Carbapenem antibiotic production in *Erwinia carotovora* is regulated by CarR, a homologue of the LuxR transcriptional activator

S. McGowan,¹ M. Sebahia,¹ S. Jones,¹ B. Yu,² N. Bainton,² P. F. Chan,² B. Bycroft,² G. S. A. B. Stewart,³ P. Williams² and G. P. C. Salmond¹

Author for correspondence: G. Salmond. Tel: +44 1203 523534. Fax: +44 1203 523701.
e-mail: GP @dna.bio.warwick.ac.uk

¹ Department of Biological Sciences, University of Warwick, Coventry CV4 7AL, UK

^{2,3} Department of Pharmaceutical Sciences² and Department of Applied Biochemistry and Food Science³, University of Nottingham, Nottingham, UK

Strain GS101 of *Erwinia carotovora* makes the carbapenem antibiotic, 1-carbapen-2-em-3-carboxylic acid. Mutants defective in antibiotic production can be assigned to two groups, group 1 and group 2. Group 2 mutants are defective in the *carI* gene encoding a protein responsible for synthesis of the Lux autoinducer *N*-(3-oxohexanoyl)-L-homoserine lactone (OHHL), which is required to induce carbapenem synthesis in strain GS101. In this paper we describe the molecular genetic analysis of the group 1 mutants which we presumed were defective in the carbapenem biosynthesis (*car*) genes. We isolated a cosmid (cWU142) that complemented the group 1 mutants of strain GS101. A small (1.03 kb) subclone of cWU142 complemented most of the group 1 mutants, and the sequence revealed that the relevant gene (*carR*) encodes a homologue of the *Vibrio fischeri* LuxR protein. A disproportionately high frequency of *carR* mutants arose in strain GS101 and this was due to *carR* acting as a 'hot spot' target for secondary transposition of a Tn5 element in this strain. The CarR protein joins a rapidly growing list of homologues, found in taxonomically unrelated bacteria, which act as positive transcriptional activators of genes encoding diverse metabolic functions, including bioluminescence, exoenzyme virulence factor synthesis, cell division, plasmid conjugation, rhizosphere-specific gene induction, surfactant synthesis and antibiotic production. Most of these LuxR-type regulators have been shown to depend, for their function, on *N*-acyl homoserine lactones, which act as chemical signals enabling co-ordination of gene expression with cell density.

Keywords: *Erwinia carotovora*, carbapenem, β -lactam antibiotic, *N*-acyl homoserine lactone, LuxR homologue

INTRODUCTION

Carbapenem antibiotics are broad-spectrum β -lactams which tend to be resistant to the clinically encountered β -lactamases and are effective against both aerobes and anaerobes, including nosocomial pathogens (Bycroft, 1988). Because of this current, and potential, clinical utility of carbapenems, and despite the very recent emergence of carbapenemase-producing clinical isolates

(Livermore, 1993; Naas & Nordmann, 1994), there has been a growing interest in the mechanisms of biosynthesis and regulation of such extended-spectrum β -lactams in the producer organisms. Some Gram-positive bacteria, such as *Streptomyces cattleya* and *Streptomyces penemifaciens*, make structurally complex carbapenems, like thienamycin, by a biosynthetic route which is thought to be distinct from that used for the penicillins and cephalosporins (Williamson *et al.*, 1985). However, although there has been some progress in the analysis of synthesis and regulation of thienamycin and some other carbapenems (e.g. Kitano *et al.*, 1985; Williamson *et al.*, 1985; Chen *et al.*, 1993) molecular genetic studies of carbapenem production have been slow because of the relatively poor genetic tractability and slow growth of the producer organisms.

Abbreviations: Car, 1-carbapen-2-em-3-carboxylic acid; OHHL, *N*-(3-oxohexanoyl)-L-homoserine lactone.

The GenBank accession number for the DNA sequence data reported in this paper is U17224.

Some strains of the Gram-negative bacteria *Erwinia* and *Serratia* make the simple carbapenem antibiotic 1-carbapen-2-em-3-carboxylic acid (Car; Parker *et al.*, 1982; Bycroft *et al.*, 1987, 1988) and we have initiated a molecular genetic study of carbapenem production in these genetically amenable, fast-growing strains. Previously we isolated carbapenem non-producing mutants (Car⁻) of *Erwinia carotovora* strain GS101 after chemical and transposon mutagenesis (Bainton *et al.*, 1992). These mutants defined two groups on the basis of 'crossfeeding' results in co-cultivation tests. Group 1 mutants were able to crossfeed group 2 mutants and restore Car production in the latter. This crossfeeding was not due to an intermediate in the carbapenem biosynthetic pathway but to a small, freely diffusible signalling molecule, *N*-(3-oxohexanoyl)-L-homoserine lactone (OHHL), which is synthesized in a growth-phase-dependent/cell-density-dependent mode (Bainton *et al.*, 1992; Williams *et al.*, 1992; Swift *et al.*, 1994). OHHL is also the Lux autoinducer of *Vibrio fischeri* and controls cell-density-dependent bioluminescence in that marine bacterium (e.g. see Meighen, 1991).

