N-(3-Oxohexanoyl)-L-homoserine lactone regulates carbapenem antibiotic production in *Erwinia carotovora*

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Erwinia carotovora A.T.C.C. 39048 produces the antibiotic 1-carbapen-2-em-3-carboxylic acid. A number of mutants with a carbapenem-non-producing phenotype were selected as part of an investigation into the molecular and genetic basis of carbapenem biosynthesis. Cross-feeding studies revealed that the mutants fell into two discrete groups. Group 1 mutants were found to secrete a diffusible low-molecular-mass compound which restored carbapenem production in group 2 mutants. This compound was isolated from the spent culture supernatant of a group 1 mutant using solvent extraction, hydrophobic-interaction chromatography and silica-gel chromatography, and finally purified by reverse-phase semi-preparative h.p.l.c. M.s. and n.m.r. spectroscopy revealed that the compound was *N*-(3-oxohexanoyl)homoserine lactone. Both D- and L-isomers were synthesized, and subsequent analysis by c.d. established that the natural product has the L-configuration. Although carbapenem production was restored by both isomers, dose-response curves indicated that the L-isomer has greater activity, with an induction threshold of about 0.5 µg/ml. *N*-(3-Oxohexanoyl)-L-homoserine lactone is, therefore, an autoregulator of carbapenem biosynthesis rather than a biosynthetic intermediate. This compound is already known for its role in autoinduction of bioluminescence in the marine bacterium *Vibrio fischeri*. It is also structurally-related to the A- and I-factors which are known to regulate production of antibiotics in some *Streptomyces* species. Its association in this work with the regulation of carbapenem biosynthesis implies a broader role for autoregulator-controlled gene expression in prokaryotes.

INTRODUCTION

The carbapenem antibiotics constitute a diverse group of β -lactam antibiotics characterized by potent antibacterial and β -lactamase-inhibitory activity. More than 40 different carbapenems are known, most of which are produced by the actinomycetes, particularly *Streptomyces* spp. (Ratcliffe & Albers-Schonberg, 1982; Brown, 1984; Williamson, 1986).

The unsubstituted bicyclic carbapenem nucleus: (5*R*)-carbapen-2-em-3-carboxylic acid (SQ27,860) [Fig. 1(i)] was isolated from the Gram-negative bacteria *Serratia marcescens* and *Erwinia carotovora* by Parker *et al.* (1982). The same compound has been identified by Kintaka *et al.* (1985) in *Azospirillum* sp. UK1521. In addition, two epimeric carbapenams, (3*S*,5*S*)- and (3*S*,5*R*)-carbapen-3-carboxylic acid, were isolated from *Serratia* and *Erwinia* (Bycroft *et al.*, 1987; Bycroft & Chhabra, 1989). The biosynthetic origin of the carbapenem ring system has been characterized in both *Serratia* (Bycroft *et al.*, 1988) and in the thienamycin producer *Streptomyces cattleya* (Albers-Schonberg *et al.*, 1976; Williamson *et al.*, 1985). In both cases the pyrroline ring is derived from glutamic acid, whereas the β -lactam carbon atoms originate from acetate. In *Serratia* and *Erwinia*, Bycroft *et al.* (1988) have demonstrated that the carbapenem and carbapenams are derived from common precursors and have proposed an intimate biosynthetic relationship between them. The regulatory mechanisms involved in the control of carbapenem biosynthesis remain largely unknown. However, Nakata *et al.* (1989) have postulated the involvement of a low-molecular-mass diffusible molecule in the regulation of thienamycin biosynthesis in

Strep. cattleya and olivanic acid production in *Strep. fulvoviridis*. Although these compounds have not been chemically characterized, the existence of regulatory factors controlling antibiotic biosynthesis in other *Streptomyces* species has been well documented. Khokhlov *et al.* (1967) isolated the regulatory factor 2-(6-methylheptanoyl)-(3*R*)-hydroxymethyl-4-butanolide (A-factor) [Fig. 1(ii)] and demonstrated its role in controlling a number of aspects of secondary metabolism in *Strep. griseus*. Mutants defective in streptomycin production, sporulation and mor-

Fig. 1. Structure of (i) (5*R*)-carbapen-2-em-3-carboxylic acid, (ii) 2-(6-methylheptanoyl)-(3*R*)-hydroxymethyl-4-butanolide (A-factor), (iii) *N*-(3-oxohexanoyl)homoserine lactone and (iv) *N*-(3-hydroxybutyryl)-L-homoserine lactone

Abbreviations used: ECP, *Erwinia* carbapenem production; EMS, ethyl methanesulphonate; MNNG, 1-methyl-3-nitro-1-nitrosoguanidine; COSY, correlation spectroscopy.

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phological development were restored to their parental phenotypes by addition of exogenous A-factor. Other structurally related butyrolactone autoregulators have subsequently been identified in *Streptomyces* species (Grafe *et al.*, 1983; Yamada *et al.*, 1987; Sato *et al.*, 1989).

In the present paper we report the purification, structural identification and chemical synthesis of an autoregulator, *N*-(3-oxohexanoyl)-L-homoserine lactone, which controls carbapenem biosynthesis in *E. carotovora*.

