* (1987)
S6	Wild-type, Car^r	Livermore (1992)
Erwinia carotovora* subsp. *carotovora		
GS101	ATCC 39048, Km^r Car⁺, restrictionless and modificationless Tn*5* insertion mutant	Bainton *et al.* (1992)
ATTn*10*	ATCC 39048, Tc^r Car⁺, restrictionless and modificationless Tn*10* insertion mutant	Laboratory collection
SCC3193	Wild-type	Pirhonen *et al.* (1988)
SCRI 193	Wild-type	Hinton & Salmond (1987)
PNP10	GS101, Km^r, NTG-induced Car⁻, *carR*::Tn*5*	McGowan *et al.* (1995)
MS1	ATTn*10*, Car⁺ Lac⁻, Tn*10* cured	Laboratory collection
ML1	PNP10, Km^r Lac⁺ Car⁻, *carR*::Tn*5*, *carB*::*lacZ*	This study
HS5	MS1, Km^r Lac⁻ Car⁻, *carR*::Tn*5*, *carB*::*lacZ*	This study
Erwinia carotovora* subsp. *betavasculorum		
SCRI 479	Wild-type	M. C. M. Pérombelon, SCRI, Dundee
Erwinia chrysanthemi		
EC 16	Wild-type	Keen *et al.* (1984)
NCPPB 1066	Wild-type	Gilbert *et al.* (1986)

are listed in Table 2. *Erwinia* and *E. coli* strains were routinely grown at 30 °C and 37 °C respectively in L-Broth (LB) medium (Sambrook *et al.*, 1989).

Carbapenem assay. Five microlitres of an overnight culture of the sample strain of *Erwinia* was spotted onto a seeded top agar lawn of *E. coli* strain ESS on an LB plate, and allowed to grow for 24 h at 30 °C. Additionally, recombinant *Erwinia* strains were supplemented with OHHL (5 μl of a 1 μg ml^{-1} solution) after spotting onto the lawn. Carbapenem antibiotics were identified by HPLC as described by Bycroft *et al.* (1987).

Inducer assay. Samples (80 μl) of an overnight culture of the test strain were put into the wells of a microtitre plate along with 80 μl of an overnight culture of *E. coli* DH1 containing the *lux*, OHHL sensor plasmid pSB401 (Throup *et al.*, 1995). The plate was incubated at 30 °C for 3 h, and the amount of light induced quantified using a luminometer (Labsystems Luminoskan). The concentration of OHHL in the culture supernatant was measured as above, along with a dilution series of OHHL (1·0, 0·1, 0·01, 0·001 and 0·0001 μg ml^{-1}). The relative light units (RLU) of bioluminescence for the OHHL dilution series were plotted against OHHL concentration and a standard curve obtained. The concentration of OHHL in the SCRI 193 culture supernatant was estimated from the standard curve.

Recombinant DNA technology. All DNA manipulation, cloning, ligation, gel electrophoresis and Southern blotting techniques were performed according to standard protocols (Sambrook *et al.*, 1989) and manufacturers' instructions. Southern hybridizations were carried out using low-stringency conditions: temperature of hybridization was 60 °C; filters were washed twice at 60 °C and twice at room temperature in 2 × SSC, 0·1 % SDS.

DNA sequencing. DNA sequences were determined using the dideoxynucleotide chain-termination method of Sanger *et al.* (1977) and the Sequenase version 2.0 kit (United States Biochemical). DNA was prepared for sequencing by sonication and ligation of the resulting fragments into M13 vector mp18 (Messing & Vieira, 1982). Alignments were done by the GCG software package (Genetics Computer Group, Madison, WI, USA).

16S ribotyping, cluster analysis of binary data. The RFLPs generated by Southern hybridization with a 16S rRNA gene probe were converted into test matrices and analysed using NTSYS-pc (Exeter Publishing, 100 North Country Road Setauket, NY 11733, USA). Graphic hard copies of the phenograms generated were obtained by exporting the file as a TIFF into PowerPoint (Microsoft).

Table 2. Bacteriophage and vectors

Bacteriophage or vector	Relevant characteristics	Source or reference
Bacteriophage		
ϕKP	*Erwinia* generalized transducing phage	Toth *et al.* (1993)
Vectors		
pSF6	Sp^r multicopy cloning vector, *cos mob*	Selvaraj *et al.* (1984)
cWU142	Car biosynthetic cluster in pSF6	McGowan *et al.* (1995)
pUW14203	3·6 kb GS101 *carR* fragment in SF6	McGowan *et al.* (1995)
pUC19	Ap^r Lac^+ multicopy cloning vector *lacZ*	Yanisch-Perron *et al.* (1985)
pMH10	GS101 *carR* in pUC19	This study
pMH20	SCRI 193 *carR* in pUC19	This study
pRK2013	Km^r conjugation 'helper' vector	Ditta *et al.* (1980)
pNJ5000	Tc^r conjugation 'helper' vector	Grinter (1983)
pKNG101	Sm^r suicide vector	Kaniga *et al.* (1991)
pSMG52	*carB*::*lacZ* in pKNG101	S. McGowan
pDAH330	Cm^r multicopy cloning vector, *lacZ*	McGowan *et al.* (1995)
pTGH10	SCRI 193 *carR* in pDAH330	This study
pACYC184	Cm^r Tc^r multicopy cloning vector	Chang & Cohen (1978)
pNED100	GS101 *carR* pACYC184	This study
pNED200	SCRI 193 *carR* in pACYC184	This study
pSB401	Tc^r, *luxR*,*CDABE* in pACYC184	Throup *et al.* (1995)

Marker exchange. Marker exchange of the *carB*::*lacZ* construct was carried out in several stages. Initially the *carB*::*lacZ* construct was cloned into the suicide plasmid pKNG101 (Kaniga *et al.*, 1991). This plasmid will only replicate in hosts supplying the product of the *pir* gene *in trans*, so the resulting plasmids were maintained in *E. coli* strain CC118(λ*pir*). Triparental matings on LB plates between CC118(λ*pir*) carrying the pKNG101 derivative, *Ecc* strain PNP10 and an *E. coli* strain carrying the helper plasmid pNJ5000 (Grinter, 1983) were carried out. Transconjugants resulting from integration of the suicide plasmid into the *Ecc* chromosome by homologous recombination were selected on M9 minimal medium agar supplemented with 0·2% glucose and streptomycin (Sm; 50 $\mu g\ ml^{-1}$). Finally, in the absence of antibiotic, strains that had undergone excision of the integrated plasmid were selected on M9 minimal medium (Sambrook *et al.*, 1989) containing 10% sucrose as the sole carbon source. Southern blot analysis of the chromosomal DNA of resulting strains was used to confirm the genotype in each case. The resulting strain was designated ML1.

Generalized transductions. Generalized transductions were carried out using ϕKP as described by Toth *et al.* (1993). A ϕKP lysate was prepared on *Ecc* strain ML1 and used to transduce the Lac^- ATTn*10* derivative, MS1. Southern blot analysis of the chromosomal DNA of resulting Km^r Car^- transductants was used to confirm the genotype in each case.

Construction of *Ecc* HS5. The first step in producing a suitable strain to assay CarR activity was to introduce a *carB*::*lacZ* fusion into the chromosome of a GS101 *carR* mutant by marker exchange. The strain chosen was PNP10, which contained a Tn5 insertion in the DNA immediately downstream of the Asn_{166} codon of CarR (McGowan *et al.*, 1995). The pKNG101 suicide vector was used to create the chromosomal replacement (see above; Kaniga *et al.*, 1991). An insert containing a *carB*::*lacZ* fusion had previously been cloned into pKNG101 (pSMG52) by S. McGowan. The cloned insert contained the *car* cluster, stretching from the *carR*–*A* intergenic region to *carH* (*Sal*I partial digest). A promoterless *lacZ* cassette from pDAH330 had been cloned into the unique *Sal*I site within *carB*. The marker-exchange construct was introduced into *E. coli* CC118(λ*pir*) by calcium chloride transformation and resulting transformants were used in the marker exchange of the construct into PNP10 (see above).

One of the *carB*::*lacZ* mutant strains resulting from marker exchange was chosen and designated ML1. This strain was Lac^+. Therefore it was necessary to transduce the *carR*::Tn5 and *carB*::*lacZ* fusions into a Lac^- background to measure fusion activity. This was achieved by using the *Erwinia* generalized transducing phage ϕKP (see above; Toth *et al.*, 1993). Transductants containing a Tn5 marker were isolated by plating on LB agar containing kananycin (Km; 50 $\mu g\ ml^{-1}$). By selecting for Km^r colonies containing a Tn5 insertion, it was hoped that most of the transductants would also have acquired the tightly linked *carB*::*lacZ* fusion by cotransduction. Transductants were screened by Southern hybridization with *carR* and *carB* probes to identify bacteria containing a Tn5 insertion in *carR*, plus the linked *carB*::*lacZ* fusion. A *carR*::Tn5, *carB*::*lacZ* transductant was identified and designated HS5.