Recently it has been demonstrated that production of OHHL and structurally related *N*-acyl homoserine lactones is widespread in bacteria (Bainton *et al.*, 1992; Williams *et al.*, 1992). These discoveries suggested that *N*-acyl homoserine lactones might act to regulate diverse cell-density-dependent physiological processes in different bacteria and recent results are consistent with this hypothesis. *N*-Acyl homoserine lactones regulate bioluminescence (*V. fischeri* and *Vibrio harveyi*), carbapenem synthesis (*E. carotovora*), elastase production (*Pseudomonas aeruginosa*), Ti plasmid conjugal transfer (*Agrobacterium*) and exoenzyme virulence factor synthesis (*E. carotovora*) (Eberhard *et al.*, 1981; Cao & Meighen, 1989; Bainton *et al.*, 1992; Passador *et al.*, 1993; Zhang *et al.*, 1993; Piper *et al.*, 1993; Jones *et al.*, 1993; Pirhonen *et al.*, 1993). For all of these bacterial hosts, except *V. harveyi*, production of the *N*-acyl homoserine lactone is mediated by a homologue of the *V. fischeri* LuxI protein (e.g. see Fuqua *et al.*, 1994; Swift *et al.*, 1994). In the carbapenem-producing *E. carotovora* strain GS101, the LuxI homologue is CarI (Swift *et al.*, 1993).

In *carI* mutants (group 2) of strain GS101, carbapenem is not produced, but, in contrast to group 1 mutants, these mutants also show co-ordinate (global) defects in the production of multiple exoenzyme (pectinases, cellulase and proteases) virulence factors (the Rex⁻ phenotype; Regulation of exoenzymes; Jones *et al.*, 1993). Exogenous addition of OHHL simultaneously restores synthesis of the antibiotic (Car⁺) and the exoenzymes (Rex⁺) in the group 2 mutants confirming that OHHL acts as the global regulatory molecule for multiple, and physiologically diverse, phenotypes in this strain (Jones *et al.*, 1993).

In strain SCRI193 of *E. carotovora* (which does not make an antibiotic) global regulatory Rex⁻ mutants were also isolated. Some of these are phenotypically similar to the group 2 mutants of strain GS101 in that OHHL restored exoenzyme synthesis in them (Jones *et al.*, 1993).

When we originally investigated Car⁻ mutants of strain GS101 we presumed that the group 1 mutants (Car⁻ Rex⁺) would be defective in the biosynthetic genes for antibiotic production. However, in this report we describe the molecular analysis of group 1 Car⁻ mutants and show that most of these mutants are, in fact, defective in a gene (*carR*) that encodes a homologue of the *V. fischeri* LuxR protein, a positive transcriptional activator protein.

METHODS

Bacterial strains and media. Strains used in this study were: *Escherichia coli* DH1 (Hanahan, 1983); *E. coli* ESS (a β -lactam supersensitive indicator strain provided by Beecham Pharmaceuticals); *Erwinia carotovora* subsp. *carotovora* strains SCRI193 (Hinton & Salmond, 1987) and GS101, a restrictionless and modificationless Tn5 insertion mutant derived from ATCC-39048 (Bainton *et al.*, 1992). Isolation of EMS-induced pleiotropic exoenzyme deficient (Rex⁻) mutants of SCRI193 (RJP116 and RJP223) has been described before (Jones *et al.*, 1993). The isolation of chemical and transposon-induced Car⁻ mutants of GS101 (Table 1) has been described previously (Bainton *et al.*, 1992). *Erwinia* and *E. coli* strains were routinely grown at 30 °C and 37 °C, respectively, in LB (Miller, 1972). For some exoenzyme assays, cultures of *Erwinia* were sometimes grown in media supplemented with OHHL (final concentration 1 μ g ml⁻¹).

Isolation of a group 1 cosmid by direct, λ -mediated cosmid complementation. Chromosomal DNA of the wild-type strain GS101 was prepared and partially digested with *Sau*III, size fractionated, then ligated with *Bam*HI-digested cosmid (pSF6; Selvaraj *et al.*, 1984) DNA. 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 mutant PNP14 carrying the *LamB* plasmid pTroy9 (see Ellard *et al.*, 1989) and cosmid-containing transductants were selected on LB agar plates containing spectinomycin at 50 μ g ml⁻¹. The transductants were screened for restoration of carbapenem production using the halo bioassay test with *E. coli* ESS as described by Bainton *et al.* (1992). The complementing cosmid (cWU142) from one such Car⁺ transductant was isolated and used to transform *E. coli* DH1 by electroporation. A high titre λ cI857 lysate was raised on the transformant, by the method of White *et al.* (1983), to efficiently package cWU142 and generate a high frequency transducing lysate for this cosmid. That lysate was used to transduce various *LamB*-containing Car⁻ mutants of *E. carotovora* with cWU142, using spectinomycin resistance (Sp^r) as the selectable marker. The general aspects of this *LamB*-based strategy are described elsewhere (Mulholland & Salmond, 1995).

Subcloning and sequence analysis. Cosmid cWU142 was digested with *Eco*RI and released multiple fragments which were cloned back into pSF6 digested with the same enzyme. The resulting subclones were used to transform various Car⁻ mutants of GS101, selecting for spectinomycin resistance. One plasmid generated by this shotgun method (pWU14203) complemented most group 1 mutants. The insert from this subclone was sequenced using the dideoxynucleotide chain-termination procedure (Sanger *et al.*, 1977) and a 'Sequenase kit' (USB). DNA was prepared for sequencing by sonication and ligation of the resulting fragments into M13mp18. Sequence analysis of the *carR* region of Car⁻ mutant strains was done by cycle sequencing of PCR-amplified chromosomal DNA. PCR amplification by *Taq* DNA polymerase (Promega) was carried

