Microbiology (1998), **144**, 201–209 Printed in Great Britain

A pheromone-independent CarR protein controls carbapenem antibiotic synthesis in the opportunistic human pathogen *Serratia marcescens*

A. R. J. Cox,[1] N. R. Thomson,[1] B. Bycroft,[2] G. S. A. B. Stewart,[3] P. Williams[2] and G. P. C. Salmond[1]

Author for correspondence: G. P. C. Salmond. Tel: +44 1223 333650. Fax: +44 1223 333345. e-mail: gpcs@mole.bio.cam.ac.uk

[1] Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1QW, UK

[2] Department of Pharmaceutical Sciences, University of Nottingham, Nottingham NG7 2RD, UK

[3] Department of Applied Biochemistry and Food Sciences, University of Nottingham Faculty of Agriculture and Food Sciences, Sutton Bonington LE12 5RD, UK

Strain ATCC 39006 of *Serratia marcescens* makes the same carbapenem, (5*R*)-carbapen-2-em-3-carboxylic acid (Car), as the *Erwinia carotovora* strain GS101. Unlike *E. carotovora*, where the onset of production occurs in the late-exponential phase of growth in response to the accumulation of the small diffusible pheromone *N*-(3-oxohexanoyl)-L-homoserine lactone (OHHL), in *S. marcescens* carbapenem is produced throughout the growth phase and does not appear to involve any diffusible pheromone molecule. Two cosmids capable of restoring antibiotic production in *E. carotovora* group I carbapenem mutants were isolated from an *S. marcescens* gene library. These cosmids were shown to contain a homologue of the *E. carotovora carR* gene, encoding a CarR protein with homology to the LuxR family of transcriptional regulators. The *S. marcescens carR* was subcloned and shown to be capable of complementing *in trans*, in the absence of OHHL, an *E. carotovora carR carI* double mutant, releasing the heterologous *E. carotovora* host from pheromone dependence for carbapenem production. The apparent OHHL-independence of the *S. marcescens* CarR explains the constitutive nature of carbapenem production in this strain of *S. marcescens*.

Keywords: *Serratia marcescens*, carbapenem, β-lactam, *N*-acylhomoserine lactone, LuxR homologue

INTRODUCTION

Carbapenems are β-lactam antibiotics, discovered in the 1970s through a mass screening of micro-organisms for new antibiotics (Sykes *et al*., 1981; Imada *et al*., 1981). They have a clinical potential that lies in their potent, broad spectrum activity against both Gram-positive and Gram-negative bacteria (Kropp *et al*., 1980, 1985) as well as resistance to a wide range of β-lactamases (Kahan *et al*., 1983; Labia *et al*., 1986). Many of the 40 or so naturally occurring carbapenems that have so far been identified are produced by *Streptomyces* spp., and radiolabelling studies on thienamycin production by *Streptomyces cattleya* (Williamson, 1986) have shown that carbapenems are produced by a biosynthetic pathway quite different from that used for penicillins and cephalosporins. However, the slow growth and poor genetic tractability of many of the carbapenem-producing organisms has hindered molecular genetic studies of the synthesis of carbapenem and the regulation of its production. Consequently all therapeutically useful carbapenems are, at present, made by total chemical synthesis.

The mass screening of different bacteria revealed that the parent carbapenem, (5*R*)-carbapen-2-em-3-carboxylic acid (Car), is produced by strains of two fast-growing, genetically amenable Gram-negative bacteria: *Serratia marcescens* and *Erwinia carotovora* (Parker *et al*., 1982). This carbapenem is easily detected by a simple plate bioassay employing a sensitive strain of *Escherichia coli*. In contrast to the more complex situation found in *Streptomyces* this simple carbapenem appears to be the only antibiotic produced by *S. marcescens* and

Abbreviations: Car, (5*R*)-carbapen-2-em-3-carboxylic acid; OHHL, *N*-(3-oxohexanoyl)-L-homoserine lactone.

The GenBank accession number for the sequence reported in this paper is AF012907.

E. carotovora, although *S. marcescens* does also produce the red pigment prodigiosin (Morrison, 1966) which is structurally related to the antibiotic undecylprodigiosin produced by *Streptomyces ceolicolor* and *Streptomyces lividans* (Tsao *et al.*, 1985; Hopwood *et al.*, 1995). Although *E. carotovora* and *S. marcescens* both produce the same antibiotic, in *S. marcescens* carbapenem is produced throughout the growth phase (Bycroft *et al.*, 1988), whereas in *E. carotovora* carbapenem production is that of a classical secondary metabolite with the onset of production occurring in the late-exponential phase of growth (Bainton *et al.*, 1992b). It was hoped that investigating carbapenem production in both *E. carotovora* and *S. marcescens* might uncover similarities in the biosynthetic pathway and differences between the regulatory mechanisms.

It had previously been shown that *E. carotovora* mutants deficient in the production of carbapenem could be divided into two groups, group 1 and group 2, and that carbapenem activity could be restored to the group 2 mutants by cross-feeding of supernatants from the group 1 mutants. Group 2 mutants are defective in the *carI* gene, which encodes a protein responsible for the synthesis of the small, diffusible pheromone *N*-(3-oxohexanoyl)-L-homoserine lactone (OHHL), which was found to be excreted by the group 1 mutants and by the wild-type (Bainton *et al.*, 1992a, b). In *E. carotovora* strain GS101, OHHL is required to induce the synthesis of carbapenem and the synthesis of multiple exoenzyme virulence factors (pectinases, cellulases and proteases) (Jones *et al.*, 1993). Consequently, the group 2 mutants have a pleiotropic, Car$^-$ (**Car**bapenem production) and Rex$^-$ (**R**egulation of **ex**oenzymes) mutant phenotype. This pleiotropy is abolished by the addition of OHHL, either exogenously or through cross-feeding, or by the provision of the *carI* gene *in trans*. Although some of the group 1 mutants were originally presumed to be defective in carbapenem biosynthesis (*car*) genes a number of them have been shown to be defective in the *carR* gene, which encodes a putative transcriptional regulator of the *car* genes, CarR (McGowan *et al.*, 1995). The group 1 CarR mutants can be complemented for carbapenem production by the provision of the *carR* gene *in trans*. CarR, the function of which is OHHL-dependent under normal physiological conditions, exhibits homology to the *Vibrio fischeri* transcriptional regulator, LuxR, and joins the rapidly growing family of LuxR homologues which control a variety of phenotypes in several different bacterial genera (Salmond *et al.*, 1995).