β-Galactosidase assay. The specific β-galactosidase activity of intact cells of the two reporter strains was determined essentially as described by Miller (1972). Protein concentrations were calculated using a method based on that of Bradford (1976).

RESULTS

Trans activation of cryptic carbapenem genes in *Ecc* SCRI 193

SCRI 193 is a wild-type *Ecc* strain which has been intensively investigated in the study of global regulation and secretion of extracellular enzymes (Hinton *et al.*, 1990; Jones *et al.*, 1993; Reeves *et al.*, 1993). This strain

Table 3. *Ecc* strains surveyed

All strains were obtained from M. C. M. Pérombelon, Scottish Crop Research Institute, Invergowrie, Dundee, UK.

Strain	Host	Original source
SCRI 101*	Tobacco	USA
SCRI 102	Avocado pear	Israel
SCRI 103	Potato tuber	Scotland
SCRI 105	Potato tuber	Australia
SCRI 106	Cucumber	UK
SCRI 109	Arum lily	S. Africa
SCRI 110	Potato stem	Brazil
SCRI 111	Tomato	Israel
SCRI 112*	Potato	Japan
SCRI 113	Onion	Japan
SCRI 114*	Potato	Denmark
SCRI 115	Potato	Scotland
SCRI 116	Soil ex. turnip crop	Scotland
SCRI 117	Potato stem	Scotland
SCRI 118	Potato stem	Scotland
SCRI 119	Potato stem	Scotland
SCRI 120	Sunflower	Uganda
SCRI 121	Sugar cane	Jamaica
SCRI 122*	Tomato	Tanzania
SCRI 123*	Potato tuber	Egypt
SCRI 124†	Potato	England
SCRI 125	Potato tuber	Scotland
SCRI 126	Potato	N. Ireland
SCRI 127	Insect	Scotland
SCRI 130	Potato tuber	Scotland
SCRI 132†	Carrot	Japan
SCRI 135	Potato	Arizona, USA
SCRI 139	Potato	Arizona, USA
SCRI 143	Potato	Tasmania
SCRI 144	Potato	Tasmania
SCRI 149	Potato	Tasmania
SCRI 152	Potato stem	Tasmania
SCRI 155	Potato tuber	Scotland
SCRI 166	Potato	Scotland
SCRI 169	Insect	Scotland
SCRI 171	Potato	Peru
SCRI 172	Potato stem	Peru
SCRI 174	Potato tuber	Peru
SCRI 178	Potato stem	Peru
SCRI 182	Potato stem	Peru
SCRI 191	Maize	Israel
SCRI 192*	Potato tuber	Scotland
SCRI 198*	Potato	Wisconsin, USA

* Strains with cryptic carbapenem production genes.

† Strains producing carbapenem constitutively.

Table 4. *Eca* strains surveyed

All strains were obtained from M. C. M. Pérombelon, Scottish Crop Research Institute, Invergowrie, Dundee, UK.

Strain	Host	Original source
SCRI 5	*Schinjanthus* spp.	Tanzania
SCRI 6	Potato	Romania
SCRI 13	Potato	N. Ireland
SCRI 18	Potato	Arizona, USA
SCRI 24	Potato	Rhodesia
SCRI 25	Potato	Scotland
SCRI 26	Potato	UK
SCRI 27	*Delphinium ajaris*	USA
SCRI 28	Potato	Scotland
SCRI 39*	Potato	USA
SCRI 41	Potato	Netherlands
SCRI 1001	Potato	Scotland
SCRI 1002	Potato	Scotland
SCRI 1034	Potato	Scotland
SCRI 1042	Potato	Scotland
SCRI 1043	Potato	Scotland
SCRI 1044	Potato	Scotland
SCRI 1055	Potato	Scotland
SCRI 1056	Potato	Scotland
SCRI 1057	Potato	Scotland
SCRI 1058*	Potato	Scotland
SCRI 1062	Potato	Scotland
SCRI 1063	Potato	Scotland
SCRI 1064	Potato	Scotland
SCRI 1065	Potato	Scotland
SCRI 1066	Potato	Scotland
SCRI 1067	Potato	Scotland
SCRI 1068	Potato	Scotland
SCRI 1069	Potato	Scotland
SCRI 1083	Potato	Netherlands
SCRI 1087	Potato	Netherlands
SCRI 1089	Potato	Netherlands
SCRI 1090	Potato	Netherlands
SCRI 1091	Potato	Netherlands
SCRI 1093	Potato	Netherlands
SCRI 1095	Potato	Netherlands
SCRI 1096	Potato	Netherlands
SCRI 1099	Potato	Netherlands
SCRI 1100	Potato	Netherlands

* Strains with cryptic carbapenem production genes.

is phenotypically negative for antibiotic production when samples are tested using the β-lactam-supersensitive indicator strain *E. coli* ESS. The introduction of the *Ecc* GS101 carbapenem cluster, *carR–H*, resulted in a Car^+ phenotype in this strain. The expression of the genes was thought to be due to endogenous OHHL synthesized by the native CarI homologue (Jones *et al.*, 1993).

The transcriptional activator of the *car* cluster of GS101, *carR*, had previously been cloned into a pSF6 cosmid (pUW14203; McGowan *et al.*, 1995). To our surprise, transfer of this construct into SCRI 193 resulted in the production of an antibiotic which was characterized as 1-carbapen-2-em-3-carboxylic acid (results not presented). Transfer of the vector pSF6 alone to SCRI 193 did not result in carbapenem biosynthesis. pUW14203 contains a 3·6 kb *Eco*RI fragment cloned into the unique

*Eco*RI site of pSF6. Approximately 1·9 kb of the 3·6 kb insert is subcloned pSF6 vector DNA, and the remaining 1·7 kb is GS101 DNA. The only open reading frame contained within the *Ecc* DNA is *carR* (735 bp). Therefore the fact that simply supplying a functional *carR* gene *in trans* to SCRI 193 led to carbapenem production strongly suggested that this strain contained the *car* biosynthetic genes but was naturally defective in the quorum-responsive regulatory mechanism.

Assay of inducer produced by *Ecc* SCRI 193 in batch culture

SCRI 193 has been shown to synthesize the bacterial pheromone OHHL, which coordinates production of multiple extracellular enzymes in a cell-density-dependent fashion (Jones *et al*., 1993). A possible cause of the Car^- phenotype of SCRI 193 was that the level of inducer produced by this strain, whilst high enough to coordinate exoenzyme production, was not high enough to stimulate carbapenem production. The restoration of the Car^+ phenotype by the introduction of pUW14203 may have been the result of *carR* multicopy suppression. This has previously been observed when studying *carI* mutants of the carbapenem-producing strain GS101 (McGowan *et al*., 1995).

Fig. 2(a) shows a growth curve of SCRI 193 and the induction of bioluminescence by SCRI 193 culture supernatant in *E. coli* containing the OHHL sensor plasmid pSB401 (Throup *et al*., 1995). During the first 2 h of growth, the concentration of inducer detected remained at a basal level. As the culture OD_{600} increased above 0·06, during the exponential phase of growth, there was an approximately 14-fold induction of luminescence. In the carbapenem-producing *Ecc* strain GS101, a rapid increase of supernatant inducer levels was seen at OD_{600} 0·2 (Swift *et al*., 1993). The concentration of inducer in the SCRI 193 culture supernatant increased until it reached a maximum when the OD_{600} reached 2·0. This coincided with the transition of growth from late exponential to early stationary phase. Previous work had shown that the threshold concentration for the induction of carbapenem production in GS101 was 0·1 μg ml^{-1} (Bainton *et al*., 1992). From the dose–response curve for the activity of OHHL in the pSB401 assay (Fig. 2b) it can be estimated that the concentration of inducer in the culture supernatant at the induction peak was approximately 0·2 μg ml^{-1}. Therefore, from the induction curve it can be seen that the threshold concentration for carbapenem production was exceeded 8 h after inoculation. This led us to conclude that the levels of OHHL production by SCRI 193 were high enough to stimulate carbapenem production, suggesting that the defect in the activation of antibiotic production resides with the transcriptional activator rather than the OHHL signal generator.