out using synthetic oligonucleotides complementary to sequences 5' and 3' of the *carR* gene, SMCAR1 (5'-GGAATTCG-GTATTATGGTTTGG-3') and SMCAR2 (5'-CGTTTGAA-TTCGTCACATATC-3'), or using one of these in combination with an oligonucleotide complementary to Tn5, TN5PR2 (5'-GGGAATTCGGAAGTCAGATCCTGG-3'). Amplification conditions were: 92 °C for 6 min; 40 °C, 2 min; 72 °C, 2 min; followed by 25 cycles of 92 °C, 1 min; 40 °C, 2 min; 72 °C, 2 min. The amplified DNA was prepared for cycle sequencing using a 'Geneclean kit' (Strattech). Cycle sequencing was done using a 'A-Taq cycle sequencing kit' (USB) and one of the above oligonucleotides. Analysis of the *carR* region of mutant PNP21 by this method enabled us to add a further 39 bp of DNA sequence data to the data already generated from pWU14203. Further subcloning of pWU14203 was done based on the sequence information gathered. A 1.03 kb *SspI* fragment from pWU14203 was cloned in pDAH330 (a chloramphenicol resistant derivative of pIC19R; D. Hodgson, personal communication) digested with *EcoRV*, to yield plasmid pSMG4. The latter was used to transform Car⁻ strains before screening for complementation.

Gene product identification. A 1.03 kb *SspI* fragment from pWU14203 was cloned in both orientations into pT7-5 (Tabor & Richardson, 1985) digested with *EcoRV*. The resulting plasmids (pSMG5 and pSMG8) were used to express the *carR* gene by the methods described by Tabor & Richardson (1985). Protein products were separated by SDS-PAGE and visualized after autoradiography.

Multicopy effects of *carR* on exoenzyme synthesis. Plasmids pWU14203 and pSMG4 and the corresponding unmodified vectors (pSF6 and pDAH330, respectively) were used to transform the wild-type strain *E. carotovora* SCRI193 and various Rex⁻ mutants described previously (Jones *et al.*, 1993). Protease, pectate lyase and cellulase production by these strains was then assayed (Reeves *et al.*, 1993) in the presence or absence of exogenously added OHHL.

RESULTS

Complementation of the group 1 carbapenem mutants

Table 1 shows a list of the Car⁻ mutants assigned to group 1 or group 2 on the basis of their crossfeeding phenotype in the carbapenem bioassay. Group 2 mutants are regulatory mutants (*carI*) that are defective in OHHL synthesis and production of Car is restored by the addition of chemically synthesized OHHL (Bainton *et al.*, 1992; Jones *et al.*, 1993). In contrast, group 1 mutants still make OHHL and Car production is not restored by exogenously supplied OHHL. Because group 1 mutants make OHHL, and, importantly, because there were significantly more group 1 mutants than group 2 (regulatory, *carI*) mutants, we presumed that the former would be defective in the biosynthetic genes for carbapenem synthesis. We therefore attempted to clone these biosynthetic genes by direct cosmid complementation of group 1 mutants.

Of 350 Sp^r cosmid transductants of the group 1 Car⁻ mutant PNP14, one had antibiotic production restored. The complementing cosmid (cWU142) was transferred into various chemically induced Car⁻ mutants and only complemented group 1 mutants (Table 1). Complementation of the *TnblaM*-induced Car⁻ mutants could not

Table 1. Construction and complementation analysis of various mutants of *E. carotovora* strain GS101

All strains were tested for the restoration of carbapenem production (+) as indicated by a zone of clearing when spotted onto a lawn of supersensitive *E. coli*. ±, only a very small halo present; NT, not tested – both complementing plasmid and mutant strain have the same antibiotic resistance marker.

Mutant	Mutagen	Group	Complementing plasmids:		
			cWU142	pWU14203	pSMG4
PNP1	NTG	1	+	+	+
PNP10	NTG	1	+	+	+
PNP14	NTG	1	+	+	+
PNP19	NTG	1	+	+	+
PNP20	NTG	1	+	+	+
PNP21	EMS	1	+	—	—
PNP22	EMS	2	—	—	±
PNP23	EMS	2	—	—	±
PNP26	EMS	1	+	+	+
MB1	<i>TnblaM</i>	2	NT	NT	±
MB2	<i>TnblaM</i>	2	NT	NT	±
MB3	<i>TnblaM</i>	1	NT	NT	—
GS2	<i>TnblaM</i>	1	NT	NT	—
JM1	<i>TnblaM</i>	1	NT	NT	—
JMJ2	<i>TnblaM</i>	1	NT	NT	+
JMJ3	<i>TnblaM</i>	1	NT	NT	—
JMJ4	<i>TnblaM</i>	1	NT	NT	—
JMJ5	<i>TnblaM</i>	1	NT	NT	+
JMJ6	<i>TnblaM</i>	1	NT	NT	—
JMJ7	<i>TnblaM</i>	2	NT	NT	±

be tested with the cWU142 cosmid because both carried the Sp^r marker.

Subcloning of the cWU142 cosmid and sequence analysis

The cWU142 cosmid contained approximately 35 kb chromosomal DNA (data not shown). An *EcoRI* fragment of approximately 3.6 kb from cWU142 restored Car production in all of the chemically induced group 1 mutants, except mutant PNP21 (Fig. 1, Table 1). The resultant plasmid was called pWU14203 and, because it complemented six independently isolated group 1 mutants, we presumed that this carried most of the biosynthetic genes (except the one represented by the PNP21 mutation). The 3.6 kb insert fragment was sequenced and DNA analysis showed that approximately 2 kb of the fragment was derived from the original vector pSF6, and only approximately 1.6 kb was *Erwinia* chromosomal DNA (Figs 1 and 2). Within the 1.6 kb, the major open reading frame (735 bp) encoded a 28136 Da protein of 244 amino acids. This predicted protein showed low, but significant (26.4%) sequence identity to the LuxR regulatory protein of *V. fischeri* and so was called CarR (Fig. 3). CarR becomes a member of the LuxR family, some of which have been shown to be positive

Fig. 1. Subcloning and localization of the *carR* gene. The complementing cosmid, cWU142, was digested with *EcoRI*, and individual fragments cloned back into pSF6 digested with the same enzyme. One of the resulting plasmids, pWU14203 containing approximately 3.6 kb of insert DNA, was found to complement all but one of the *Erwinia* mutants. The insert of this plasmid was sequenced completely and found to contain one major open reading frame, *carR*. A 1.03 kb *SspI* fragment was then cloned into pDAH330 digested with the same enzyme, to give plasmid pSMG4.

transcriptional activators which are involved in the regulation of diverse physiological processes in various taxonomically unrelated bacteria (Fig. 3).