Most of the regulators in the LuxR family exert their control in a cell-density-dependent manner and, as with *E. carotovora* CarR, many of them are known to, or are predicted to, act in concert with OHHL or similar diffusible *N*-acylhomoserine lactones. In this process (now known as quorum sensing) it appears that availability of a threshold concentration of the pheromone is responsible for initiating significant gene expression, presumably by binding to and activating the appropriate transcriptional regulator. In *Agrobacterium tumefaciens* cell-density-dependent expression of the *tra* genes, which are involved in the conjugal transfer of the Ti plasmid, is regulated by *luxI/R* homologues, *traI* and *traR*, and involves the pheromone, *N*-3-(oxo-octanoyl)-L-homoserine lactone (Zhang *et al.*, 1993; Piper *et al.*, 1993). In the human pathogen *Pseudomonas aeruginosa*, a LuxR homologue, LasR, acts as a positive transcriptional regulator for the expression of *lasB* (Gambello *et al.*, 1991), which is responsible for the production of elastase, a major virulence determinant of this organism. A *luxI* homologue, *lasI*, is necessary for the production of the pheromone *N*-3-(oxo-dodecanoyl)-L-homoserine lactone (Passador *et al.*, 1993; Pearson *et al.*, 1994), which is thought to activate the LasR protein. In *Pseudomonas aureofaciens* the phenazine antibiotic genes are transcriptionally regulated by a LuxR homologue, PhzR (Pierson, 1994), in response to the accumulation of a pheromone produced by *phzI* (Wood & Pierson, 1996). In *Erwinia stewartii* pathogenicity is effected by capsular polysaccharide, the biosynthetic genes for which are transcriptionally controlled by the product of the *esaR* gene, EsaR. OHHL, resulting from expression of the *esaI* gene, is required as the pheromone (Beck von Bodman & Farrand, 1995). In *Rhizobium leguminosarum* the LuxR homologue RhiR exerts positive transcriptional control over the *rhiABC* operon (Cubo *et al.*, 1992). However, there are LuxR homologues, such as SdiA in *Escherichia coli*, which specifically increases transcription of the *ftsQAZ* cell division gene cluster (Wang *et al.*, 1991), for which no specific pheromone molecule has yet been associated.

In this report we describe the isolation and identification of an *S. marcescens carR* gene, encoding a LuxR homologue, CarR, which appears to function in a pheromone-independent fashion and so is capable of constitutively activating carbapenem synthesis in *S. marcescens*. The *S. marcescens* CarR is also capable of releasing a heterologous *E. carotovora* host from pheromone dependency for carbapenem production.

METHODS

Bacterial strains and media. Strains, plasmids and phages used in this study are listed in Table 1. *Erwinia carotovora* and *Serratia marcescens* strains were routinely grown at 30 °C; *Escherichia coli* was grown at 37 °C. The *E. coli* ESS bioassay was carried out at 25 °C. LB medium (Miller, 1972) was used with OHHL supplements added where required, to a final concentration of 1 μg ml^{-1}

Isolation of *S. marcescens* carbapenem cosmids by direct, λ-mediated cosmid complementation. Chromosomal DNA of the wild-type *S. marcescens* strain ATCC 39006 was prepared and partially digested with *Sau*3A, size-fractionated and then ligated with *Bam*HI-digested DNA of cosmid vector pSF6 (Selvaraj *et al.*, 1984). The ligation mixture was packaged into coliphage λ heads *in vitro* using the Giga pack gold II kit (Stratagene). The packaged λ cosmid library was used to infect *E. carotovora* group 1 Car$^-$ mutants carrying the LamB plasmid pTroy9 (see Ellard *et al.*, 1989) and cosmid-containing transductants were selected on LB agar plates containing spectinomycin at 50 μg ml^{-1}. The transductants were screened for restoration of carbapenem production using the halo