MATERIALS AND METHODS

Bacterial strains and growth conditions

All *E. carotovora* strains used in the present study were derived from strain A.T.C.C. 39048. *Escherichia coli* strain ESS, a β -lactam-sensitive strain, was kindly provided by SmithKline Beecham Pharmaceuticals. *Erwinia* strains were routinely subcultured on nutrient-agar plates at 26 °C for 48 h. *E. coli* strains were subcultured on nutrient agar at 37 °C for 24 h. For experiments in liquid media, *Erwinia* strains were grown in either a complex medium, consisting of 2.0% Oxoid neutralized soya peptone and 0.2% (w/v) sucrose (adjusted to pH 7.0 with NaOH) (Bycroft *et al.*, 1988) or *Erwinia* carbapenem production (ECP) medium, which consisted of 0.2% (w/v) L-glutamic acid, 0.1% (w/v) $(\text{NH}_4)_2\text{SO}_4$, 0.37% (w/v) K_2HPO_4 , 0.62% (w/v) KH_2PO_4 and 0.02% (w/v) NaCl, adjusted to pH 7.4 with NaOH; the following were added after autoclaving: 0.4% (w/v) glucose, 0.2% (w/v) casamino acids, 0.001% (w/v) FeSO_4 and 0.01% (w/v) MgSO_4 .

Transposon mutagenesis

To render *Erwinia* susceptible to λ -phage infection, *E. carotovora* strain A.T.C.C. 39048 was transformed with plasmid pTROY9 (DeVries *et al.*, 1984) carrying the *lamB* gene of *E. coli*. Transposon mutagenesis was then carried out as described by Salmond *et al.* (1986) using λ ::Tn5 and λ ::Tnblam (Tadayyon & Broome-Smith, 1992).

After mutagenesis, single colonies were picked off nutrient-agar plates containing 50 μg of spectinomycin/ml (to select for Tnblam insertions) and screened for carbapenem-non-producing mutants on ESS agar plates. These consisted of DST agar (Oxoid) plates seeded with *E. coli* ESS and were prepared by adding 3 ml of an overnight culture of strain ESS grown in BHI broth (LabM, Amersham International) at 37 °C, to 1 litre of DST agar.

Chemical mutagenesis

E. carotovora strain GS101, a restrictionless Tn5-induced mutant derivative of A.T.C.C. 39048, was grown in Luria-Bertani broth on a rotary shaker at 26 °C overnight. When the A_{600} was greater than 2.0, 1.0 ml of inoculum was added to 50 ml of *Erwinia* complex medium and incubated on a rotary shaker at 26 °C until the A_{600} reached 0.7. Aliquots (1 ml each) were subjected to chemical mutagenesis using ethyl methane-sulphonate (EMS) and 1-methyl-3-nitro-1-nitrosoguanidine (MNNG). EMS mutagenesis was performed by the method of Kohalmi & Kunz (1988), except that a concentration of 2.0% (v/v) EMS was used. MNNG mutagenesis was carried out as described by Richardson *et al.* (1987).

Carbapenem-non-producing mutants were screened as described for transposon mutagenesis.

Cross-feeding bioassays

To screen carbapenem-non-producing mutants for putative secreted biosynthetic intermediates, mutant cross-feeding bioassays were developed.

Preliminary experiments were carried out in which overnight cultures of two different carbapenem-non-producing mutants grown on nutrient agar at 26 °C for 24 h, were mixed and inoculated on to the surface of plates seeded with *E. coli* ESS. If cross-feeding occurred, an inhibition of growth of ESS in a zone around the mixed *Erwinia* inoculum was observed. Pairs of cross-feeding mutants were identified and further analysed for secretion of putative intermediates.

Each mutant from a cross-feeding pair was inoculated around a well cut in a seeded ESS plate. Samples of up to 50 μl of filter-sterilized supernatant (from an overnight culture of *Erwinia* grown in ECP medium) from the other mutant of the pair were added to the well. Induction of carbapenem biosynthesis resulted in inhibition of growth of the *E. coli* ESS and indicated which mutant secreted a diffusible factor.

Purification of diffusible factor

Mutant PNP26 was grown in *Erwinia* complex medium overnight on a rotary shaker at 26 °C. A 0.1% inoculum was added to 5 litres of ECP medium and incubated at 26 °C for 16 h. Cells were harvested by centrifuging at 15300 *g* for 10 min. The supernatant was decanted and extracted twice with 0.2 vol. of ethyl acetate (previously distilled over anhydrous K_2CO_3). The volume of the ethyl acetate extract was reduced to approx. 50 ml by rotary evaporation at 30 °C, an equal volume of distilled water was added and evaporation was continued until all the ethyl acetate had been removed.

The aqueous solution was passed through a column containing hydrophobic resin (styrene-divinylbenzene copolymer CH3P; Mitsubishi Ltd.) pre-equilibrated with distilled water (column dimensions: length, 10 cm; internal diameter, 10 mm), then eluted sequentially with 60 ml volumes of water, methanol/water (3:7, v/v) and methanol/water (7:3, v/v).