Identification of the CarR protein

To confirm that the *carR* gene alone was responsible for complementation of the group 1 mutants, and for the purposes of gene product identification, part of the 1.6 kb fragment of pWU14203 was subcloned into plasmid pDAH330 to generate pSMG4 (Fig. 1). The resultant plasmid was used to transform group 1 mutants and transformants were tested for complementation of the Car phenotype. The results confirmed that only *carR* was required for complementation *in trans* (Table 1). The 1.03 kb *SspI* fragment and others were cloned into a T7 expression system and used to identify the CarR product as a 28 kDa protein, in agreement with the predicted size from sequence data (Fig. 4).

The results above confirmed that the six group 1 putative Car biosynthetic mutants induced by chemical muta-

genesis were in fact regulatory mutants defective in the *carR* gene. We also tested for complementation of the TnblaM-induced Car⁻ mutants after transformation with plasmid pSMG4. As shown in Table 1, the plasmid complemented only a subset (two/eight) of the mutants that had been assigned to group 1 by crossfeeding analysis. This suggested that these complemented TnblaM-induced mutants also had insertions in the *carR* gene and confirmed that the group 1 mutants were divisible into *carR* regulatory mutants and putative *car* biosynthetic mutants. The *carR* and *carI* regulatory genes (Swift *et al.*, 1993) lie on different cosmids and so are genetically unlinked.

Molecular characterization of the *carR* mutations

We attempted to isolate the chemically induced *carR* mutations from the group 1 mutants by PCR amplification using primers which annealed to sequences upstream and downstream of the *carR* gene (Fig. 2). Curiously, we were unable to amplify any *carR* DNA from the chemically induced *carR* mutants, yet could amplify the correctly sized fragment (approximately 1.1 kb) from the wild-type chromosome, from group 2 Car⁻ (*carI*) mutants and from group 1 mutant PNP21 (which is not defective in *carR*) (data not shown). As the TnblaM-induced *carR* mutations also failed to amplify in the PCR reactions, we considered the possibility that the *carR* mutants isolated after chemical mutagenesis were actually due to large deletions or insertions, perhaps from a mobile genetic element.

All of the chemically induced Car⁻ mutants were derived from strain GS101. This strain is a restrictionless and modificationless derivative of the progenitor ATCC39048 and was generated by random Tn5 mutagenesis (Bainton *et al.*, 1992). Because GS101 carries a Tn5 insertion we considered the possibility that the Car⁻ mutants may have arisen due to secondary transposition of Tn5 into the *carR* gene. To test this hypothesis we attempted PCR-based amplification of *carR* sequences using a *carR* primer and a primer which hybridized to the terminus of Tn5 (see Methods). As predicted, using these primers we were able to amplify discrete fragments of *carR* from the various *carR* mutants. The sites of Tn5 insertion were confirmed by sequence analysis across each insertion point and are shown in Fig. 2. Interestingly, five of these Tn5 insertions (in the independently isolated mutants PNP1, 10, 14, 19 and 20) are at exactly the same point (after the DNA encoding codon Asn₁₆₆), with another (in mutant PNP26) 33 bases upstream of this common site. Thus, although the mutants carrying these Tn5 insertions had been independently isolated after EMS and NTG mutagenesis, they were due, in fact, to Tn5 'hot spotting' into *carR* by secondary transposition. Probing *EcoRI*-digested chromosomal DNA from GS101, group 1 and group 2 Car⁻ strains for sequence homology to Tn5 showed that there were multiple (two to four) copies of Tn5 inserted in their respective chromosomes (data not presented), revealing a marked proclivity towards secondary hopping of the transposon in GS101 and its derivatives. However, neither of the two chemically induced group 2 (*carI*) mutants had Tn5 insertions within *carI* because they

Fig. 2. Nucleotide sequence of the *carR* region of *E. carotovora* strain GS101. The sequence was generated by DNA sequence analysis of pWU14203 (to the *EcoRI* site at position 1554) and extended by cycle sequencing of a PCR-amplified portion of the chromosome of the mutant PNP21. The deduced amino acid sequence (single letter code) of the major open reading frame *carR* is shown below the nucleotide sequence. The positions of insertion of various transposons (as shown by an inverted triangle) have been indicated by convention upstream of the 9 bp repeated sequence generated upon insertion (underlined). The positions of two oligonucleotides, SMCAR1 and SMCAR2, have also been indicated. A pair of direct repeats and a pair of inverted repeats are located immediately downstream of *carR* (1 and 2, respectively).

produced the correct sized fragment by PCR analysis (data not presented). Thus, although the 'target' sizes of *carI* and *carR* are similar (651 and 734 bp, respectively), and the phenotype scored in the bioassay screen, following mutagenesis was identical (*Car*⁻), there was a disproportionate number of *carR* mutations isolated which were due to Tn5 secondary hops.