Interestingly, after the peak in the measured OHHL levels at OD_{600} 2·0, the levels decreased steadily as the SCRI 193 culture moved into stationary phase. Thirty hours after the culture was inoculated, the concentration

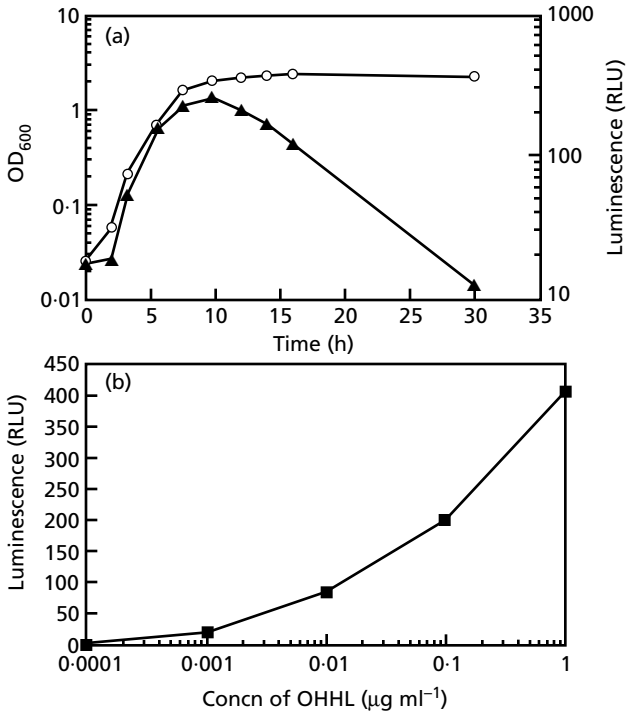

Fig. 2. (a) Production of OHHL by *Ecc* SCRI 193 in batch culture. The levels of inducer were measured in culture supernatant by monitoring light induction, measured in relative light units (RLU), of *E. coli* carrying pSB401. ○, OD_{600}; ▲, luminescence. (b) Dose–response curve of OHHL induction of light in *E. coli* carrying pSB401.

of inducer had fallen back to basal levels. The occurrence of a peak of inducer in the culture supernatant was also observed in the carbapenem-producing strain GS101. The possibility that the fall in the levels of OHHL was due to chemical instability was tested by incubating OHHL (0·1 μg ml^{-1}) in un-inoculated LB under the same conditions. The mixture was sampled over time without observing a similar fall in inducer levels, suggesting that the decrease in OHHL levels in the culture was due to a microbial process (data not presented). Therefore, for *Ecc* strains it would appear that the OHHL levels are not solely cell density dependent, but are closely linked to growth phase.

Distribution of OHHL production in two subspecies of *Erwinia carotovora*

Over 80 strains of *Erwinia carotovora* were assayed for the presence of an inducer in spent culture supernatants using the pSB401 assay. The majority of strains were obtained from the culture collection at the Scottish Crop Research Institute (SCRI), Invergowrie. This collection included 43 *Ecc* strains and 39 *Erwinia carotovora* subsp. *atroseptica* (*Eca*) strains (Tables 3 and 4). The strains assayed were originally isolated from a range of plant hosts and from many sites around the world. All of the *Ecc* and *Eca* strains tested produced detectable levels of OHHL (data not presented). This result is perhaps

not surprising as OHHL has been shown to have an essential role in the regulation of extracellular enzymes which are a key taxonomic marker for bacteria of this species (Pérombelon & Kelman, 1980).

Trans activation of cryptic carbapenem genes in the *Ecc* and *Eca* strains

Prior to the introduction of pUW14203 into the *Erwinia carotovora* strains, all of the strains were screened for constitutive antibiotic production using the β-lactam-supersensitive *E. coli* indicator strain ESS. Two of the SCRI *Ecc* strains (SCRI 124 and 132) produced antibiotics constitutively. These antibiotics were identified by HPLC as 1-carbapen-2-em-3-carboxylic acid, the same molecule produced by GS101 (data not presented).

pUW14203 was introduced into all of the strains by conjugation with *E. coli* DH1 containing the donor plasmid and the helper plasmid pRK2013 (Ditta *et al*., 1980). When the transconjugants were assayed for β-lactam antibiotic production, 16% of the *Ecc* strains (SCRI 101, 112, 114, 122, 123, 192 and 198, and SCRI 193 as described above) and 5% of the *Eca* strains (SCRI 39 and 1058) produced antibiotic after the introduction of pUW14203. The levels of antibiotics produced by these strains, as detected by the ESS assay, were comparable with that produced by GS101.

Some of the *Ecc* and *Eca* strains appeared to produce less inducer than others when tested using the pSB401 OHHL assay (data not presented). Therefore to investigate if any of the pUW14203 transconjugants were unable to produce antibiotic because of low endogenous levels of inducer, exogenous OHHL was supplied (5 μl of 1 μg ml^{-1}) when the strains were assayed. No additional strains produced antibiotic when containing pUW14203. Interestingly, three of the strains containing pUW14203 that produced antibiotics in the absence of exogenous OHHL did not produce antibiotic in the presence of excess OHHL, suggesting that the additional inducer supplied was enough to cause inhibition of carbapenem production. Previous work with GS101 has indicated that concentrations of OHHL above 1·0 μg ml^{-1} can inhibit carbapenem biosynthesis in this strain (Bainton *et al*., 1992).

Identification of *car* homologues in *Erwinia carotovora* chromosomal DNA

A 4548 bp *Stu*I fragment from GS101 containing the carbapenem genes *carA–H* (Fig. 1) was used to probe chromosomal DNA from *Ecc* strains that produced carbapenems constitutively, or contained *trans*-activatable carbapenem genes, or which exhibited no antibiotic activity (Fig. 3a). Homologues of the carbapenem biosynthetic genes could clearly be identified in *Ecc* strains. A fragment giving a strong signal appeared in the positive control GS101 chromosomal DNA at the predicted size for an *Eco*RI digest of the carbapenem cluster (5·3 kb), thus confirming the specificity of the probe. *car* homologues could be identified in strains that

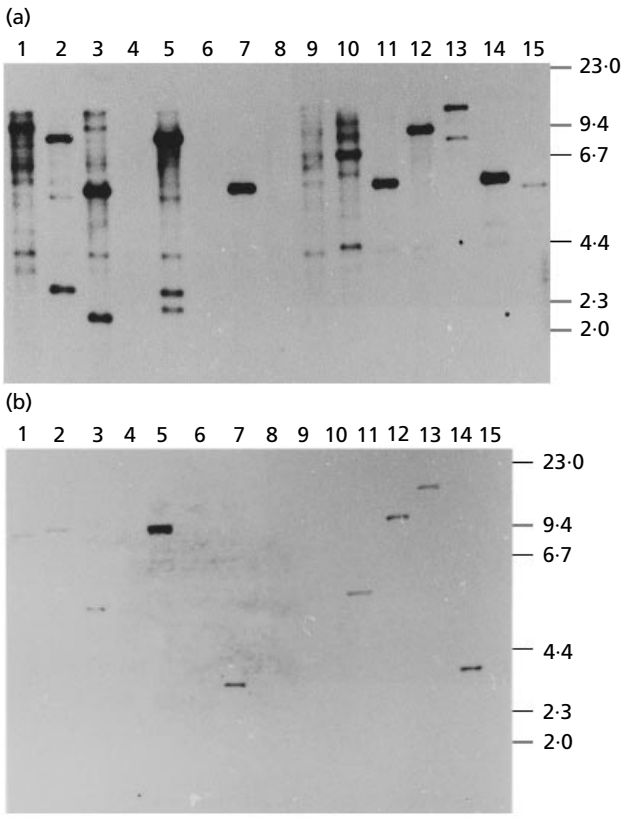

Fig. 3. Southern hybridization: *Ecc* chromosomal DNA digested with *Eco*RI and probed with the GS101 *car Stu*I fragment (a), or the *carR* PCR product (b). Size markers are indicated on the right of the figure (kb). Lanes: 1, SCRI 112; 2, SCRI 114; 3, SCRI 122; 4, SCRI 172; 5, SCRI 124; 6, SCRI 125; 7, SCRI 132; 8, SCRI 149; 9, SCRI 166; 10, SCRI 123; 11, SCRI 192; 12, SCRI 193; 13, SCRI 198; 14, GS101; 15, SCC3193.

were constitutive for the production of carbapenem (SCRI 124 and 132), and cryptic (SCRI 112, 114, 122, 123, 192, 193 and 198). There was a degree of polymorphism exhibited by these strains, suggesting that evolutionary divergence had occurred not only between cryptic or constitutive strains, but also between these two phenotypic groups. With the conditions of hybridization used, hybridizing fragments with strong signals were not detected in the strains which were negative for either constitutive or cryptic antibiotic production (SCRI 125, 149, 166, 172 and SCC3193).