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

Strain/plasmid/phage	Characteristics	Reference
Strain		
Escherichia coli K-12		
ESS	*β*-Lactam super-sensitive	Bainton *et al.* (1992a)
DH1	F$^-$ *supE44 recA1 endA1 gyrA96* (Nal^r) *thi-1 hsdR17* ($r_k^-m_k^+$) *relA1*	Hanahan (1983)
Erwinia carotovora subsp. *carotovora*		
ATCC 39048	Wild-type (Car$^+$)	Bainton *et al.* (1992a)
GS101	ATCC 39048(pTROY9)::Tn*5* restrictionless	Bainton *et al.* (1992a)
ATTN10	ATCC 39048::Tn*10* restrictionless (Tn*10* cured Tc^s)	McGowan *et al.* (1996)
PNP11	GS101 *carR*	McGowan *et al.* (1995)
PNP14	GS101 *carR*	McGowan *et al.* (1995)
TCR5	ATTN10 *carR*::Tn*5*	This study
PNP21	GS101 Car$^-$	McGowan *et al.* (1995)
PNP22	GS101 *carI*	McGowan *et al.* (1995)
PNP23	GS101 *carI*	McGowan *et al.* (1995)
PRTX1	ATTN10 *carI*::Tn*phoA′2*	This study
TCRI 51	TCR*5 carI*::Tn*phoA′2*	This study
Serratia marcescens		
ATCC 39006	Wild-type (Car$^+$)	Bycroft *et al.* (1988)
SC13	ATCC 39006 (pMUT13)	This study
SC9	ATCC 39006 (pTROY9)	This study
WT1	SC9 Pig$^-$	This study
WM5	SC13 Pig$^-$	This study
CWT11	WT1 Car$^-$	This study
CWT12	WT1 Car$^-$	This study
CWT13	WT1 Car$^-$	This study
CWT14	WT1 Car$^-$	This study
CWT15	WT1 Car$^-$	This study
CWT18	WT1 Car$^-$	This study
CWT31	WT1 Car$^-$	This study
CWM52	WM5 Car$^-$	This study
Plasmids		
pSF6	Low-copy-number mobilizable cosmid cloning vector	Selvaraj *et al.* (1984)
pNRT1	pSF6, *car*$^+$ (*S. marcescens*)	This study
pNRT20	pSF6, *car*$^+$ (*S. marcescens*)	This study
cWU142	pSF6, *car*$^+$ (*E. carotovora*)	McGowan *et al.* (1995)
pWU14203	pSF6 (cWU142 *carR*$^+$ subclone)	McGowan *et al.* (1995)
pTON28	pSF6 (pNRT1 *carR*$^+$ subclone)	This study
pDAH330	High-copy-number (pIC19R derived) cloning vector	Unpublished results
pTON28M	pDAH330 (pNRT1 *carR*$^+$ subclone)	This study
pT7-5	T7*ϕ*10 promoter	Tabor & Richardson (1985)
pT7-15	pT7-5 (pNRT1 *carR*$^+$ subclone)	This study
Phages		
Coliphages		
M13 mp18	DNA sequencing vector	Messing *et al.* (1977)
*λ*cI857	Thermoinducible	Vollenweider *et al.* (1980)
λ::Tn*5*-B20	Carries Tn*5*–*lacZ*	Krebs & Reznikoff (1988)
λ::Tn*phoA′-2*	Carries Tn*10*–*lacZ*	Wilmes-Riesenberg & Wanner (1992)
E. carotovora phages		
*ϕ*KP	*E. carotovora* generalized transducing phage	Toth *et al.* (1993)

bioassay test with *E. coli* ESS as described by Bainton *et al.* (1992b). Complementing cosmids were isolated and used to transform *E. coli* DH1 by electroporation. High-titre *λ*cI857 lysates were raised on the transformants by the method of White *et al.* (1983) to efficiently package the cosmid DNA and generate high-frequency transducing lysates for these cosmids.

The lysates were used to transduce various LamB-containing Car$^-$ mutants of *E. carotovora* and *S. marcescens* using spectinomycin resistance (Sp^r) as the selectable marker. The general aspects of LamB-based cloning and complementation strategies are described elsewhere (Mulholland & Salmond, 1995).

Southern blot hybridization and subcloning. Southern blots were carried out on *Bam*HI restriction digests of *S. marcescens* cosmids pNRT1 and pNRT20 which had been subjected to agarose gel electrophoresis. The agarose gels were photographed with a scale before the DNA was transferred and immobilized on nitrocellulose filters. Filters were blotted at 65 °C with a ^{32}P-labelled probe made from the *E. carotovora carR* subclone pWU14032, then washed in SSC and visualized by autoradiography. A partial *Bam*HI digest was used to construct an initial restriction map of the *S. marcescens* cosmids pNRT1 and pNRT20 (Fig. 1). The 2·8 kb *Bam*HI fragment of pNRT1 was cloned into the *Bam*HI site of both pSF6 and pDAH330 (a chloramphenicol-resistant derivative of pIC19R; D. Hodgson, personal communication) to yield the subclones pTON28 (Sp^r) and pTON28M (Cm^r) respectively. These subclones were transferred into various Car$^-$ mutants of *E. carotovora* and *S. marcescens* by electroporation, using spectinomycin and chloramphenicol resistance as the selectable markers.

Sequence analysis. Random subclones, ligated into M13mp18, were generated from the insert DNA of pTON28 by the ligation and sonication method described by Bankier *et al.* (1986). The subclones were sequenced by the dideoxynucleotide chain-termination procedure (Sanger *et al.*, 1977) using a Sequenase kit (USB).

Gene product identification. A 3·4 kb *Sal*I–*Pst*I fragment derived from pNRT1 and containing the *S. marcescens carR* gene was ligated into pT7-5 also digested with *Sal*I and *Pst*I. The resulting plasmid (pT7-15) was used to express the *S. marcescens carR* by the methods described by Tabor & Richardson (1985). Protein products were separated by SDS-PAGE and visualized after autoradiography.

Isolation of *S. marcescens* carbapenem mutants. Work done on carbapenem non-producing strains of *S. marcescens* had revealed that the prodigiosin pigment produced by *S. marcescens* had a slight anti-bacterial effect on the sensitive *E. coli* strain used in the carbapenem bioassy. Therefore, a Pig$^-$ derivative of the ATCC 39006 strain was used for the isolation of *S. marcescens* carbapenem mutants by chemical mutagenesis. Chemical mutagenesis, performed using EMS, was adapted from the protocol described by Forbes & Perombelon (1985).

Construction of an *E. carotovora carR carI* double mutant. A lysate was made on *E. carotovora carI* mutant PRTX1 (Tc^r) using the ϕKP generalized transducing phage (Toth *et al.*, 1993). This lysate was used to infect *E. carotovora carR* mutant TCR5 (Kn^r). An *E. carotovora carR carI* double mutant, TCRI51, was isolated by selecting transductants on LB agar plates containing both tetracycline at 10 μg ml^{-1} and kanamycin at 50 μg ml^{-1}.