Methanol was removed by rotary evaporation at 30 °C, and the aqueous solution extracted with an equal volume of ethyl acetate, which was in turn, dried (over anhydrous MgSO_4) and rotary-evaporated to dryness at 30 °C. The residue was dispersed in 2 ml of ethyl acetate/hexane (7:3, v/v) and loaded on to a silica-gel column (column dimensions: length, 20 cm; internal diameter, 2.3 cm; silica supplied by Woelm TSC, Woelm Pharma G.m.b.H., Eschwege, Germany), previously equilibrated in the same solvent. The column was eluted with an ethyl acetate/hexane (7:3, v/v) mobile phase and 10 ml fractions collected. Fractions (21–41) were pooled and evaporated to approx. 20 ml. Water (2 ml) was added and the residual ethyl acetate and hexane removed by rotary evaporation at 30 °C.

The final purification was carried out using semi-preparative h.p.l.c. (h.p.l.c. equipment: Gilson 105 u.v. detector; Gilson 305 pump controller with Gilson 302 slave pump; Hewlett-Packard HP3394A integrator/chart recorder; Hichrom S50DS2 reverse-phase semi-preparative column). The mobile phase was methanol/water (1:4, v/v). The monitoring wavelength was 210 nm. Methanol was removed by rotary evaporation at 30 °C and samples were freeze-dried to yield a white powder.

Biological activity in the various fractions was monitored throughout the purification procedure using the cross-feeding bioassay, employing *Erwinia* mutant PNP22, which does not produce either carbapenem or a diffusible factor able to induce carbapenem biosynthesis in any other mutant, but can be restored to carbapenem production itself by addition of exogenous factor from other mutants such as PNP26.

Characterization of the diffusible factor

Mass spectra (electron impact) and accurate mass measurement of molecular ions were determined by using an AEI MS902

Fig. 2. Scheme for the synthesis of *N*-(3-oxohexanoyl)-L-homoserine lactone

instrument. N.m.r. spectra were recorded on a Bruker AM400 spectrometer operating at 400 MHz. I.r. spectra were recorded using a Perkin-Elmer 257 spectrophotometer. C.d. spectra were obtained from optical rotations measured on a JASCO J-600 spectropolarimeter.

Chemical synthesis of *N*-(3-oxohexanoyl)homoserine lactone

Fig. 2 shows the synthetic route adopted. Triethylamine (140 μ l, 1 mmol) was added to a stirred solution of L- or D-homoserine lactone hydrochloride (137.5 mg, 1 mmol) in water (2 ml), followed by the addition of the ethylene glycol ketal of 3-oxohexanoic acid (174 mg, 1 mmol) and 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide hydrochloride (191.5 mg, 1 mmol). The mixture was stirred at room temperature for 20 h, then evaporated to dryness at 35 °C using a rotary evaporator. The residue was extracted five times with warm ethyl acetate (5 $\times$ 5 ml). The extracts were pooled and washed successively with water (1 $\times$ 3 ml), 5% NaHCO_3 solution (1 $\times$ 3 ml), 1.0 M- KHSO_4 solution (1 $\times$ 3 ml) and finally a saturated solution of NaCl in distilled water (1 $\times$ 5 ml). The ethyl acetate extract was dried with anhydrous MgSO_4 and solvent evaporated *in vacuo* to give the ethylene glycol ketal of *N*-(3-oxohexanoyl)-L- or -D-homoserine lactone (yield 45%).

HClO_4 (0.25 ml of 60% solution) was added to an ice-cold solution of ethylene glycol ketal of *N*-(3-oxohexanoyl)homoserine lactone (128.5 mg, 0.5 mmol) in dichloromethane (15 ml). The mixture was stirred at 0 °C for 30 min, then at room temperature for a further 90 min. Solvent was removed *in vacuo* and the residue redissolved in ethyl acetate (20 ml). The solution was washed twice with cold water (2 $\times$ 5 ml) and once with a solution of saturated NaCl in water (5 ml), dried with anhydrous MgSO_4 and rotary-evaporated to obtain the desired *N*-(3-oxohexanoyl)-L- or -D-homoserine lactone isomers (yield 58%).

Induction of carbapenem biosynthesis in liquid culture

Induction of carbapenem in carbapenem-non-producing mutants using exogenous *N*-(3-oxohexanoyl)-L- or -D-homoserine lactone was carried out in ECP medium to facilitate subsequent

h.p.l.c. analysis. The carbapenem-non-producing mutant PNP22 was used for these experiments.

A seed culture of mutant PNP22 was grown overnight in BHI broth at 26 °C. After the addition of all other ECP media components, filter-sterilized *N*-(3-oxohexanoyl)-L-homoserine lactone was added to give a range of final concentrations between 0 and 100 μ g/ml and *N*-(3-oxohexanoyl)-D-homoserine lactone added to give a range of final concentrations between 0 and 75 μ g/ml. Immediately afterwards a 2% inoculum (v/v) of the seed culture was added to 50 ml of fresh ECP medium. This was incubated on a rotary shaker (220 rev./min) at 26 °C for 16 h (until the A_{600} reached 6.0).