Southern hybridization with a GS101 *carR* probe confirmed hybridizing fragments in the DNA from most of the strains identified as containing *car* biosynthetic homologues (Fig. 3b). Homologues were identified in the cryptic strains SCRI 112, 114, 122, 123, 192, 193 and 198, suggesting that the silencing of the *car* cluster in these strains was not the result of gross deletion of the *carR* homologue.

In addition to hybridizing with the *car* biosynthetic and *carR* probes, the *Ecc* chromosomal DNA was hybridized with a *carI* probe (data not presented). All of the strains

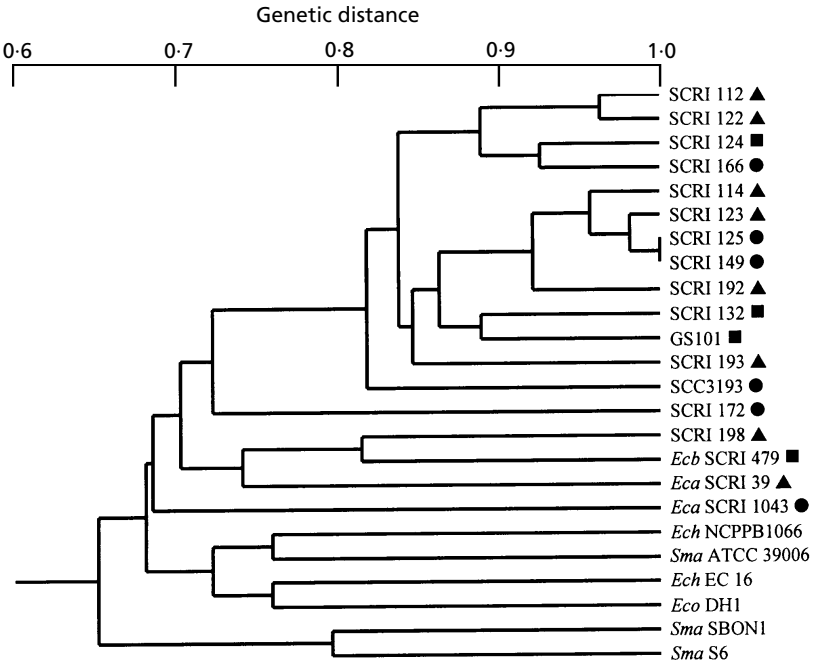

Fig. 4. Ribotyping of *Erwinia carotovora* strains. Phenogram of the RFLPs generated from hybridization of *Eco*RI-digested chromosomal DNA with an SCRI 193 16S rDNA probe (data not presented). Also included in the analysis were two strains of the related 'soft-rot' *Erwinia*, *Erwinia chrysanthemi* (*Ech*), three strains of *Serratia marcescens* (*Sma*) and an *E. coli* (*Eco*) strain. ■, Constitutive carbapenem-producing strains; ▲, cryptic carbapenem-producing strains; ●, carbapenem-nonproducing strains.

tested contained homologues, as would be expected from the results of the OHHL screenings previously described. Interestingly, the homologues identified exhibited very little polymorphism, which contrasts with the results of the hybridizations using the *carR* and *car* biosynthetic probes.

Previously it has been reported that some strains of *Erwinia carotovora* subsp. *betavasculorum* produce an unknown antibiotic (Axelrood *et al*., 1988). During the course of this study an isolate (SCRI 479) from the SCRI culture collection was found to produce an antibiotic that was detected using the *β*-lactam-supersensitive *E. coli*. Chromosomal DNA from this *Erwinia carotovora* subsp. *betavasculorum* strain was also hybridized with the *Stu*I biosynthetic probe (data not presented) and found to contain hybridizing fragments that gave strong signals. This suggests that the antibiotic detected is likely to be a carbapenem, thus providing further evidence of the widespread distribution of carbapenem genes in *Erwinia carotovora* subspecies.

Ribotyping of *Ecc* strains

On the basis of their geographical origins (Tables 3 and 4), the *Ecc* strains that were identified as containing constitutively expressed, or cryptic, carbapenem genes are clearly unrelated. However, to gain a further insight into any relatedness of *Ecc* strains and the distribution of cryptic *car* genes, 16S ribotyping was used to examine the phylogenetic relationships of some of these SCRI strains. Comparison of the RFLPs generated when chromosomal DNA is hybridized with a 16S DNA probe can be used to characterize phylogenetically distinct bacteria (Grimont & Grimont, 1991). In addition to the *Ecc* strains previously hybridized with the *car* probes, DNA from a selection of other strains was analysed. These included a constitutive Car-producing strain of *Serratia marcescens* (ATCC 39006); a naturally Car-resistant clinical isolate of *S. marcescens* (S6); the *Erwinia carotovora* subsp. *betavasculorum* strain previously shown to contain *car* homologues (*Ecb* SCRI 479); a cryptic Car-producing strain of *Eca* (SCRI 39); a Car-nonproducing strain of *S. marcescens* (SBON1); two wild-type *Erwinia chrysanthemi* strains (NCPPB-1066 and EC 16), a Car-nonproducing *Eca* strain (SCRI 1043) and an *E. coli* strain (DH1). The phenogram (Fig. 4) generated from the RFLP patterns shows that most of the *Ecc* strains exhibit a high degree of relatedness and cluster together, as would be expected from their taxonomic classification. However, there is no correlation between the distribution of strains with regard to the production of carbapenem antibiotics and their general genetic relatedness as defined by 16S ribotyping. Interestingly, this is a similar result to that found by Mehling *et al*. (1995), when investigating the distribution of streptomycin and lincomycin antibiotic production patterns in *Streptomyces* spp.

Sequence of the *Ecc* SCRI 193 *carR* homologue

We attempted to determine the molecular basis of the *carR* defect responsible for the Car$^-$ phenotype of SCRI 193. A 2011 bp fragment from SCRI 193 DNA was identified as containing a *carR* open reading frame and the first 229 bp of the *carA* homologue. The *carR* open reading frame was identical in size to the GS101 homologue and encoded a protein of 244 amino acids, with a predicted molecular mass of 28056 Da and a pI of 6·96. The overall identity with the GS101 homologue at the DNA level was 89·5%, with a GC content of 39·9 mol%, a similar value to that of the GS101 homologue (McGowan *et al*., 1995). At the amino acid

```
  1 MDHEIHSFIKRKLKGVGDVWFSYFMMSKNSTSQPYIISNYPEAWMKEYIK
    ||||||||||||||||||||||||||||.|||:|||||||||||||.||:|
  1 MDHEIHSFIKRKLKGVGDVWFSYFMMSKTSTSHPYIISNYPEAWMNEYLK

 51 KEMFLSDPIIVASLARITPFSWDDNDIVTLRAKNQDVFISSVQHDISSGY
    ||||||||||||||||||||||||||:||||||||||||||||||||||||
 51 KEMFLSDPIIVASLARITPFSWDDNDLVTLRAKNQDVFISSVQHDISSGY

101 TFVLHDHDNNVATLSIANHLEDANFEKCMKNHENDLQMLLVNVHEKVMAY
    ||||||||||||||||||||||||:||||||.||||||||||||||||||
101 TFVLHDHDNNVATLSIANHLEDADFEKCMKSHENDLQMLLVNVHEKVMAY

151 QRAINDQDNPPDNSRNALLSPRETEVLFLVSSGRTYKEVSRILGISEVTV
    ||||.||..|||.||||||||||||||||||||||||||||||||||||||
151 QRAIKDQAKPPDSSRNALLSPRETEVLFLVSSGRTYKEVSRILGISEVTV

201 KFHINNSVRKLDVINSRHAITKALELNLFHSPCEPVVMKHMDAR 244
    ||||||||||||||||||||||||||||:||||||||||:.|
201 KFHINNSVRKLDVINSRHAITKALELNLFNSPCEPVVMKHMESR 244
```

Fig. 5. Alignment of *Ecc* GS101 (top) and SCRI 193 (bottom) CarR homologues. Amino acid substitutions are underlined.

```
-141 TGTTGACTATTTGTGTGTTAATTGATATTTTTCGTCCCTGTTAGATATTA -92
     ||||||||  |||| | ||||||||||  ||||| || ||||| ||  ||
-147 TGTTGACTGATTGTATATTAATTGATAACTTTCGACCTTGTTAAATCCTA -98

-91  ATGATTATATTCTGTATAACTAAAAATAATTACATTTACTCTTTGGGAGA -42
      ||||||||||  || ||||||||||||||| ||||||||| ||| | |
-97  GTGATTATATTTGGTGTAACTAAAAATAATTATATTTACTCTCTGGTAAA -48

-41  TAGT......TTTTTATCATTGAGCAAAGTCGGTAAGAGGGTGTAATATG +3
      |||      |||| ||| |||||||||||||||||||| | ||||||||
-47  GAGTTGAATCTTTTAATCTTTGAGCAAAGTCGGTAAGAGAGGGTAATATG +3
```