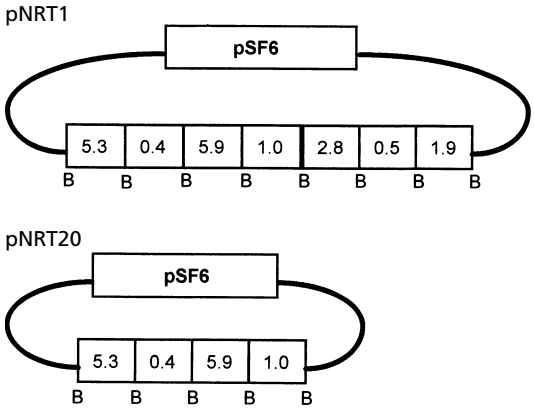

Fig. 1. A schematic map of *Bam*HI (B) restriction enzyme sites present in the *S. marcescens* cosmids pNRT1 and pNRT20. The size, in kb, of the individual fragments is indicated. Both cosmids, due to *Bam*HI/*Sau*3A ligation, have an indeterminate amount of *S. marcescens* DNA linked to the pSF6 vector DNA.

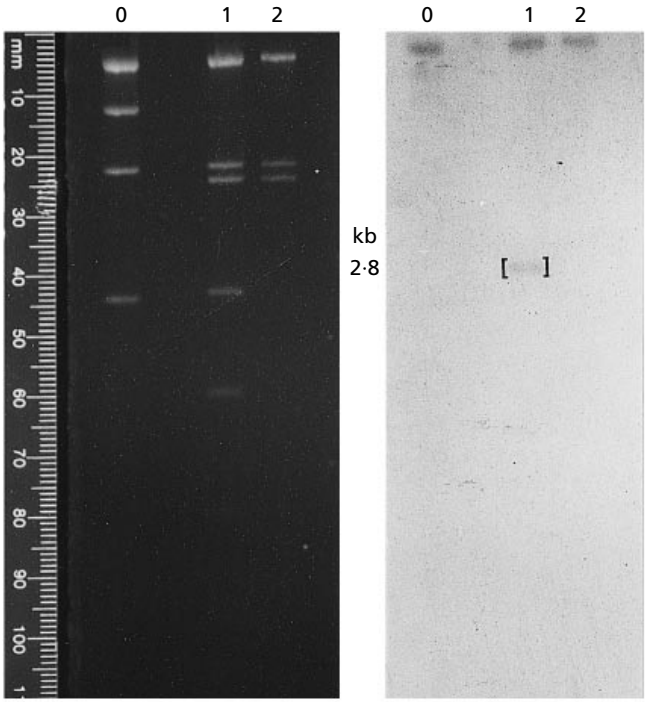

Fig. 2. A Southern blot, of *Bam*HI restriction digests, of the *S. marcescens* cosmids pNRT1 (lane 1) and pNRT20 (lane 2), and the *E. carotovora* cosmid cWU142 (lane 0), using the *E. carotovora carR* subclone pWU14203 as a probe. The non-vector homology exhibited by the 2·8 kb *Bam*HI fragment of pNRT1 is indicated.

RESULTS AND DISCUSSION

Complementation of the *E. carotovora* group I carbapenem mutants

The screening of 750 Sp^r colonies resulting from the transduction of the *S. marcescens* cosmid gene library into *E. carotovora* group I carbapenem mutants PNP1 and PNP20 revealed two cosmids, pNRT1 and pNRT20, capable of restoring antibiotic production in the respective mutants. A restriction map (Fig. 1) was con-