Carbapenem h.p.l.c. assay

Bacteria grown as described above were harvested in a Microfuge at 9700 *g* for 5 min and the supernatant removed and filter-sterilized. Routinely a 100 μ l volume of supernatant was analysed by h.p.l.c. Samples were eluted from a reverse-phase semi-preparative column (S5 ODS2; Hi-chrom.) with a mobile phase of 100 mM- KH_2PO_4 , pH 7.0, and a flow rate of 2 ml/min. The monitoring wavelength was 254 nm.

Where β -lactamase was added, 2 μ l of a 0.1 mg/ml solution of β -lactamase (EC 3.5.2.6; Calbiochem Novabiochem) was added to 100 μ l of sample.

RESULTS

Mutant classes and cross-feeding studies

Carbapenem-non-producing mutants were isolated by chemical and transposon mutagenesis (*TnblaM*). Four *spc*^r carbapenem-non-producing transposon mutants were selected with a frequency of 0.8×10^{-3} compared with *spc*^r transposants. EMS- and MMNG-induced carbapenem-non-producing mutants were selected at a frequency of 0.9×10^{-3} and 2.5×10^{-3} respectively. By mixing two different mutants on plates seeded with *E. coli* ESS, the mutants were analysed for their ability to cross-feed each other and restore carbapenem production. By using this technique, a collection of 24 mutants could be subdivided into two distinct cross-feeding groups: group 1 (two transposon and 17 chemical mutants) and group 2 (two transposon and three chemical mutants). The results from cross-feeding experiments involving transposon mutants were indistinguishable from those observed with EMS- and MMNG-induced mutants.

Using filter-sterilized supernatants, we were able to demonstrate that group 1 mutants secreted a diffusible factor which restored carbapenem production in group 2 mutants, but not vice versa. In addition, this diffusible factor was found to be present in the β -lactamase-treated (to inactivate endogenous carbapenem) supernatants of the parental A.T.C.C. 39048 strain. Adjustment of the pH of supernatants from group 1 mutants to pH 9.0 with NaOH, followed by neutralization to pH 7.0 with HCl, abolished the ability to cross-feed group 2 mutants. Thus it was concluded that group 1 mutants secreted an alkali-sensitive diffusible factor.

Purification of diffusible factor

The ability of a component of the supernatant obtained from a group 1 mutant (PNP26) to restore carbapenem biosynthesis in a group 2 mutant (PNP22) was used as the basis of an assay for monitoring the presence of the biologically active factor.

The factor was initially solvent-extracted from supernatants of mutant PNP26 and A.T.C.C. 39048 using ethyl acetate. Analysis of the residual aqueous phase from the extracted supernatants revealed that no detectable activity remained. After extraction, the factor was further purified by loading on to a column containing CH3P hydrophobic resin and eluted with a methanol/

Fig. 3. Electron-impact mass spectrum of natural *N*-(3-oxohexanoyl)-L-homoserine lactone

M.s. gave the following data: m/z (%) 213.0989 (47, M^+ , $C_{10}H_{15}NO_4$ requires m/z 213.1001), 185(13), 170(8), 155(7), 143(33), 128(8), 113(19), 102(56), 101(35), 71(82), 57(100).

Fig. 4. High-resolution ^1H -n.m.r. spectrum of *N*-(3-oxohexanoyl)-L-homoserine lactone

The spectrum was measured in $[\text{D}_2]\text{chloroform}$ at 400 MHz (chemical shifts in p.p.m. from tetramethylsilane) and gave the following data: δ_{H} 0.9 (3H, t, CH_3), 1.64 (2H, sextet, $\text{CH}_2 \cdot \text{CH}_2$), 2.22 (1H, dddd, 4 α -H), 2.51 (2H, t, $\text{CH}_2 \cdot \text{CO}$), 2.77 (1H, ddd, 4 β -H), 3.47 (2H, s, $\text{CO} \cdot \text{CH}_2 \cdot \text{CO}$), 4.28 (1H, ddd, 5 α -H), 4.48 (1H, dd, 5 β -H), 4.59 (1H, ddd, 3-H), 7.65 (1H, bs, NH).

water mobile phase. The cross-feeding assay indicated the factor was contained exclusively in the 30–60% methanol fraction. Further purification was achieved by using silica-gel chroma-

tography which resolved the active factor in fractions 21–41. Before spectroscopic studies, final purification was carried out using reverse-phase semi-preparative h.p.l.c. The purified com-

Fig. 5. Proton-decoupled ^{13}C -n.m.r. spectrum of *N*-(3-oxohexanoyl)-L-homoserine lactone

The spectrum was measured in $[^2\text{H}]\text{chloroform}$ at 100 MHz (chemical shifts in p.p.m. from tetramethylsilane) and gave the following data: $\delta_{13\text{C}}$ 13.58 (CH_3), 16.91 (CH_2), 29.94 (CH_2), 45.85 (CH_2), 48.13 (CH_2), 49.12 (CH), 65.94 (CH_2), 166.38 ($\text{CO}\cdot\text{NH}$), 174.83 (ring $\text{C}=\text{O}$), 206.54 ($\text{C}=\text{O}$).