Fig. 6. Comparison of the nucleotide sequences of the putative promoters and RBSs of *Ecc* SCRI 193 (top) and GS101 (bottom) *carR*s. The start codons of the *carR* open reading frames are marked in bold. The putative RBSs are boxed and putative GS101 −35 and −10 promoter sequences are underlined.

sequence level there was 94% identity and 97% similarity, with 14 amino acid substitutions (Fig. 5). Comparison of the SCRI 193 protein sequence with the GS101 CarR and other LuxR homologues showed that the amino acid substitutions did not affect residues conserved amongst the LuxR family of transcriptional activators (data not presented). Equally, none of the substitutions affected residues which are considered to be functionally critical residues of LuxR (Shadel *et al*., 1990; Slock *et al*., 1990). Therefore, none of the amino acid substitutions identified in the SCRI 193 CarR homologue could be identified as likely key substitutions from sequence comparisons with the GS101 CarR, LuxR, or other homologues. Equally, the effect of the substitutions on the structure and function of the protein can not be predicted, as little is known about the structure of this protein or any member of the LuxR family of transcriptional activators.

Seven basepairs upstream of the start codon of the open reading frame is a putative Shine–Dalgarno ribosome-binding site (RBS) sequence (Fig. 6). This purine-rich sequence differs in only one basepair from that of the

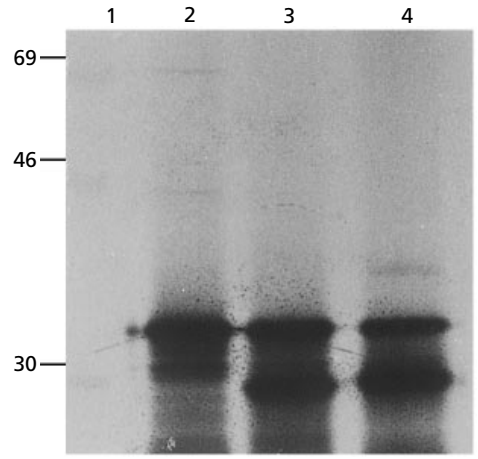

Fig. 7. Prokaryotic cell-free *in vitro* expression of *Ecc* GS101 and SCRI 193 *carR* homologues. Recombinant plasmids containing *carR* homologues of GS101 and SCRI 193 were expressed *in vitro* using an *E. coli* S-30 cell-free extract (De Vries & Zubay, 1967; Zubay, 1973). ^{35}S-labelled products of the *in vitro* expression were separated using SDS-PAGE. The photograph shows an autoradiograph of the *in vitro* Zubay expression of pMH10 (GS101 *carR*) and pMH20 (SCRI 193 *carR*). Lanes: 1, molecular mass standards (kDa); 2, pUC19 (vector control); 3, pMH20; 4, pMH10.

GS101 homologue. The substitution of a guanine for an adenine residue in the SCRI 193 RBS brings the sequence closer to the *E. coli* consensus (Stormo, 1986). Therefore it was thought unlikely that this substitution would be detrimental to the translational efficiency of the SCRI 193 homologue. Previous localized mutagenesis experiments with the GS101 *carR* have identified putative −35 and −10 promoter regions which exhibit homology with the *E. coli* consensus (unpublished observation; Harley & Reynolds, 1987). Alignment of the upstream sequences of the two homologues shows that the putative −35 regions are conserved (Fig. 6). Seventeen basepairs downstream of this there is divergence in the putative −10 sequences. The SCRI 193 sequence contains two nucleotide substitutions which result in movement away from the *E. coli* consensus. In an attempt to assess the effect of the putative promoter substitutions on the transcriptional efficiency, an *in vitro* prokaryotic transcription and translation assay was used to express the SCRI 193 CarR. Fig. 7 shows the products of the expression of the *carR* homologues of GS101 and SCRI 193 cloned into pUC19 (pMH10 and pMH20 respectively). Two protein products of the same size (~28 kDa) were observed in both the SCRI 193 and GS101 samples. A 28 kDa product was not seen in the vector control lane. A product of the same size has previously been observed for the GS101 CarR using the T7 expression system (McGowan *et al*., 1995). Therefore as the SCRI 193 *carR* homologue was able to direct *in vitro* transcription and translation in the *E. coli* S-30 cell extract, it seemed unlikely that mutations in the regulatory sequences were responsible for the *carR* defect in SCRI 193.

Table 5. Induction of the *carB*::*lacZ* fusion of *Ecc* HS5 by SCRI 193 and GS101 CarR homologues

HS5 containing either pNED100 (GS101 *carR*), pNED200 (SCRI 193 *carR*) or the control plasmid pACYC184 was grown in LB at 30 °C. The assay was carried out on triplicate samples from one growth curve experiment. Units of β-galactosidase activity are expressed in specific units (ΔA_{420} min^{-1} ml^{-1} mg^{-1}).

Plasmid carried by HS5	β-Galactosidase activity	
	10 h	14 h
pACYC184	0·0024	0·0023
pNED100	0·2746	0·2883
pNED200	0·2557	0·2747

Multicopy suppression of the cryptic carbapenem phenotype of *Ecc* SCRI 193

During the cloning and sequencing of the SCRI 193 *carR* homologue, the 2·0 kb *Hin*dIII fragment containing *carR* was cloned into the medium-copy-number vector pDAH330 (McGowan *et al*., 1995), to give pTGH10. Surprisingly, when this plasmid was transferred back into SCRI 193, the recombinant strain produced detectable levels of carbapenem. Even more remarkably, the same result was obtained using the SCRI 193 *carR* gene cloned into the low-copy-number vector pACYC-184 (pNED200). These results show that when the SCRI 193 *carR* transcriptional activator is supplied in multiple copies, even at low gene dosage, there is a clear suppression of the cryptic carbapenem phenotype.

Relative activities of the CarR homologues of *Ecc* GS101 and SCRI 193

There were no detectable differences in the relative activities of the *carR* homologues of SCRI 193 and GS101 in pDAH330 and pACYC184 vectors when activity was determined by using the relatively crude β-lactam-supersensitive *E. coli* ESS assay, measuring the activation of carbapenem production in SCRI 193. In an attempt to discern different levels of transcriptional activation directed by the *carR* homologues present in multiple copies, a more sensitive transcriptional fusion approach was utilized. A GS101 derivative containing a *carB*::*lacZ* fusion, with a Tn5 insertion in *carR*, was generated (HS5) in which Car transcriptional activation could be assayed. This strain was used to measure the levels of *carB*::*lacZ* activation directed *in trans* by GS101 and SCRI 193 *carR*s cloned into pACYC184 (pNED100 and pNED200 respectively).

Comparisons of β-galactosidase activities of HS5 carrying either pNED100 or pNED200 through the growth curve showed that there was no discernible difference (data not presented). Table 5 shows the levels of activity measured after 10 and 14 h. The 10 h sample was taken in the transition from late-exponential to early-stationary phase, when OHHL levels in the culture supernatants of SCRI 193 and GS101 are near maximal. The 14 h sample was taken when the culture had entered stationary phase. When the activities of the two homologues *in vivo* were measured repeatedly in separate experiments, comparable levels of transcriptional activation of the *carB*::*lacZ* fusion were found. When the experiment was repeated with the addition of exogenous OHHL (1 μg ml^{-1}), similar levels of activity were seen (data not presented). These results confirm the previous observation that multiple copies of the SCRI 193 *carR* *in trans* can suppress the cryptic Car phenotype.

DISCUSSION

It has long been assumed that the production of β-lactam antibiotics by Gram-negative soil bacteria is rare (Sykes *et al*., 1981). When originally describing the isolation of carbapenem-producing bacteria, Parker *et al*. (1982) reiterated this by noting that the production of 1-carbapen-2-em-3-carboxylic acid appeared to be rather limited in the natural environment. Consequently, when considering *Erwinia carotovora*, we thought it reasonable to assume that only very few strains would have the capacity to produce carbapenem antibiotics. The results presented here show that while a few *Ecc* strains do constitutively produce carbapenem, a larger proportion retain functional, but cryptic, biosynthetic genes.