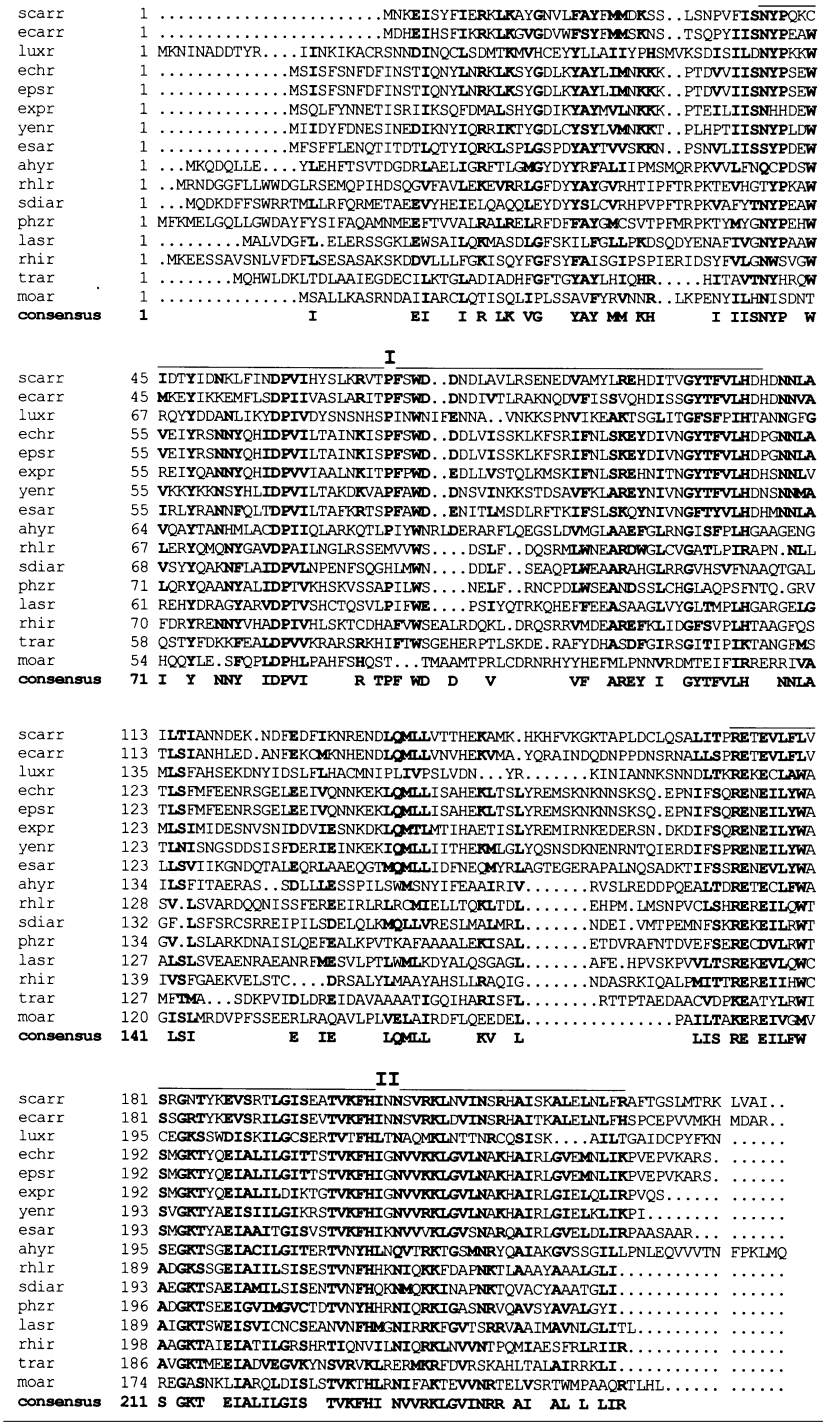

Fig. 3. Alignments of the predicted amino acid sequences of (S)CarR (*S. marcescens*; this work), (E)CarR (*E. carotovora*; McGowan *et al*., 1995; accession no. U17224), LuxR (*V. fischeri*; Gray & Greenberg, 1992; accession no. M96844), EchR (*E. chrysanthemi*; accession no. U45854), EspR (*P. solanacearum*; accession no. M61197), ExpR (*E. carotovora*; accession no. X80457), YenR (*Y. enterocolitica*; Throup *et al*., 1995; accession no. X76082), EsaR (*E. stewartii*; Beck von Bodman & Farrand, 1995; accession no. L32184), AhyR (*A. hydrophila*; accession no. X89469), RhlR (*P. aeruginosa*; Oschner *et al*., 1994; accession no. L08962), SdiA (*E. coli*; Wang *et al*., 1991; accession no. P07026), PhzR (*P. fluorescens*; Pierson *et al*., 1994; accession no. L32729), LasR (*P. aeruginosa*; Gambello & Iglewski, 1991; accession no. M59425), RhiR (*R. leguminosarum*; Cubo *et al*., 1992; accession no. M98835), TraR (*A. tumefaciens*; Zhang *et al*., 1993; accession no. P33909), MoaR (*E. aerogenes*; Azakami *et al*., 1993; accession no. D49928). Positions with eight or more similar residues have been highlighted and indicated as a consensus sequence. The proposed autoinducer binding region and the DNA binding region (regions I and II respectively) are indicated.

structed for both cosmids and showed that pNRT20 was effectively a subclone of pNRT1. Both cosmids were transferred into various chemically induced *E. carotovora* Car$^-$ mutants and were shown to complement both *carR* and putative *car* biosynthetic mutants. This suggested that both pNRT1 and pNRT20 might contain at least some of the *S. marcescens* genes necessary for carbapenem biosynthesis in addition to a homologue of the *E. carotovora carR* gene.

Subcloning and cross-hybridization of the pNRT1 and pNRT20 cosmids

Complementation of *E. carotovora* group I carbapenem mutants by the two *S. marcescens* cosmids pNRT1 and pNRT20 was identical to the complementation achieved with the *E. carotovora* cosmid cWU142, which was known to contain the *E. carotovora carR* gene and has recently been shown to contain the *E. carotovora car*

biosynthetic genes (McGowan *et al*., 1996). This suggested that the carbapenem genes of *E. carotovora* and *S. marcescens* might be functionally conserved and might also have significant sequence homology.

A Southern blot hybridization was carried out using the *E. carotovora carR* subclone pWU14203 as the probe (Fig. 2). Non-vector homology was observed in the 2·8 kb *Bam*HI fragment of pNRT1. Any similar homology in pNRT20 is masked by vector homology due to the attachment of this region of DNA to the vector in this cosmid.

This 2·8 kb *Bam*HI fragment of pNRT1 was subcloned back into the low-copy-number vector pSF6 to give pTON28 (Sp^r) and into the high-copy-number vector pDAH330 to give pTON28m (Cm^r). Both plasmids complemented all the *E. carotovora* CarR mutants (Table 2) but failed to complement the putative *E. carotovora* Car biosynthetic mutants.

Sequence analysis of the pTON28 subclone

Analysis of the sequence data revealed a complete ORF of 735 bp, with a potential ribosome-binding site and a putative start site for transcription with homology to the −10 and −35 consensus of the *Escherichia coli* promoter. This ORF showed significant DNA homology to the *Erwinia carotovora carR* gene (62·3%) and was predicted to encode a 28·216 kDa protein of 244 amino acids. The predicted protein had strong sequence identity with the predicted *E. carotovora* CarR protein (59·3%; similarity 72·0%) and was therefore also designated CarR. As with *E. carotovora* CarR, the *S. marcescens* CarR had significant similarity to the *Vibrio fischeri* transcriptional activator LuxR and other members of the LuxR family of transcriptional regulators (Fuqua *et al*., 1994) (Fig. 3). The sequence identity between the CarR proteins and these transcriptional regulators is particularly strong in the carboxy-terminal putative DNA-binding domain (Choi *et al*., 1991, 1992), consistent with the model that the CarR proteins act as DNA-binding proteins which are transcriptional regulators of the *car* biosynthetic genes. There is also strong identity in the putative autoinducer binding domain (Adar *et al*., 1992). This was expected in the *E. carotovora* CarR, as carbapenem production in *E. carotovora* is known to be cell-density-dependent and involves OHHL (Bainton *et al*., 1992b), but is perhaps more surprising in *S. marcescens* CarR, as carbapenem production in this strain of *S. marcescens* is independent of growth phase and does not appear to involve any diffusible pheromone molecule in this strain (unpublished data).