Fig. 6. I.r. spectrum of *N*-(3-oxohexanoyl)-L-homoserine lactone (KBr disc)

Prominent absorptions were at $\nu_{\max}$ (KBr): 3295 (NH), 1780 (ring C=O), 1710 (ketone C=O), 1650 (amide C=O), 1550, 1170 cm^{-1} .

pound eluted from the h.p.l.c. column with a retention time of 11.8 min at a flow rate of 2 ml/min.

Elucidation of the structure of the diffusible factor

On the basis of results of high-resolution electron-impact m.s. (see Fig. 3), a molecular formula of $C_{10}H_{15}NO_4$ was deduced for the pure compound.

By using detailed high-field ^1H and ^{13}C n.m.r. (Figs. 4 and 5 respectively); connectives for the various hydrogen and carbon atoms were determined from a combination of ^1H , ^1H correlation spectroscopy (COSY) and ^1H , ^{13}C COSY experiments. The i.r. spectrum (Fig. 6) (prominent absorptions at 1780, 1710, 1650 and

1550 cm⁻¹) was indicative of the presence of a five-membered ring lactone, a ketone and an amide group. The inference of ring lactone was consistent with the observed base-susceptibility of the product. Thus, on the basis of comprehensive spectroscopic analysis, the naturally occurring product was assigned the structure: *N*-(3-oxohexanoyl)homoserine lactone (Fig. 1). The final proof of the structure was obtained by its synthesis and its absolute configuration determined by c.d. spectroscopy.

Synthesis

L- and D-Homoserine lactones were acylated with the ethylene glycol ketal of 3-oxohexanoic acid by using a water-soluble

Fig. 7. C.d. spectra of natural (-----) versus synthetic L- (—) and D- (·····) isomers of *N*-(3-oxohexanoyl)homoserine lactone

Fig. 8. Induction of carbapenem biosynthesis in mutant PNP22: dose-response curve for synthetic *N*-(3-oxohexanoyl)-L-homoserine lactone

After the addition of all other ECP media components, filter-sterilized *N*-(3-oxohexanoyl)-L-homoserine lactone was added to give a range of final concentrations between 0 and 100 $\mu\text{g/ml}$. Immediately afterwards a 2% inoculum (v/v) of an overnight seed culture of PNP22 was added to 50 ml of fresh ECP medium. This was incubated on a rotary shaker (220 rev./min) at 26 °C for 16 h, then the supernatants were analysed for the presence of carbapenem by using h.p.l.c. Except for the last point ($n = 5$) results are means of six replicates.

carbodi-imide. Removal of the ketal protection with a catalytic amount of HClO_4 in dichloromethane at room temperature furnished the desired *N*-(3-oxohexanoyl)-L-homoserine lactone and *N*-(3-oxohexanoyl)-D-homoserine lactone in 28–30% overall yield (Fig. 2). The products were approximately 95% pure and could be used without further purification for bioassay. Analytical samples were obtained by h.p.l.c. purification. $[\alpha]_D^{25}$ values for the L and D isomers were -26.5° (c 0.4, water) and $+25.9^\circ$

(c 0.4, water) respectively. The synthetic materials displayed n.m.r., m.s. and i.r. spectra identical with those obtained for the natural product. In addition, the h.p.l.c. profiles were identical, and the two materials, when co-injected, were eluted from the column as a single peak with a retention time of 11.8 min with a mobile phase of methanol/water (1:4, v/v).

Determination of chirality

Comparison of the c.d. spectra (Fig. 7) of the natural product and the two synthetic isomers established that the natural material had the L-configuration.

Induction of carbapenem biosynthesis by *N*-(3-oxohexanoyl)-L-homoserine lactone

The ability of synthetic *N*-(3-oxohexanoyl)-L-homoserine lactone to induce carbapenem production in the group-2 mutants was tested on bioassay plates containing *E. coli* ESS. All were restorable to carbapenem production with similar induction thresholds.

Extracts from *E. carotovora* A.T.C.C. 39048, grown in ECP medium were analysed for the presence of carbapenem using h.p.l.c. When a mobile phase of 100 mM- KH_2PO_4 , pH 7.0, with a flow rate of 2 ml/min was used, the carbapenem had a retention time of approx. 7.8 min. This peak can be abolished by incubating the sample with β -lactamase. No peak with a comparable retention time was observed in supernatants of mutant PNP22. However, inclusion of synthetic *N*-(3-oxohexanoyl)-L-homoserine lactone at a concentration of 10 mg/ml results in the restoration of a carbapenem peak with the correct retention time which can be abolished using β -lactamase.