The initial observation that sparked this study was the surprising discovery that an active *carR* *in trans* caused production of Car in SCRI 193. This suggested that SCRI 193, a strain phenotypically Car^-, contained functional carbapenem biosynthetic genes. To find out whether *car* genes were present in other *Erwinia* strains, *Ecc* strains from a culture collection were screened for antibiotic production after the introduction of *carR* *in trans*. A significant proportion (16%) of *Ecc* strains from the SCRI culture collection were Car^+ due to *trans*-activation by *carR*. Two other classes of strains were identified: those which were naturally Car^+ (4%) and those which remained Car^- after the introduction of *carR*. Notably, the number of strains that contained cryptic *car* genes was greater than that which were constitutively Car^+.

One of the questions raised by this discovery is how these gene clusters became cryptic. In strains containing cryptic carbapenem clusters, homologues of the *car* biosynthetic genes and *carR* were identified by Southern hybridization. This result suggested that the biosynthetic genes were not cryptic due to the loss of the *carR* transcriptional activator, but contained mutant *carR*s, a defect complemented by the introduction of the GS101 *carR*.

The high frequency of cryptic clusters, apparently defective in the same gene, suggested that there might be a discrete genetic switch responsible for silencing. To investigate this we analysed the well-studied wild-type strain, SCRI 193. However, we found no gross alterations such as insertions, inversions or deletions in the *carR* gene of this strain. Rearrangements such as these

have been associated with phenotype switching effecting a range of bacterial phenotypes (for a review see Dybvig, 1993).

Analysis of the predicted amino acid sequence of the mutant CarR showed that there were multiple changes, but none were in residues conserved in the LuxR family of transcriptional activators, and no potential effects on CarR function could be identified. Multiple copies of the SCRI 193 *carR* could suppress the Car$^-$ phenotype. Therefore the defect in *carR*, whilst clear in the context of a chromosomal single copy, could be overridden by artificially supplying multiple copies of the gene. This effect was so dramatic that differences in the relative activities of the GS101 and SCRI 193 CarR proteins could not be accurately measured, even when using the more sensitive assay of the transcriptional activation of a *carB*::*lacZ* fusion. In the light of this, the conclusion drawn from the *in vitro* expression experiment may not be entirely valid. Whilst a product of the correct size was seen, it does not reflect the ability of the SCRI 193 chromosomal locus to be effectively transcribed and translated under natural physiological conditions *in vivo*. Further experiments need to be carried out to determine if the mutation that renders the *car* genes of SCRI 193 cryptic, is at the level of the protein, or at the level of transcription and translation.

Suppression by multiple copies of the SCRI 193 *carR* illustrates the physiological importance of the natural stoichiometry of the regulatory components in carbapenem production. The use of multiple copies of *carR* to study the function and mode of action of this transcriptional activator clearly gives results which are physiologically artefactual. Previous work with the GS101 *carR* cloned into a high-copy-number vector has shown that it is possible to make carbapenem production in GS101 OHHL independent (McGowan *et al*., 1995). In recent regulatory studies with members of the LuxR family of transcriptional activators, it has been common practice to use plasmid-borne genes in multiple copies (Gray *et al*., 1994; Fuqua & Winans, 1994; Hwang *et al*., 1995; Ochsner *et al*., 1994; Ochsner & Reiser, 1995; Passador *et al*., 1993). The dramatic effect seen with the plasmid-borne SCRI 193 *carR* shows that there is a danger of generating artefactual results by using multiple copies of a gene which is found at only one locus on the chromosome, i.e. normally has a copy number of one.

Cryptic genes affecting a variety of phenotypes have previously been identified in a range of organisms. Hall *et al*. (1983) proposed that cryptic genes were phenotypically silent DNA sequences not normally expressed during the life cycle of an individual, but capable of activation as a rare event in a few members of a large population by mutation, recombination, insertion elements or other genetic mechanisms. One of the best-characterized sets of cryptic genes is that involved in sugar utilization in *E. coli*. The *E. coli* strain K12 has been shown to contain three cryptic operons, *asc*, *cel* and *bgl*, which encode three distinct permease and phospho-*β*-glucosidase systems (Schnetz *et al*., 1987; Kricker & Hall, 1987; Hall & Xu, 1992). In addition to the attenuated laboratory strain, high frequencies of wild-type natural isolates have been found to contain cryptic operons (Hall & Betts, 1987). The maintenance of these cryptic operons has been proposed to be the result of periodic cycles of decryptification and cryptification, which are closely linked to the selective advantage conferred under particular growth conditions.

The observation that antibiotic-producing strains of bacteria can act as biological control agents, protecting plants from soil-borne fungal infections, has been seen as evidence that antibiotics have an offensive role in the rhizosphere, acting as competitive chemical weapons (Cavalier-Smith, 1992; Maplestone *et al*., 1992). These molecules are therefore likely to be important determinants of the interactions of micro-organisms in the environment. For the plant pathogen *Erwinia carotovora*, the production of antibiotics by some strains has been shown to confer a competitive advantage *in planta* (Axelrood *et al*., 1988). The production of antibiotics enables the phytopathogenic bacterium to capitalize on the nutrient-rich niche created by its attack on the host plant. Conversely, in the nutrient-limited environment of the rhizosphere, the growth rate of the bacterium decreases and physiological demands on the cell change dramatically. For bacteria adapting to these conditions, the production of antibiotics may confer little, if any, advantage.

Why are the cryptic carbapenem genes maintained by the bacterial host? It is seductive to speculate that carbapenem genes are present in a high proportion of *Erwinia carotovora* strains because of the selective advantage these genes confer under certain environmental conditions. However, in the majority of the strains that were found to contain carbapenem genes, the presumed advantage conferred had not been effective in the relative short term, thus resulting in silencing by random mutation. In these strains the mutation appears to affect *carR* and the transcriptional activation of the Car biosynthetic cluster. Further work remains to be carried out to assess both the long- and short-term expression of carbapenem genes in *Erwinia* to investigate if *Ecc* uses this coarse genetic control to cope with environmental challenges.

Whilst this report describes, to the best of our knowledge, the first example of cryptic *β*-lactam antibiotic genes, cryptic antibiotic genes have been reported before. Evidence of silent antibiotic genes has been noted in *Streptomyces*, a genus noted for prolific production of secondary metabolites (Berdy, 1988). An intriguing case of 'silent' genetic information was reported in *Streptomyces* spp. by Jones & Hopwood (1984a). Two separate fragments of DNA from the actinomycin-producing *S. antibioticus* caused *S. lividans* to produce the enzyme phenoxazinone synthase (PHS), although neither of the clones carried the structural gene for this enzyme. PHS is an enzyme thought to catalyse the penultimate step in the actinomycin biosynthetic pathway in *S. antibioticus*. Significantly, *S. lividans* does

not normally produce either PHS or actinomycin D (Jones & Hopwood, 1984b).

The detection of an antibiotic biosynthetic gene in a species of *Streptomyces* that is phenotypically negative for that antibiotic has parallels with the observation of cryptic antibiotic genes in *Ecc* SCRI 193. However there are distinct differences between the two systems. Firstly, *S. lividans* does not normally produce PHS or actinomycin D, whereas *Ecc* strains have been documented as producing carbapenem antibiotics (Jones & Hopwood, 1984b; Parker *et al*., 1982). Secondly, additional antibiotic biosynthetic genes have not been found that would direct the production of actinomycin, suggesting that the PHS gene is an isolated locus.

The carbapenem clusters identified by Southern hybridization exhibited polymorphism when probed with the *carA–H* probe, but not with the *carI* probe. This observation suggested that the two loci have evolved independently, and the polymorphism could perhaps have arisen by genetic transfer of the *car* cluster between *Erwinia* strains. We have shown that the carbapenem cluster (*carR–H*) can direct the synthesis of carbapenem in a range of quorum-sensing bacteria, its transcription being dependent essentially on an $OHHL^+$ background (unpublished observation). The carbapenem cluster acts as a cassette when transferred, 'plugging in' to the native quorum-sensing background to coordinate regulation of the antibiotic cluster in concert with cell density. Significantly, all of the *Ecc* and *Eca* strains assayed produced detectable levels of an inducer. In addition, other *Erwinia* species have also been found to contain *luxI* homologues, *echI* in *Erwinia chrysanthemi* and *esaI* in *Erwinia stewartii*, suggesting that they too produce inducer molecules (accession number U45854; Beck von Bodman & Farrand, 1995). These observations raise the question as to the potential distribution of *car* cluster genes in other members of the genus *Erwinia*. More light will be shed on the origins and evolution of the carbapenem biosynthetic cluster when *car* genes from other producers, such as streptomycetes, are identified and sequenced.