Identification of the *S. marcescens* CarR protein

Using [^{35}S]methionine labelling in the T7 expression system, a protein of approximately 28 kDa was identified (Fig. 4). This corresponds well to the size of 28·216 kDa predicted for the *S. marcescens* CarR from the sequence data.

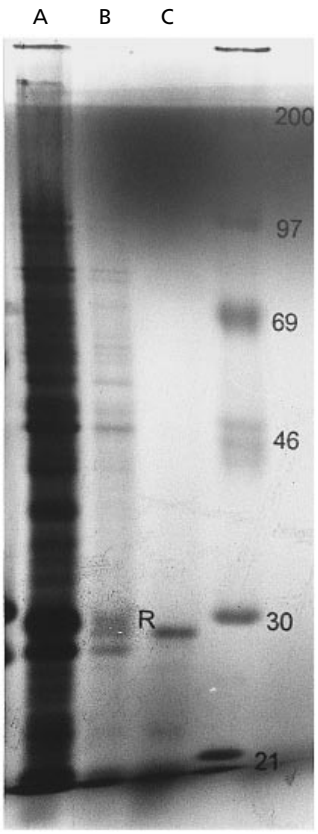

Fig. 4. Identification of CarR in a T7 expression system. A 3·4 kb fragment carrying *carR* was cloned into pT7-5 in the correct orientation for expression of *carR* (pT7-15). Lane A, 30 °C; lane B, 42 °C; lane C, 42 °C + rifampicin (final concn 200 μg ml^{-1}). Molecular size markers (kDa) are indicated in the right-hand lane. In the presence of rifampicin only one major protein of approximately 28 kDa was labelled (marked R in lane C).

Evidence of OHHL-independent function of the *S. marcescens* CarR

A number of *S. marcescens* carbapenem non-producing mutants were isolated by chemical mutagenesis of WT1 and WM5 Pig^- derivatives of the ATCC 39006 strain. All of these mutants were complemented for antibiotic production by the pNRT1 and pNRT20 cosmids (again suggesting that both these cosmids might contain some of the genes necessary for carbapenem production). The *carR* subclones pTON28 and pTON28m restored carbapenem production to two of these mutants (Table 2), CWT12 and CWM52, indicating that these were probably CarR mutants. Unlike *E. carotovora* (Bainton *et al*., 1992b) the *S. marcescens* Car^- mutants could not be divided into different cross-feeding groups and the addition of exogenous OHHL did not restore carbapenem activity or have any other observable phenotypic effect on any of the *S. marcescens* Car^- mutants.

Having previously been shown to complement the *E. carotovora* group 1 CarR mutants, the *S. marcescens carR* subclones were transferred into *E. carotovora* group 2 ($CarI^-$) mutants, PNP22 and PNP23 (Table 2),

Table 2. Complementation analysis of various *S. marcescens* and *E. carotovora* Car⁻ mutants by the *carR* genes of *S. marcescens* and *E. carotovora*

All strains were tested for the restoration of wild-type carbapenem production (+) as indicated by a 5–6 mm zone of clearing when spotted onto a lawn of super-sensitive *Escherichia coli*. Less than wild-type levels of carbapenem production are shown by halo size in parentheses.

Strain/mutant	Effect on carbapenem production of complementation with plasmids/ addition of exogenous OHHL				
	+OHHL	pWU14203	pWU14203+ OHHL	pTON28	pTON28m
PNP11	NT	+	NT	+	+
PNP14	NT	+	NT	+	+
PNP21	NT	−	NT	−	−
CWT11*	NT	NT	NT	−	−
CWT12	NT	NT	NT	+	+
CWT13*	NT	NT	NT	−	−
CWT14*	NT	NT	NT	−	−
CWT15*	NT	NT	NT	−	−
CWT18*	NT	NT	NT	−	−
CWT31*	NT	NT	NT	−	−
CWM52	NT	NT	NT	+	+
PNP22	+	−	NT	+(1–2 mm)	+(3–4 mm)
PNP23	+	−	NT	+(1–2 mm)	+(3–4 mm)
TCRI51	−	−	+	+	+

NT, Not tested.

*Putative Car biosynthetic mutant.