With PNP22, dose-response curves were constructed for the induction of carbapenem by *N*-(3-oxohexanoyl)-L-homoserine lactone and *N*-(3-oxohexanoyl)-D-homoserine lactone at a range of concentrations from 0 to 100 $\mu\text{g/ml}$ (Fig. 8) and 0 to 75 $\mu\text{g/ml}$ respectively. Fig. 8 shows the threshold for induction of carbapenem production is approx. 0.5 $\mu\text{g/ml}$ with an optimum concentration of 1.0 $\mu\text{g/ml}$. The induction threshold for the D isomer was about 1 $\mu\text{g/ml}$, but the maximal level of carbapenem induced was 7% of that observed for the L isomer (results not shown; N. J. Bainton, B. W. Bycroft & P. Williams, unpublished work).

DISCUSSION

We have discovered that *N*-(3-oxohexanoyl)-L-homoserine lactone regulates carbapenem antibiotic biosynthesis in *E. carotovora*. Chemical and transposon mutagenesis were used to produce carbapenem-non-producing mutants. Of these mutants, those which were unable to synthesize *N*-(3-oxohexanoyl)-L-homoserine lactone could be restored to the parental carbapenem-producing phenotype on incubation with *N*-(3-oxohexanoyl)-L-homoserine lactone. Chemical and transposon mutants with single inserts showed the same characteristics, indicating that the phenotype of the chemical mutants did not result from multiple point mutations. No cross-feeding was observed between any of the group 1 mutants. However, in order to achieve such an effect, any accumulated intermediate would have to be both stable and freely diffusible. In addition, Bycroft *et al.* (1988) have suggested the pathway for carbapenem biosynthesis from glutamate and acetate consists of relatively few steps.

N-(3-Oxohexanoyl)homoserine lactone has already been established as the autoinducer of bioluminescence in the marine bacterium *Vibrio fischeri* (Meighen, 1991). In *V. fischeri*, the two regulatory (*luxI* and *luxR*) and five structural genes (*luxC*, *D*, *A*, *B*, *E*) involved in bioluminescence are encoded by the *lux* operon. The *luxI* gene, which is located upstream of the

structural genes, codes for a 22 kDa polypeptide associated with autoinducer biosynthesis. The autoinducer molecule combines homoserine lactone (an intermediate of amino acid metabolism) and 3-oxohexanoic acid (which is related to intermediates of fatty acid metabolism) and may function as a nutritional signal based on these two major biosynthetic pathways (Eberhard *et al.*, 1981). This has been reinforced by recent work describing autoinducer biosynthesis *in vitro*. The substrates of the autoinducer synthetase (postulated to be the *luxI* gene product) were tentatively identified as *S*-adenosylmethionine and 3-oxohexanoyl-CoA (Eberhard *et al.*, 1991).

N-(3-Oxohexanoyl)-L-homoserine lactone functions as an autoinducer initiating transcription of the *lux* operon after binding to the *luxR*-gene product. It is likely that the regulation of carbapenem biosynthesis involves functionally analogous genes and gene products. In *V. fischeri*, the induction of bioluminescence follows a dose-response curve with a threshold of ~1 ng of autoinducer/ml (Eberhard *et al.*, 1981; Meighen, 1991) and an optimum of 10 ng/ml. The induction of carbapenem biosynthesis by *N*-(3-oxohexanoyl)-L-homoserine lactone also follows a dose-response curve. In contrast, however, the threshold and optimum concentrations of autoinducer for induction of carbapenem biosynthesis are higher, being approx. 0.1 and 1.0 µg/ml respectively.

The ability of the natural *Erwinia* autoregulator and synthetic D and L isomers to induce bioluminescence was evaluated using a recombinant derivative of the *lux* system. The resultant plasmid construct, pSB237 provides *E. coli* with a bioluminescent phenotype only in response to exogenous *N*-(3-oxohexanoyl)homoserine lactone (Bainton *et al.*, 1992). By using this bioassay, both the natural *Erwinia* product and the synthetic L isomer, but not the D isomer, were found to be active (G. S. A. B. Stewart, unpublished work). Thus, compared with the carbapenem-regulatory system, the induction of bioluminescence appears to show a higher level of stereospecificity.

The autoregulation of bioluminescence is mediated by a LuxR protein-autoinducer complex which acts as a transcriptional activator of the *lux* operon. The LuxR protein has been characterized as a member of a superfamily of two-element transcriptional regulatory systems. A region near the C-terminus of LuxR, involved in binding to the *lux* operator sites, shows a marked similarity to an *E. coli* 28 kDa regulatory protein upstream of the *uvrC* gene. In addition, similarity has been observed to the sequences of the gene products of: ComA, DegU, FixJ, LasR, NarL, UhpA, UvrC-23k, UvrC-28k, BvgA, GerA, MalT and BrpA (Henicoff *et al.*, 1990; Gambello & Iglewski, 1991; Raibaud *et al.*, 1991). Though the roles of these proteins in transcriptional regulation have been well studied, the physiological signals that they detect remain uncertain. Whether structurally related regulatory proteins are to be found controlling production of carbapenem requires investigation. In addition, the potential significance of *N*-(3-oxohexanoyl)-L-homoserine lactone as a cell-density-dependent regulator of gene expression in diverse prokaryotes has recently been described (Bainton *et al.*, 1992).