An interesting finding of this study was the occurrence of dramatic changes in OHHL levels in culture supernatants of SCRI 193. A peak in OHHL levels coincided with the transition from the late-exponential to the early-stationary phase of growth, as has previously been shown with the antibiotic-producing strain of *Ecc*, GS101 (Swift *et al*., 1993). However, we found a significant drop in OHHL levels as cultures of SCRI 193 progressed into stationary phase. Experiments are currently under way to investigate if the 'damping' of the pheromone signal is the result of catabolism or some other cellular process.

In conclusion, the results presented here show that carbapenem genes are common in *Erwinia carotovora* but remain cryptic due to a defective transcriptional activator. Previous work has identified CarR and OHHL as being the primary regulatory components that allow continuous modulation of carbapenem production in *Ecc* (Bainton *et al*., 1992; McGowan *et al*., 1995). This physiological regulation generates a homogeneous response to environmental change. In contrast, cryptic gene activation would operate at a higher hierarchical level, affecting only a few members of a population. By its very nature, this form of regulation would be less responsive, being reliant on mutational events for modulation of activity, therefore providing a bacterial population with diversity. A consequence of this is that the number of possible antibiotic-producing strains would be underestimated using conventional screening approaches, thus leaving a potential reservoir of novel carbapenem derivatives untapped. So, on a practical level, facile transcription of cryptic carbapenem genes by a functional CarR might have utility in uncovering many strains which can elaborate simple, and modified, carbapenems.

ACKNOWLEDGEMENTS

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REFERENCES

Axelrood, P. E., Rella, M. & Schroth, M. N. (1988). Role of antibiosis in competition of *Erwinia* strains in potato infection courts. *Appl Environ Microbiol* **54**, 1222–1229.

Bainton, N. J., Bycroft, B. W., Chhabra, S. R., Stead, P., Gledhill, L., Hill, P., Rees, C. E. D., Winson, M. K., Salmond, G. P. C., Stewart, G. S. A. B. & Williams, P. (1992). A general role for the *lux* autoinducer in bacterial cell signalling: control of antibiotic biosynthesis in *Erwinia*. *Gene* **116**, 87–91.

Beck von Bodman, S. & Farrand, S. K. (1995). Capsular polysaccharide biosynthesis and pathogenicity in *Erwinia stewartii* require induction by an *N*-acylhomoserine lactone autoinducer. *J Bacteriol* **177**, 5000–5008.

Berdy, J. (1988). New ways to obtain antibiotics. *Chin J Antibiot* **7**, 272–290.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding. *Anal Biochem* **72**, 248–254.

Bycroft, B. W., Maslen, C., Box, S. J., Brown, A. & Tyler, J. W. (1987). The isolation and characterisation of (3*R*,5*R*)-carbapenem-3-carboxylic acid (3*S*,5*R*)-carbapenem-3-carboxylic acid from *Serratia* and *Erwinia* species and their putative biosynthetic role. *J Chem Soc Chem Commun* **21**, 1623–1625.

Bycroft, B. W., Maslen, C., Box, S. J., Brown, A. & Tyler, J. W. (1988). The biosynthetic implication of acetate and glutamate incorporation into (3*R*,5*R*)-carbapenem-3-carboxylic acid (5*R*)-carbapen-2-em-3-carboxylic acid by *Serratia* sp. *J Antibiot* **41**, 1231–1242.

Cavalier-Smith, T. (1992). Origins of secondary metabolism. In *Secondary Metabolites: Their Function and Evolution*, pp. 64–87. Edited by D. J. Chadwick & J. Whelan. Chichester: Wiley.

Chang, A. C. Y. & Cohen, S. N. (1978). Construction and characterisation of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. *J Bacteriol* **134**, 1141–1156.

Demain, A. L. (1995). Why do microorganisms produce antimicrobials? In *Fifty Years of Antimicrobials: Past Perspectives*

and Future Trends (Society for General Microbiology Symposium no. 53), pp. 205–228. Edited by P. A. Hunter, G. K. Darby & N. J. Russell. Cambridge: Cambridge University Press.

De Vries, J. K. & Zubay, G. (1967). DNA-directed peptide synthesis. II. The synthesis of the α-fragment of the enzyme β-galactosidase. *Proc Natl Acad Sci USA* **57**, 1010–1012.

Ditta, G., Stanfield, S., Corbin, D. & Helinski, D. R. (1980). Broad host range DNA cloning systems for Gram-negative bacteria: construction of a gene bank of *Rhizobium meliloti*. *Proc Natl Acad Sci USA* **77**, 7347–7351.

Dybvig, K. (1993). DNA rearrangements and phenotypic switching in prokaryotes. *Mol Microbiol* **10**, 465–471.

Foster, H. A., Yasouri, F. N. & Daould, N. N. (1992). Antibiotic activity of soil myxobacteria and its ecological implication. *FEMS Microbiol Ecol* **101**, 27–32.

Fuqua, W. C. & Winans, S. C. (1994). A LuxR–LuxI type regulatory system activates *Agrobacterium* Ti plasmid conjugal transfer in the presence of a plant tumour metabolite. *J Bacteriol* **176**, 2796–2806.

Fuqua, W. C., Winans, S. C. & Greenberg, P. E. (1996). Census and consensus in bacterial ecosystems: the LuxR–LuxI family of cell quorum-sensing regulators. *Annu Rev Microbiol* **50**, 727–751.

Gilbert, H., Blazek, J., Bullman, R. & Minton, N. P. (1986). Cloning and expression of the *Erwinia chrysanthemi* asparaginase gene in *Escherichia coli* and *Erwinia carotovora*. *J Gen Microbiol* **132**, 151–160.

Gray, K. M., Passador, L., Iglewski, B. H. & Greenberg, E. P. (1994). Interchangeability and specificity of components from the quorum-sensing regulatory systems of *Vibrio fischeri* and *Pseudomonas aeruginosa*. *J Bacteriol* **176**, 3076–3080.

Grimont, F. & Grimont, P. A. D. (1991). DNA fingerprinting. In *Nucleic Acid Techniques*, pp. 249–279. Edited by E. Stackebrandt & M. Goodfellow. London: Wiley.

Grinter, N. J. (1983). A broad-host-range cloning vector transposable to various replicons. *Gene* **21**, 133–143.

Hall, B. G. & Betts, P. W. (1987). Cryptic genes for cellobiose utilization in natural isolates of *Escherichia coli*. *Genetics* **115**, 431–439.

Hall, B. G. & Xu, L. (1992). Nucleotide sequence, function, activation, and evolution of the cryptic *asc* operon of *Escherichia coli* K12. *Mol Biol Evol* **9**, 688–706.

Hall, B. G., Yokoyama, S. & Calhoun, D. H. (1983). Role of cryptic genes in microbial evolution. *Mol Biol Evol* **1**, 109–124.

Harley, C. B. & Reynolds, R. P. (1987). Analysis of *E. coli* promoter sequences. *Nucleic Acids Res* **15**, 2343–2361.

Hanahan, D. (1983). Studies of transformation of *Escherichia coli* with plasmids. *J Mol Biol* **166**, 557–580.

Herrero, M., de Lorenzo, V. & Timmis, K. N. (1990). Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in Gram-negative bacteria. *J Bacteriol* **172**, 6557–6567.

Hinton, J. C. D. & Salmond, G. P. C. (1987). Use of Tn*phoA* to enrich for extracellular mutants of *Erwinia carotovora* subspecies *carotovora*. *Mol Microbiol* **1**, 381–386.

Hinton, J. C. D., Gill, D. R., Lalo, D., Plastow, G. S. & Salmond, G. P. C. (1990). Sequence of the *peh* gene of *Erwinia carotovora*: homology between *Erwinia* and plant enzymes. *Mol Microbiol* **4**, 1029–1036.

Hwang, I., Cook, D. M. & Farrand, S. K. (1995). A new regulatory element modulates homoserine lactone-mediated autoinduction of Ti plasmid conjugal transfer. *J Bacteriol* **177**, 449–458.

Jones, G. H. & Hopwood, D. A. (1984a). Activation of phenoxazinone synthase expression in *Streptomyces lividans* by cloned DNA sequences from *Streptomyces antibioticus*. *J Biol Chem* **259**, 14158–14164.

Jones, G. H. & Hopwood, D. A. (1984b). Molecular cloning and expression of the phenoxazinone synthase gene from *Streptomyces antibioticus*. *J Biol Chem* **259**, 14151–14157.

Jones, S. E., Yu, B., Bainton, N. J. & 11 other authors (1993). The *lux* autoinducer regulates the production of exoenzyme virulence determinants in *Erwinia carotovora* and *Pseudomonas aeruginosa*. *EMBO J* **12**, 2477–2482.