which do not produce OHHL and consequently have a pleiotropic phenotype which includes the inability to make carbapenem and the down-regulation of exoenzyme synthesis (Jones *et al*., 1993). The introduction of the *S. marcescens carR* subclones restored some antibiotic activity to these mutants. A low level of antibiotic activity was achieved with the low-copy-number subclone pTON28. The high-copy-number subclone pTON28m restored a higher level of antibiotic activity, but this was still less than in the wild-type strain. The effect of the *S. marcescens* CarR appears to be specific for carbapenem as neither subclone had any observable effect on exoenzyme synthesis (data not shown). It is possible that in the absence of OHHL the *E. carotovora* CarR may even partially inhibit transcription of the *E. carotovora* carbapenem genes. The *E. carotovora* group 2 mutants still have a wild-type copy of the *E. carotovora carR* gene (resulting in the production of *E. carotovora* CarR, the OHHL-dependent transcriptional activator of the *E. carotovora* carbapenem genes; McGowan *et al*., 1995). Therefore, it was reasoned that the copy-number-dependent complementation observed with the *S. marcescens carR* in *E. carotovora* group 2 mutants, in the absence of OHHL, might be explained by competition between the OHHL-dependent *E. carotovora* CarR and the OHHL-independent *S. marcescens* CarR for a DNA-binding site. To test this hypothesis an *E. carotovora carR carI* double mutant, TCRI51, was constructed. The *E. carotovora carR* subclone pWU14203 was transferred into this *carR carI* double mutant (Table 2). Carbapenem activity was not restored until exogenous OHHL was also added. When the low-copy-number *S. marcescens carR* subclone pTON28 was transferred into this *carR carI* double mutant (Table 2) carbapenem activity was restored to wild-type levels without the need for the addition of OHHL. It was also noted that carbapenem production was detectable at an earlier stage in the growth curve in the recombinant strain than in the wild-type *E. carotovora* strain (data not shown). The *S. marcescens* CarR appears to be capable of acting as a functional replacement for the mutant *E. carotovora* CarR without the need for OHHL. The wild-type levels of carbapenem activity brought about by the low-copy *S. marcescens carR* subclone pTON28 in the *E. carotovora carR carI* double mutant (compared with the lower, copy-number-dependent levels obtained in the *E. carotovora carI* mutants) support the idea of some interaction/competition between the OHHL-dependent *E. carotovora* CarR and the OHHL-independent *S. marcescens* CarR in an *E. carotovora* background (possibly forming mixed dimers/multimers or competing for the same target site).

Because there are multiple amino acid differences between the *E. carotovora* and *S. marcescens* CarR proteins, and as both exhibit strong amino acid identity in the putative autoinducer binding domain (Adar *et al*.,

1992), it is not at present obvious what the molecular basis for OHHL-independence is in the *S. marcescens* CarR. Perhaps mixing and matching of domains to form chimeric CarR proteins may allow the identification of the key residues involved in the release of CarR protein function from pheromone dependence.

ACKNOWLEDGEMENTS

This work was supported by the BBSRC. We thank D. Hodgson for plasmid pDAH330.

REFERENCES

Adar, Y. Y., Simaan, M. & Ulitzur, S. (1992). Formation of the LuxR protein in the *Vibrio fischeri lux* system is controlled by the HtpR through the GroESL proteins. *J Bacteriol* **174**, 7138–7143.

Azakami, H., Sugino, H., Iwata, N., Yamashita, M. & Murooka, Y. (1993). *moaR*, a gene that encodes a positive regulator of the monoamine regulon in *Klebsiella aerogenes*. *J Bacteriol* **175**, 6287–6292.

Bainton, N. J. & ten others (1992a). A general role for the *lux* autoinducer in bacterial cell signalling: control of antibiotic biosynthesis in *Erwinia*. *Gene* **116**, 87–91.

Bainton, N. J., Stead, P., Chhabra, S. R., Bycroft, B. W., Salmond, G. P. C., Stewart, G. S. A. B. & Williams, P. (1992b). *N*-(3-Oxohexanoyl)-L-homoserine lactone regulates carbapenem antibiotic production in *Erwinia carotovora*. *Biochem J* **288**, 997–1004.

Bankier, A. T., Weston, K. M. & Barrell, B. G. (1986). *Random Cloning and Sequencing by the M13/Dideoxynucleotide Chain Termination Method*. Cambridge: MRC Laboratory of Molecular Biology.

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.

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

Choi, S. H. & Greenberg, E. P. (1991). The C-terminal region of the *Vibrio fischeri* LuxR protein contains an inducer-independent *lux* gene activating domain. *Proc Natl Acad Sci USA* **88**, 11115–11119.

Choi, S. H. & Greenberg, E. P. (1992). Genetic dissection of DNA binding and luminescence gene activation by the *Vibrio fischeri* LuxR protein. *J Bacteriol* **174**, 4064–4069.

Cubo, M. T., Economou, A., Murphy, G., Johnston, A. W. B. & Downie, J. A. (1992). Molecular characterisation and regulation of the rhizosphere-expressed genes *rhiABCR* that can influence nodulation by *Rhizobium leguminosarum* biovar *viciae*. *J Bacteriol* **174**, 4026–4035.

Ellard, F. M., Cabello, A. & Salmond, G. P. C. (1989). Bacteriophage lambda-mediated transposon mutagenesis of phytopathogenic and epiphytic *Erwinia* species is strain dependent. *Mol Gen Genet* **218**, 491–498.

Forbes, K. J. & Perombelon, M. C. M. (1985). Chromosomal mapping of *Erwinia carotovora* subspecies *carotovora* with the IncP plasmid R68::Mu. *J Bacteriol* **164**, 1110–1116.

Fuqua, W. C., Winans, S. C. & Greenberg, E. P. (1994). Quorum sensing in bacteria: the LuxR–LuxI family of cell density-responsive transcriptional regulators. *J Bacteriol* **176**, 269–275.

Gambello, M. J. & Iglewski, B. H. (1991). Cloning and characterisation of the *Pseudomonas aeruginosa las*R gene, a transcriptional activator of elastase expression. *J Bacteriol* **173**, 3000–3009.

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

Hopwood, D. A., Chater, K. F. & Bibb, M. J. (1995). Genetics of antibiotic production in *Streptomyces coelicolor* A3(2), a model streptomycete. *Biotechnology* **28**, 65–102.

Gray, K. M. & Greenberg, E. P. (1992). Sequencing and analysis of *luxR* and *luxI*, the luminescence regulatory genes from the squid light organ symbiont *Vibrio fischeri* ES114. *Mol Marine Biol Biotechnol* **1**, 414–419.

Imada, A., Kitato, K., Kintaka, K., Muroi, M. & Asai, M. (1981). Sulphacecin and isosulphazecin, novel β-lactam antibiotics of bacterial origin. *Nature* **289**, 590–591.