Compounds structurally similar to *N*-(3-oxohexanoyl)-L-homoserine lactone have been identified in *V. harveyi* and among the actinomycetes. In *V. harveyi* the autoregulator has been identified as *N*-(3-hydroxybutyryl)homoserine lactone [Fig. 1, structure (iv)] (Cao & Meighen, 1989). In the actinomycetes all the factors identified contain a γ -butyrolactone ring structure. In various *Streptomyces* species autoregulators have been implicated in the regulation of different aspects of secondary metabolism, including virginiamycin production in *Strep. virginiae* (Yamada *et al.*, 1987), anthracycline biosynthesis in *Strep. viridochromogenes*, cytodifferentiation and anthracycline biosynthesis in

Strep. bikiniensis and *Strep. cyaneofuscatus*. The most extensively studied butyrolactone regulator is A-factor (Khoklov *et al.*, 1967), studies of which have been largely confined to streptomycin-producing strains of *Strep. griseus* (A-factor-producing *Strep. bikiniensis* strains having been reclassified as streptomycin-producing *Strep. griseus*) in which A-factor regulates streptomycin biosynthesis, streptomycin resistance and sporulation. An A-factor-binding protein has been described by Miyake *et al.* (1989, 1990), which may be a transcriptional regulator analogous to LuxR.

Hara & Beppu (1982) have presented evidence that the genetic determinant of A-factor biosynthesis (AfsA) is encoded by an unstable extrachromosomal element, but to date no plasmid has been identified (Kinashi *et al.*, 1987; Kinashi, 1988). The cloned *afsA* gene has been shown to restore A-factor biosynthesis, restoring mutants to normal streptomycin biosynthesis, streptomycin resistance and sporulation phenotypes (Horinouchi *et al.*, 1984; Beppu & Horinouchi, 1987). Horinouchi *et al.* (1985) have demonstrated a gene-dosage effect with the *afsA* gene and concluded *afsA* encodes a single biosynthetic enzyme.

Ohnuki *et al.* (1985) first described the *str* gene cluster, which encodes the genes for streptomycin biosynthesis and resistance. This permitted the investigation of the role of A-factor in the transcription of the biosynthetic genes involved. Distler *et al.* (1985, 1987) have shown the *str* operon contains AphD (streptomycin 6-phosphotransferase), StrB (aminocyclitol amidinotransferase), StrR (putative regulatory protein) and an uncharacterized open reading frame, possibly encoding a hexose nucleotidylating enzyme. Transcription of the genes in the operon is controlled by A-factor (Distler *et al.*, 1985, 1987; Horinouchi & Beppu, 1987), and it is possible that the *strR* gene product controls transcription after binding of an A-factor molecule.

Thus the autoregulators from both *Strep. griseus*, *V. fischeri* and *V. harveyi* apparently act as transcriptional regulators. It would seem likely, therefore, that the autoregulator of carbapenem biosynthesis in *Erwinia* functions in the same way. *N*-(3-Oxohexanoyl)-L-homoserine lactone, thus belongs to an expanding group of structurally related autoregulatory compounds controlling diverse and important aspects of bacterial secondary metabolism.

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REFERENCES

- Albers-Schonberg, G., Arison, B. H., Kaczka, E., Kahan, F. M., Kahan, J. S., Lago, B., Maiese, W. M., Rhodes, R. E. & Smith, J. L. (1976) Intersci. Conf. Antimicrob. Ag. Chemother. 16th, Chicago abstr. no. 228
- Bainton, N. J., Bycroft, B. W., Chhabra, S. R., Stead, P., Gledhill, L., Hill, P. J., Rees, C. E. D., Winson, M. K., Salmond, G. P. C., Stewart, G. S. A. B. & Williams, P. (1992) *Gene* **116**, 87-91
- Beppu, T. & Horinouchi, S. (1987) in *Perspectives in Biotechnology* (Duarte, J. M. C., Archer, L. J., Bull, A. T. & Holt, G., eds.), p. 155, Plenum publishing, New York
- Brown, A. G. (1984) in *Encyclopedia of Chemical Technology*, suppl. vol., 3rd edn., 83-131, John Wiley and Sons, New York
- Bycroft, B. W. & Chhabra, S. R. (1989) *J. Chem. Soc. Chem. Commun.* 423-425
- Bycroft, B. W., Maslen, C., Box, S. J., Brown, A. & Tyler, J. W. (1987) *J. Chem. Soc., Chem. Commun.* 1623-1625
- Bycroft, B. W., Maslen, C., Box, S. J., Brown, A. & Tyler, J. W. (1988) *J. Antibiot.* **41**, 1231-1242
- Cao, J.-G. & Meighen, E. A. (1989) *J. Biol. Chem.* **264**, 21670-21676
- DeVries, G. E., Raymond, C. K. & Ludwig, R. A. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 6080-6084