The group 1 *Car*⁻ mutant PNP21 was complemented by cWU142, but not by pSMG4, and therefore was not a *carR* mutant. However, PCR amplification of the PNP21 chromosomal DNA with the 5' *carR* primer and a Tn5 terminal primer did yield a product. On sequencing, the position of the Tn5 insertion in mutant PNP21 was shown to be in the region 3' of the *carR* termination codon and approximately 70 bases downstream of the *SspI* site (Fig. 2). Computer translation of the DNA sequence around this region did not reveal any candidate translational start sites or obvious genes, so it is unclear

whether or not this insertion lies within a *car* biosynthetic gene.

Two *TnblaM*-induced mutants (JMJ2 and JMJ5) were complemented by pSMG4. Cycle sequencing of PCR-amplified chromosomal DNA revealed the JMJ5 insertion to be within the putative ribosome-binding site, 5' of *carR*, and the JMJ2 insertion to be after the DNA encoding Leu₆₄. Interestingly, although six of the seven Tn5 secondary transposition-induced group 1 mutants were in *carR*, only two of the eight group 1 *TnblaM*-induced mutants were defective in *carR*. This apparently more random transposition of the *TnblaM* element could be an intrinsic product of its structure. However, as *TnblaM* is a derivative of Tn5 (Tadayyon & Broome-Smith, 1992) it seems unlikely that it would transpose preferentially to different sites from the latter. Alternatively, because the *TnblaM* insertions are due to λ -mediated 'superinfection' of the transposon into the