Kahan, J. S., Kahan, F. M., Goegelman, R. & 10 other authors (1979). Thienamycin, a new β-lactam antibiotic. I. Discovery, taxonomy, isolation and physical properties. *J Antibiot* **32**, 1–12.

Kaniga, K., Delor, I. & Cornelis, G. R. (1991). A wide-host-range suicide vector for improving reverse genetics in Gram-negative bacteria: inactivation of the *blaA* gene of *Yersinia enterocolitica*. *Gene* **109**, 137–141.

Keel, C., Schnider, U., Maurhofer, M., Voisard, C., Laville, J., Burger, P., Wirthner, P., Haas, D. & Défago, G. (1992). Suppression of root diseases by *Pseudomonas fluorescens* CHAO: importance of the secondary metabolite 2,4-diacetylphloroglucinol. *Mol Plant–Microbe Interact* **5**, 4–13.

Keen, N. T., Dalhbeck, D., Staskawicz, B. & Belser, W. (1984). Molecular cloning of pectate lyase genes from *Erwinia chrysanthemi* and their expression in *Escherichia coli*. *J Bacteriol* **159**, 825–831.

Kricker, M. & Hall, B. G. (1987). Biochemical genetics of the cryptic gene system for the cellobiose utilization in *Escherichia coli* K12. *Genetics* **115**, 419–429.

Livermore, D. M. (1992). Leading article: carbapenemases. *J Antimicrob Chemother* **29**, 609–616.

McGowan, S., Sebaihia, M., Jones, S. & 7 other authors (1995). Carbapenem antibiotic production in *Erwinia carotovora* is regulated by CarR, a homologue of the LuxR transcriptional activator. *Microbiology* **141**, 541–550.

McGowan, S., Sebaihia, M., Porter, L. E., Stewart, G. S. A. B., Williams, P., Bycoft, B. W. & Salmond, G. P. C. (1996). Analysis of bacterial carbapenem antibiotic genes reveals a novel β-lactam biosynthesis pathway. *Mol Microbiol* **22**, 415–426.

Maplestone, R. A., Stone, M. J. & Williams, D. H. (1992). The evolutionary role of secondary metabolites – a review. *Gene* **115**, 115–157.

Mehling, A., Wehmeier, U. F. & Piepersberg, W. (1995). Nucleotide sequences of streptomycete 16S ribosomal DNA: towards a specific identification system for streptomycetes using PCR. *Microbiology* **141**, 2139–2147.

Messing, J. & Vieira, J. (1982). A new pair of M13 vectors for selecting either DNA strand of double-digest restriction fragments. *Gene* **19**, 269–276.

Miller, J. H. (1972). *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Moellering, R. C., Eliopoulos, G. M. & Setochnik, D. E. (1989). The carbapenems: new broad spectrum β-lactam antibiotics. *J Antimicrob Chemother* **24**, 1–7.

Neu, H. C. (1994). Why carbapenems? *Curr Opin Infect Dis* **7**, 3–10.

Ochsner, U. A. & Reiser, J. (1995). Autoinducer-mediated regulation of rhamnolipid biosurfactant synthesis in *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA* **92**, 1490–1494.

Ochsner, U. A., Kock, A. K., Fiechter, A. & Reiser, J. (1994).

Isolation and characterization of a regulatory gene affecting rhamnolipid biosurfactant synthesis in *Pseudomonas aeruginosa*. *J Bacteriol* **176**, 2044–2054.

Parker, W. L., Rathnum, M. L., Wells, J. S., Trejo, W. H., Principe, P. A. & Sykes, R. B. (1982). SQ27850, a simple carbapenem produced by species of *Serratia* and *Erwinia*. *J Antibiot* **35**, 653–660.

Passador, L., Cook, J. M., Gambello, M. J., Rust, L. & Iglewski, B. H. (1993). Expression of *Pseudomonas aeruginosa* virulence genes requires cell-to-cell communication. *Science* **260**, 1127–1130.

Pérombelon, M. C. M. & Kelman, A. (1980). Ecology of the soft rot erwinias. *Annu Rev Phytopathol* **18**, 361–387.

Pirhonen, M., Heino, P., Helander, I., Harju, P. & Palva, E. T. (1988). Bacteriophage T4 resistant mutants of the plant pathogen *Erwinia carotovora*. *Microbiol Path* **4**, 359–367.

Pirhonen, M., Felgo, D., Heikinheimo, R. & Palva, E. T. (1993). A small diffusible molecule is responsible for the global control of virulence and exoenzyme production in *Erwinia carotovora*. *EMBO J* **12**, 2467–2476.

Reeves, P. J., Whitcombe, D., Wharam, S. & 9 other authors (1993). Molecular cloning and characterization of 13 *out* genes from *Erwinia carotovora* subsp. *carotovora*: genes encoding members of a general secretion pathway (GPS) widespread in Gram-negative bacteria. *Mol Microbiol* **8**, 433–456.

Salmond, G. P. C., Bycroft, B. W., Stewart, G. S. A. B. & Williams, P. (1995). The bacterial 'enigma': cracking the code of cell–cell communication. *Mol Microbiol* **16**, 615–624.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Sanger, F., Nicklen, S. & Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* **74**, 5463–5467.

Schnetz, K., Toloczyki, C. & Rak, B. (1987). β-Glucoside (*bgl*) operon of *Escherichia coli* K-12: nucleotide sequence, genetic organization, and possible evolutionary relationship to regulatory components of two *Bacillus subtilis* genes. *J Bacteriol* **169**, 2579–2590.

Selvaraj, G., Fong, Y. C. & Iyer, V. N. (1984). A portable DNA sequence carrying the cohesive site (*cos*) of bacteriophage λ and the *mob* (mobilization) region of the broad-host-range plasmid RK2: a module for the construction of new cosmids. *Gene* **32**, 235–241.

Shadel, G. S., Young, R. & Baldwin, T. O. (1990). Use of regulated cell lysis in a lethal genetic selection in *Escherichia coli*: identification of the autoinducer-binding region of the LuxR protein from *Vibrio fischeri* ATCC 7744. *J Bacteriol* **172**, 3980–3087.

Slock, J., VanRift, D., Kolibachuk, D. & Greenberg, E. P. (1990). Critical regions of the *Vibrio fischeri* LuxR protein defined by mutational analysis. *J Bacteriol* **172**, 3974–3979.

Stormo, G. (1986). Translation initiation. In *Maximizing Gene Expression*, pp. 195–224. Edited by W. Reznikoff & L. Gold. Boston: Butterworths.

Swift, S., Winson, M. K., Chan, P. F. & 11 other authors (1993). A novel strategy for the isolation of *luxI* homologues: evidence for the wide spread distribution of a LuxR:LuxI superfamily in enteric bacteria. *Mol Microbiol* **10**, 511–520.

Swift, S., Throup, J. P., Salmond, G. P. C., Williams, P. & Stewart, G. S. A. B. (1996). Quorum sensing: a population-density component in the determination of bacterial phenotype. *Trends Biochem Sci* **21**, 214–219.

Sykes, R. B., Cimarusti, C. M., Bonner, D. P. & 11 other authors (1981). Monocyclic β-lactam antibiotics produced by bacteria. *Nature* **291**, 489–491.

Throup, J. P., Camara, M., Briggs, G. S., Winson, M. K., Chhabra, S. R., Bycroft, B. W., Williams, P. & Stewart, G. S. A. B. (1995). Characterisation of the *yenI*/*yenR* locus from *Yersinia enterocolitica* mediating the synthesis of two *N*-acylhomoserine lactone signal molecules. *Mol Microbiol* **17**, 345–356.

Toth, I., Pérombelon, M. C. M. & Salmond, G. P. C. (1993). Bacteriophage φKP mediated generalised transduction in *Erwinia carotovora* subsp. *carotovora*. *J Gen Microbiol* **139**, 2705–2709.

Ubukata, K., Hikida, M., Yoshida, M., Yishiki, Y., Furukawa, Y., Tashiro, K., Konno, M. & Mitsuhashi, S. (1990). *In vitro* activity of LJC-10,627, a new carbapenem antibiotic with high stability to dehydropeptidase I. *Antimicrob Agents Chemother* **34**, 994–1000.

Williamson, J. M., Inamine, E., Wilson, K. E., Douglas, A. W., Liesch, J. M. & Albers-Schönberg, G. (1985). Biosynthesis of the β-lactam antibiotic, thienamycin by *Streptomyces cattleya*. *J Biol Chem* **260**, 4637–4647.

Yanisch-Perron, C., Vieira, J. & Messing, J. (1985). Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33**, 103–119.

Zubay, G. (1973). *In vitro* synthesis of protein in microbial systems. *Annu Rev Genet* **7**, 267–287.

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