- Distler, J., Mansouri, K. & Piepersberg, W. (1985) *FEMS Microbiol. Lett.* **30**, 151–154
- Distler, J., Ebert, A., Mansouri, K., Pissowotski, K., Stockman, M. & Piepersberg, W. (1987) *Nucleic Acids Res.* **15**, 8041–8056
- Eberhard, A., Burlingame, A. L., Eberhard, C., Kenyon, G. L., Neelson, K. H. & Oppenheimer, N. J. (1981) *Biochemistry* **20**, 2444–2449
- Eberhard, A., Lonngin, T., Widrig, C. A. & Stranick, S. J. (1991) *Arch. Microbiol.* **155**, 294–297
- Gambello, M. J. & Iglewski, B. H. (1991) *J. Bacteriol.* **173**, 3000–3009
- Grafe, U., Reinhardt, G., Schade, W., Eritt, I., Fleck, W. F. & Radics, L. (1983) *Biotechnol. Lett.* **5**, 591–595
- Hara, O. & Beppu, T. (1982) *J. Antibiot.* **35**, 349–357
- Henikoff, S., Wallace, J. C. & Brown, J. P. (1990) *Methods Enzymol.* **183**, 111–132
- Horinouchi, S. & Beppu, T. (1987) *Genetics of Industrial Microorganisms (Proceedings of the Fifth International Symposium on the Genetics of Industrial Micro-organisms, part B) (Alacevic, M., Hranueli, D. & Toman, Z., eds.), p. 41, Ognjen Prica Printing Works, Karlovac, Yugoslavia*
- Horinouchi, S., Kumada, Y. & Beppu, T. (1984) *J. Bacteriol.* **158**, 481–487
- Horinouchi, S., Nishimaya, M., Suzuki, H., Kumada, Y. & Beppu, T. (1985) *J. Antibiot.* **38**, 636–641
- Khokhlov, A. S., Tovarova, I. I., Borisova, L. N., Pliner, S. A., Shevchenko, L. A., Kornitskaya, E. Y., Ivkina, N. S. & Rapoport, I. A. (1967) *Dokl. Akad. Nauk. SSSR*, **177**, 232–235
- Kinashi (1988) in *Biology of Actinomycetes '88 (Proceedings of the Seventh International Symposium on the Biology of Actinomycetes) (Okami, Y., Beppu, T. & Ogawara, H., eds.), p. 117–121, Japan Scientific Societies Press, Tokyo*
- Kinashi, H., Shimeji, M. & Sakai, A. (1987) *Nature (London)* **328**, 454–456
- Kintaka, K., Harada, S., Ono, H. & Okazaki, H. (1985) *J. Takeda Res. Lab.* **44**, (1/2), 17–21
- Kohalmi, S. E. & Kunz, B. A. (1988) *J. Mol. Biol.* **204**, 561–568
- Miyake, K., Horinouchi, S., Yoshida, M., Chiba, N., Mori, K., Nogawa, N., Morikawa, N. & Beppu, T. (1989) *J. Bacteriol.* **171**, 4298–4302
- Miyake, K., Kuzuyama, T., Horinouchi, S. & Beppu, T. (1990) *J. Bacteriol.* **172**, 3003–3008
- Meighen (1991) *Microbiol. Rev.* **55**, 123–142
- Nakata, K., Horinouchi, S. & Beppu, T. (1989) in *Trends in Actinomycetology in Japan (Koyama, Y., ed.), p. 79, Society for Actinomycetes, Japan*
- Ohnuki, T., Imanaka, T. & Aiba, S. (1985) *J. Bacteriol.* **164**, 85–94
- Parker, W. L., Rathum, M. L., Scott Wells, J., Trejo, W. H., Principe, P. A. & Sykes, R. B. (1982) *J. Antibiot.* **35**, 653–660
- Raibaud, A., Zalacain, M., Holt, T. G., Tizard, R. & Thompson, C. J. (1991) *J. Bacteriol.*, **173**, 4454–4463
- Ratcliffe, R. W. & Albers-Schonberg, G. (1982) in *The Chemistry and Biology of β -Lactam Antibiotics, vol. 2 (Morin, R. B. & Gorman, M., eds.), pp. 1–49, Academic Press, New York*
- Richardson, K. R., Crosby, R. M., Richardson, F. C. & Skopek, T. R. (1987) *Mol. Gen. Genet.* **209**, 526–532
- Salmond, J. P. C., Hinton, J. C. D., Gill, D. R. & Perombelon, M. C. M. (1986) *Mol. Gen. Genet.* **203**, 524–528
- Sato, K., Nihira, T., Sakuda, S., Yanagimoto, M. & Yamada, Y. (1989) *J. Ferment. Bioeng.* **68**, 170–175
- Tadayyon, M. & Broome-Smith, J. K. (1992) *Gene* **111**, 21–26
- Williamson, J. M. (1986) *CRC Crit. Rev. Biotechnol.* **4**, 111–131
- Williamson, J. M., Inamine, E., Wilson, K. E., Douglas, A. W., Liesch, J. M. & Albers-Schonberg, G. (1985) *J. Biol. Chem.* **260**, 4637–4647
- Yamada, Y., Sugamura, K., Kondo, K., Yanagimoto, M. & Okada, H. (1987) *J. Antibiot.* **40**, 496–504

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