	1		50
CarR (E. carotovora)		..MDHEINS	FIKRLKGVG DWVFSYFMS
EsaR (E. stewartii)		..MFSFLE	NGTITDITQT YIORKLSPLG SPDYATYVVS
TraR (A. vitis)		..MQMLDKLT	DLTAIEGDGC ILKTLGLADVA .DHFGTGYA
TraR (A. tumefaciens)		..MQMLDKLT	DLAAIEGDEC ILKTLGLADIA .DHFGTGYA
LasR (P. aeruginosa)		..MA	LVDGFL.ELE RSSGKLEUSA ILQOMASDLG SHKILFGLLP
SdiA (E. coli)		..MQDDFFS	WRRITLLRFO RMETAEVYH EIELQAQGLE YDYTSLCVRH
PhzR (P. aureofaciens)		..MELGQGLG	WDAYFYSAF RTMDQGEFTA VALRALRELR DDFRYGMS
RhlR (P. aeruginosa)		..MRNDGGFL	WDLGLREMG PINDSQGVFA VLEKEVRRGL DDTYATGVRH
LuxR (V. fischeri)		..MNIKINANE	KIIDKIKTCN NKIDINQCLS EIAKIIINC.E YYLFAIITPH
RhlR (R. leguminosarum)		..MKEESSAVSN	LVDFDLESA SAKSKDDVLL LFGKISQYFG FSYFAISGIP
Consensus		FL--L-	-----E-- IL-K-L--LG FDYFA----
	51	Region 1	100
CarR (E. carotovora)	KNS..TSQPY	IISNYPEALN	KEYIKKEMFL SDPIIVASLA RITPFSMDON
EsaR (E. stewartii)	KKN..PSNVL	IISNYPEDEI	RLYRANFQL TDPVILTAFK RTPSFALDEN
TraR (A. vitis)	YLHIGKHI	AVTNYHNDWR	SLYDFKKFDA LDPVVKRARS RQVFAVMSGE
TraR (A. tumefaciens)	YLHIGKHI	AVTNYHNDWR	SLYDFKKFEA LDPVVKRARS RQVFAVMSGE
LasR (P. aeruginosa)	KDSQDYENAF	IVGNYPAAWR	EHYDRAGYV VDPVSHCTQ SVLPFIHAPS
SdiA (E. coli)	PVPFTRPKVA	FYTHNPEAW	SYQAQNFLL IDPVLNPFN SGNLHMDND
PhzR (P. aureofaciens)	VTFPMRPTV	NYGNYPEDIV	GRYQAANYAV IDPTVKHSKY SSSPILASNE
RhlR (P. aeruginosa)	TIPFTRPKTE	VHGTYPKAA	ERYQWQNYGA VDPAILNGLR SSENVIASDS
LuxR (V. fischeri)	SIKQVVS..	IIDNYPEKMR	KYDDAGGLE YDPVVDYSK. SHSPILNMDV
RhlR (R. leguminosarum)	SPIERIDSYF	VLGNHVSQWF	DRYENNYVM ADPIVHLSKT CDHAFVMSA
Consensus		IVGNYPEAW	--Y--NF-A -DPV--S-- S--PF-WS--
	101		150
CarR (E. carotovora)	DIVTLRAK.N	QGVFISSVQH	DISSGYTFLV HNDHNVATL S.IAHNLEDA
EsaR (E. stewartii)	ITLMSDLR.F	TKIFLSLQY	NIVNGFTYVL HDMHNLALL SVIKGMDGT
TraR (A. vitis)	QERPKLSEEE	RAFYAQAADF	GIRSGITPI RTANGSMHF TL..ASERT
TraR (A. tumefaciens)	HERPTLSKDE	RAFYDASDF	GIRSGITPI RTANGSMHF TM..ASDKP
LasR (P. aeruginosa)	IYG...TRQG	NEFFEEASAA	GLVYGLTNP NGARGELGAL SLVSEANRA
SdiA (E. coli)	LF.....SEA	QPLMEAAAH	GLRRGVHSVF NAAQTALGF .LSFRCRR
PhzR (P. aureofaciens)	LF.....RGC	PDLVSEAND	NLRGLAOPS FMTQ.GRVGV .LSLARDNP
RhlR (P. aeruginosa)	LF.....DGS	RMLNLEARD	GLCVGATLPI RAPH.MLLSV .LSVARDQON
LuxR (V. fischeri)	FEKTKIKES	PNVKEAQES	GLITGFSFPI HTASNGFG..MLSFANDKD
RhlR (R. leguminosarum)	LRDQKLDQGS	RRVMEAREF	KLIDGFSVPL HTAAGFGS..IVSFGAEKVE
Consensus		L-----R--	R-F--EA-DF GLR-GFT-PI HTA-G----F -LS--A-D--
	151		200
CarR (E. carotovora)	NFEKCHKNHE	NDLQMLLVNV	HEKVMAYGRA INDDNPPDN SRNA...LLS
EsaR (E. stewartii)	ALQRLAAEQ	GTQMILLIDF	HEQM..YRLA GTEGERAPAL NQSDAKTIFS
TraR (A. vitis)	ATPLDREIDA	VAAAAGVGL	HARISFLRIT PTAEDAH..LD
TraR (A. tumefaciens)	VIDLDREIDA	VAAAATIGGI	HARISFLRTT PTAEDAAC..VD
LasR (P. aeruginosa)	EANRFHESVL	PTLMLDKDYA	LQSG.....AG..LAFEN PV.SKPVVLT
SdiA (E. coli)	EIPILSDELQ	LKMLLVRES	LMAL.....MR..LNDEI .VMTPEMNF
PhzR (P. aureofaciens)	ISLQEFALK	VTKAFAAAV	HEKI.....SE..LESVD RVFNTDVEFS
RhlR (P. aeruginosa)	ISSFEREEIR	LRLRCHIELL	TQKL.....TD..LEHNP .LMSNPVCLS
LuxR (V. fischeri)	IYT...DSL	LHASTHVPPL	LPSL.....VDHYKINT TRICKSDSILT
RhlR (R. leguminosarum)	LSTCDRSALY	LMAAYANSL	RAGI.....GMD.....AS RKIQAIPMIT
Consensus		DRE-L- L-A-LV--L	H--I-----T--LA-----LS
	201	Region 2	250
CarR (E. carotovora)	PRETEVLFLV	SSGRTYKEVS	RILGISETVV KFHINNSVRK LDVINSRHAI
EsaR (E. stewartii)	SRNEVLYMA	SMGKTYAEIA	AITGISVSTV KFHINNVVVK LGVSHARQAI
TraR (A. vitis)	PKEATYLRWI	AVGKTMEEIA	DVEEVKYNV RVKLEAMKR FVRSKAHLT
TraR (A. tumefaciens)	PKEATYLRWI	AVGKTMEEIA	DVEGVKYNV RVKLEAMKR FVRSKAHLT
LasR (P. aeruginosa)	SREKEVLQWC	AGKTSMEIS	VICNCSEANV NFHMGNIRK FGVTSRRVAA
SdiA (E. coli)	KREKEILRWT	AEKTSAEIA	MILSISENTV NFHMGNIRK INAPKTKQVA
PhzR (P. aureofaciens)	GRECEVLRTV	ADGKTSSEIG	VINGVCTDTV NYHMGNIRK IGASHRVQAS
RhlR (P. aeruginosa)	HREREILQWT	ADGKTSSEIA	IILSISESTV NFHMGNIRK FAPAKTLAA
LuxR (V. fischeri)	KREKECLAWA	SEGKSTMDIS	KILGCSERTV TFLNTHQNK LNTNHCQSI
RhlR (R. leguminosarum)	TREREITHMC	AGKTAIEIA	TILGRSHRTI QNVILNIRK LNVVTPQMI
Consensus		RE-EVLRV- A-GKTS-EIA	-ILGISE-TV NFH--NIQRK FDV-NK-QAI
	251		274
CarR (E. carotovora)	TKALELNLFH	SPCEPVVWKH	MDAR
EsaR (E. stewartii)	RLGVLDLIR	PAASAAR	
TraR (A. vitis)	ALAIKRLI.		
TraR (A. tumefaciens)	ALAIKRLI.		
LasR (P. aeruginosa)	IMAVNLGIT		
SdiA (E. coli)	CYAAATGLI.		
PhzR (P. aureofaciens)	RYAVANGYI.		
RhlR (P. aeruginosa)	AYAAALGLI.		
LuxR (V. fischeri)	SKAAILTGAIN	CPYLK.	
RhlR (R. leguminosarum)	AESFRLRIIR		
Consensus		A-AI-LGLI-	

Fig. 3. Alignments of the predicted amino acid sequences of CarR (this work), EsaR (S. Beck von Bodman & S. K. Farrand, unpublished; accession no. L32184), TraR (*A. tumefaciens*; W. C. Fuqua, C. M. Senzou & S. C. Winans, unpublished; accession no. L08596), TraR (*A. vitis*; Fournier *et al.*, 1994; accession no. P33909), LasR (Gambello & Iglewski, 1991; accession no. M59425), SdiA (Sharma *et al.*, 1986; accession no. P07026), PhzR (Pierson *et al.*, 1994; accession no. L32729), RhlR (Ochsner *et al.*, 1994; accession no. L08962), LuxR (Gray & Greenberg, 1992; accession no. M96844) and RhlR (Cubo *et al.*, 1992; accession no. M98835). Positions with four or more identical residues have been indicated as a consensus sequence. The proposed autoinducer-binding region and the DNA-binding region (regions 1 and 2, respectively) have been marked.