Jones, S. & 12 others (1993). The *lux* autoinducer regulates the production of exoenzyme virulence determinants in *Erwinia carotovora* and *Pseudomanas aeruginosa*. *EMBO J* **12**, 2477–2482.

Kahan, F. M., Kropp, H., Sundelof, J. G. & Birnbaum, J. (1983). Theinamycin: development of imipenem-cilastatin. *J Antimicrob Chemother* **12**, 1–35.

Krebs, M. P. & Reznikoff, W. S. (1988). Use of a Tn5 derivative that creates *lacZ* translational fusions to obtain a transposition mutant. *Gene* **63**, 277–285.

Kropp, H., Sundelof, J. G., Kahan, J. S., Kahan, F. M. & Birnbaum, J. (1980). MK0787 (*N*-formimidoyl thienamycin): evaluation of *in vitro* and *in vivo* activities. *Antimicrob Agents Chemother* **17**, 993–1000.

Kropp, H., Gerkens, L., Sundelof, J. G. & Kahan, F. M. (1985). Antibacterial activity of imipenem: the first thienamycin antibiotic. *Rev Infect Diseases* **7**, 389–410.

Labia, R., Morand, A. & Guionie, M. (1986). β-Lactamase stability of imipenem. *J Antimicrob Chemother* **18**, 1–8.

McGowan, S. & nine others (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., Bycroft, B. & Salmond, G. P. C. (1996). Analysis of bacterial carbapenem antibiotic production genes reveals a novel β-lactam biosynthesis pathway. *Mol Microbiol* **22**, 415–426.

Messing, J., Gronenborn, B., Muller-Hill, B. & Hofschneider, P. (1977). Filamentous coliphage M13 as a cloning vehicle: insertion of a *Hin*dII fragment of the *lac* regulatory region in M13 replicative form *in vitro*. *Proc Natl Acad Sci USA* **74**, 3642–3646.

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

Morrison, D. A. (1966). Prodigiosin synthesis in mutants of *Serratia marcescens*. *J Bacteriol* **91**, 1599–1603.

Mulholland, V. & Salmond, G. P. C. (1995). Use of coliphage λ and other bacteriophages for molecular genetic analysis of *Erwinia* and related Gram-negative bacteria. *Methods Mol Genet* **6**, 439–454.

Oschner, U. L., Koch, A., Fiechter, A. & Reiser, J. (1994). Isolation and characterisation 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., Jr, Trejo, W. H., Principe, P. A. & Sykes, R. B. (1982). SQ 27860, 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.

Pearson, J. P., Gray, K. M., Passador, L., Tucker, K. D., Eberhard, A., Iglewski, B. H. & Greenberg, E. P. (1994). Structure of the autoinducer required for expression of *Pseudomonas aeruginosa* virulence genes. *Proc Natl Acad Sci USA* **91**, 197–201.

Pierson, L. S., III, Keppenne, V. D. & Wood, D. W. (1994). Phenazine antibiotic biosynthesis in *Pseudomonas aeruginosa* 30–84 is regulated by PhzR in response to cell density. *J Bacteriol* **176**, 3966–3974.

Piper, K. R., Beck von Bodman, S. & Farrand, S. K. (1993). Conjugation factor of *Agrobacterium tumefaciens* regulates Ti plasmid transfer by autoinduction. *Nature* **362**, 448–450.

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

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

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.

Sykes, R. B. & 12 others (1981). Monocyclic β-lactam antibiotics produced by bacteria. *Nature* **291**, 489–491.

Tabor, S. & Richardson, C. C. (1985). A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. *Proc Natl Acad Sci USA* **82**, 1074–1078.

Throup, J. P., Camara, M., Briggs, G. S., Winson, M. K., Chhabra, 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 2 *N*-acylhomoserine lactone signal molecules. *Mol Microbiol* **17**, 345–356.

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

Tsao, S. W., Rudd, B. A. M., He, X. G., Chang, C. & Floss, H. G. (1985). Identification of a red pigment from *Streptomyces coelicolor* A3(2) as a mixture of prodigiosin derivatives. *J Antibiotics* **38**, 128–131.

Vollenweider, H. J., Fiandt, M., Rosenvold, E. C. & Szybalski, W. (1980). Packaging of plasmid DNA containing the cohesive ends of coliphage lambda. *Gene* **9**, 171–174.

Wang, X., de Boer, P. A. J. & Rothfield, L. I. (1991). A factor that positively regulates cell division by activating transcription of the major cluster of essential cell division genes of *E. coli*. *EMBO J* **10**, 3363–3372.

White, F. F., Klee, H. J. & Nester, E. W. (1983). *In vivo* packaging of cosmids in transposon mediated mutagenesis. *J Bacteriol* **153**, 1075–1078.

Williamson, J. M. (1986). The biosynthesis of theinomycin and related carbapenems. *CRC Crit Rev Biotechnol* **4**, 111–131.

Wilmes-Riesenberg, M. R. & Wanner, B. L. (1992). Tn*pho*A and Tn*pho*A′ elements for making and switching fusions for study of transcription , translation and cell surface localisation. *J Bacteriol* **174**, 4558–4575.

Wood, D. W. & Peirson, L. S., III (1996). The *phzI* gene of *Pseudomonas aureofaciens* 30–84 is responsible for the production of a diffusible signal required for phenazine antibiotic production. *Gene* **168**, 49–53.

Zhang, L., Murphy, P. J., Kerr, A. & Tate, M. E. (1993). *Agrobacterium* conjugation and gene regulation by *N*-acyl-L-homoserine lactones. *Nature* **362**, 446–448.

Received 25 April 1997; revised 5 September 1997; accepted 15 September 1997.