Fig. 4. Identification of the CarR protein in a T7 expression system. A 1.03 kb fragment encoding *carR* was cloned into pT7-5 in both orientations. In the correct orientation for expression of *carR* (pSMG8) and in the presence of rifampicin (rif; final concentration of 200 µg ml⁻¹), only one major protein of approximately 30 kDa was labelled (arrowed in lane 3). Under the same conditions, no proteins were labelled when either the fragment was cloned in the opposite orientation (pSMG5) or just vector alone was used (lanes 4 and 5, respectively). Molecular size markers were run in lane 6 and their sizes in kDa have been indicated.

endogenous Tn5-containing background (as opposed to secondary transposition), this may have resulted in targeting of the incoming transposon to different sites.

Effects of *carR* on the exoenzyme production phenotype

Although the wild-type *carR* gene (pSMG4) complements the *carR* group 1 mutants of strain GS101 in the carbapenem bioassay, it did not complement group 2 Car⁻ mutants for either antibiotic production or for exoenzyme synthesis (Rex⁻). (Interestingly, however, with pSMG4 in the latter strains, very small/'intermediate' haloes were occasionally noted in the antibiotic bioassay; Table 1.) Similarly, both cWU142 and pWU14203 failed to restore exoenzyme production in either group 1 (OHHL⁺) or group 2 (OHHL⁻) Rex⁻ mutants of strain SCRI193 (data not presented). Thus CarR appears to be a LuxR-type regulator which is specific for control of carbapenem antibiotic production.

Although *carR* in low copy number had no noticeable effect on the Rex mutants of SCRI193, when *carR* was

Fig. 5. Effect of *carR* in multicopy on exoenzymes in *E. carotovora* strain SCRI193. Protease (▨), pectate lyase (□) and cellulase (■) production was assayed in triplicate in the presence of either pDAH330 (1) or pSMG4 (3). The same strains were also assayed for exoenzyme production in the presence of exogenously added OHHL (2 and 4, respectively). The values obtained for SCRI193 (pDAH330) were taken as 100%. Other values were then calculated as a percentage of this control value.

present on a high copy number plasmid (pSMG4) it reduced exoenzyme synthesis levels in the wild-type strain (Fig. 5) and exacerbated the Rex phenotype in Rex⁻ mutants (data not shown). However, this effect was at least partially remedied by the addition of exogenous OHHL to the test strains. These results suggest that excessive amounts of the CarR protein can interfere with the global regulation of the exoenzymes, at least in strain SCRI193 and its derivatives.

DISCUSSION

The majority of the group 1 Car⁻ mutants of *E. carotovora* were not defective in the antibiotic biosynthetic genes but were mutant for *carR*. The 28 kDa CarR protein is homologous with the transcriptional activator LuxR of *V. fischeri* (Devine *et al.*, 1989; Engebrecht & Silverman, 1984). LuxR acts in concert with OHHL to activate the *lux* genes in that organism. Thus it seems likely that a similar CarR:OHHL interaction is also required for carbapenem antibiotic gene expression in *E. carotovora*.

Although CarR function is clearly OHHL-dependent under normal physiological conditions, on occasion we did observe that pSMG4 enabled group 2 mutants of GS101 to produce very slight haloes in the carbapenem bioassay (e.g. 1–2 mm, cf. 8–10 mm in the wild-type) (Table 1). Although this effect was sporadic and obviously was not a complementation of the *carI* defect, this observation implies that the CarR protein, in very high copy, can function partially in the absence of OHHL.

CarR joins a rapidly growing family of LuxR homologues which are required for regulation of diverse physiological traits, e.g. LasR (elastase regulation in *Pseudomonas*); TraR (regulation of Ti plasmid conjugal transfer in *Agrobacterium*); SdiA (regulation of cell division gene expression in *E. coli*); RhlR (regulation of rhizosphere-specific genes

in *Rhizobium*); RhlR (regulation of rhamnolipid biosurfactant synthesis in *Pseudomonas*); and, recently, PhzR (regulation of phenazine antibiotic synthesis in *Pseudomonas*) (Fuqua *et al.*, 1994; Swift *et al.*, 1994; Pierson *et al.*, 1994). All of these results strongly suggest that the CarR protein acts as a positive transcriptional activator of the carbapenem biosynthetic genes. Our results also show that *carR* must function *in trans* and so must lie in a different transcriptional unit from the biosynthetic genes. Also, because CarR does not complement the Rex⁻ phenotype in either the cognate strain or another *Erwinia* strain (SCRI193) it seems likely that CarR is specific to the activation of the carbapenem biosynthesis genes. However, in multicopy, CarR caused a reduction in the production of pectinases, cellulases and proteases in wild-type SCRI193, effectively mimicking the Rex⁻ phenotype (Fig. 5). In addition, multicopy CarR exacerbated the Rex⁻ phenotype in Rex⁻ mutants although growth rate was unaffected (data not presented). High copy CarR might produce these effects by sequestering some of the OHHL that would normally function in the regulation of the Rex phenotype or, alternatively, CarR protein in excess could interfere directly with genes or proteins of the Rex regulatory system. However, the exoenzyme down regulation phenotypes of the wild-type SCRI193 (pSMG4) or the Rex⁻ mutants carrying pSMG4 were partially restored by exogenously supplied OHHL (Fig. 5), consistent with the model in which the naturally produced OHHL is sequestered by excess CarR. These results also imply that at least one other homologue of LuxR exists in the Rex regulon and that artificially high amounts of CarR compete with this (these) for available OHHL. [It is interesting in this context that we have recently identified another LuxR homologue in SCRI193 encoded by a gene which is contiguous with *carI* and transcribed convergently with it (S. Jones, unpublished) and similar results have been found for another *E. carotovora* strain in which the *expI* gene (*carI* homologue) is linked to another *luxR* homologue, *expR* (EMBL database accession number X72891).] Surprisingly, in contrast to the effects of multicopy CarR in SCRI193, in the cognate wild-type strain (GS101) there was no significant effect on exoenzyme synthesis implying that there must be subtle differences in the overall mode of global regulation of the virulence factors in the two strains. The molecular basis of these differences is currently unknown.

For several of the *N*-acyl homoserine lactone regulated phenotypes described above, a LuxI homologue partner has been identified in the respective host, e.g. LuxI/LuxR (*V. fischeri*), LasI/LasR (*P. aeruginosa*), TraI/TraR (*Agrobacterium tumefaciens*) and, very recently, PhzI/PhzR (*P. aureofaciens*) (Fuqua *et al.*, 1994; Swift *et al.*, 1994; Pierson *et al.*, 1994). However, neither a LuxI homologue 'partner', nor a defined signalling molecule, has been identified to date for either the *E. coli* SdiA or *Rhizobium leguminosarum* RhlR systems, and whether or not the LasI regulator gene plays a role in RhlR-mediated activation of rhamnolipid synthesis in *P. aeruginosa* was not discussed by Ochsner *et al.* (1994).

Unlike *luxI* and *luxR* in *V. fischeri* (e.g. see Meighen,

1991; Meighen & Dunlap, 1993), the *carI* and *carR* regulatory genes in *E. carotovora* are not linked. Although *carR* mutants are only apparently affected in carbapenem production, *carI* mutants have very pleiotropic phenotypes (down regulation in synthesis of pectinases, cellulases and proteases, and loss of carbapenem production) confirming that the ultimate product (OHHL) of a single *carI* locus acts to control multiple, physiologically unrelated phenotypes by (indirect) transcriptional activation of many genes. The only common feature of these *N*-acyl homoserine lactone regulated phenotypes is a growth-phase/cell-density dependency, and that arises naturally from the cell-density dependency of OHHL synthesis (Williams *et al.*, 1992; Swift *et al.*, 1993).

How can OHHL regulate multiple genes? A simple way in which to link the cell-density-dependent (OHHL-dependent) control of multiple, diverse phenotypes would be to have multiple LuxR homologues in a single cell which are all responsive to the same signalling molecule. At least some of these LuxR homologues might be 'dedicated' transcriptional activators in that they act, directly or indirectly, specifically on their respective target genes, e.g. carbapenem synthesis genes (controlled by CarR) or exoenzyme genes (a hypothetical RexR?) in *E. carotovora*. The validity of this model is currently being assessed.

LuxR is essentially a two domain protein (Slock *et al.*, 1990; Fuqua *et al.*, 1994), and based on the LuxR model we expect that the LuxR homologues will also bind their respective *N*-acyl homoserine lactones in the N-terminal domain and will bind to their respective target DNA sequences via their C-terminal domains. In this context, it is interesting that the most conserved residues of the LuxR homologues lie within the putative *N*-acyl homoserine lactone and DNA-binding domains (Fig. 3; see Fuqua *et al.*, 1994).

The recent observation (Pierson *et al.*, 1994) that phenazine antibiotic production by *P. aureofaciens* is regulated by the LuxI/LuxR homologues, PhzI/PhzR, suggests some interesting analogies and comparisons with carbapenem antibiotic regulation in *E. carotovora*. Both antibiotics are secondary metabolites, their synthesis being growth-phase-dependent because they are regulated by prior production of a small signalling molecule. In the case of *P. aureofaciens*, the *phzI* and *phzR* regulatory genes are linked, although they are not contiguous (Pierson *et al.*, 1994). In contrast, the *carI* and *carR* regulatory genes of *E. carotovora* are unlinked. The phenazine biosynthetic genes are linked to the *phzI/R* locus (Pierson *et al.*, 1994), and the preliminary genetic results described here suggest that at least some of the carbapenem biosynthetic genes may be linked to *carR*. This is currently under investigation.

Finally, it is very interesting that, although most of the *carR* mutants were isolated after chemical mutagenesis, they arose because of Tn5 secondary transposition in the GS101 parent strain. In contrast, the chemically induced Car mutants with *carI* mutations did not carry Tn5 insertions and were, presumably, due to mis-sense mutations. The 'target' size of the *carR* gene (735 bp) is similar

to that of *carI* (651 bp) yet in all of our mutagenesis experiments we have found a disproportionate number of *carR* mutants (relative to *carI* mutants). Therefore, these data suggest strongly that the *carR* gene acts as a 'hot spot' for Tn5 insertion, at least in secondary transpositions in GS101. We presume that chemical mutagenesis indirectly stimulates the general level of secondary transposition in this strain thereby revealing the *carR*::Tn5 derivatives at high frequency among survivors, when screened in the carbapenem bioassay. Interestingly, the common site for Tn5 insertion in *carR* lies within the sequence encoding the 'hinge' region of the CarR protein upstream of the conserved DNA-binding motifs (Figs 2 and 3). Based on information derived from the LuxR protein (Slock *et al.*, 1990; Fuqua *et al.*, 1994), the OHHL-binding site is likely to be in the N-terminal domain and the DNA-binding site in the C-terminal domain of CarR. If we view the LuxR family of proteins as effectively modular in this way, then is it possible that a highly recombinogenic region in the corresponding gene could act as a source of generation of 'R' protein diversity and evolution? This would be especially important if the genes encoding the LuxR homologues could move horizontally. In this respect it is interesting that the *carR* gene has a GC ratio of 39.2% (data not presented), which is significantly different from the overall GC content of *E. carotovora* (52.1%; Starr & Chatterjee, 1972).

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