Mutations in *rpsL* that confer streptomycin resistance show pleiotropic effects on virulence and the production of a carbapenem antibiotic in *Erwinia carotovora*

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Spontaneous streptomycin-resistant derivatives of *Erwinia carotovora* subsp. *carotovora* strain ATTN10 were isolated. Sequencing of the *rpsL* locus (encoding the ribosomal protein S12) showed that each mutant was missense, with a single base change, resulting in the substitution of the wild-type lysine by arginine, threonine or asparagine at codon 43. Phenotypic analyses showed that the *rpsL* mutants could be segregated into two groups: K43R mutants showed reduced production of the β -lactam secondary metabolite 1-carbapen-2-em-3 carboxylic acid (Car), but little effect on exoenzyme production or virulence in potato tuber tests. By contrast, the K43N and K43T mutations were pleiotropic, resulting in reduced exoenzyme production and virulence, as well as diminished Car production. The effect on Car production was due to reduced transcription of the quorum-sensing-dependent *car* biosynthetic genes. The effects of K43N and K43T mutations on Car production were partially alleviated by provision of an excess of the quorum-sensing signalling molecule *N*-(3-oxohexanoyl)-L-homoserine lactone. Finally, a proteomic analysis of the K43T mutant indicated that the abundance of a subset of intracellular proteins was affected by this *rpsL* mutation.

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INTRODUCTION

Erwinia carotovora subsp. *carotovora* (*Ecc*) is a Gram-negative phytopathogen which causes soft rot disease of a variety of plants hosts (including potato), by the production of an arsenal of plant cell wall degrading exoenzymes, such as pectate lyases, cellulases and proteases (reviewed by Toth & Birch, 2005). In addition, some strains of *Ecc*, including strain ATCC 39048 and its derivatives, ATTN10 and MS1, produce the secondary metabolite antibiotic 1-carbapen-2-em-3-carboxylic acid (Car; Bainton *et al.*, 1992a, b). Production of exoenzymes and Car is under the control of the quorum-sensing (QS) system in *Ecc*, such that these factors are only produced during the late-exponential and stationary phases of growth in laboratory culture. The QS signal is *N*-(3-oxohexanoyl)-L-homoserine lactone (3-oxo-C6-HSL); its synthesis is catalysed by the CarI synthase (Bainton *et al.*, 1992a, b; Jones *et al.*, 1993). QS signals are normally sensed by members of the LuxR family of transcriptional regulators (reviewed by Whitehead *et al.*, 2001; Barnard & Salmond, 2005; Waters

& Bassler, 2005). In *Ecc* strain ATTN10, three *luxR* homologues have been identified. The *expR* gene is located adjacent to *carI* in the genome; however, the function of ExpR is unclear since an *expR* mutant appears to have no obvious phenotype in this strain (Rivet, 1998). Exoenzymes are controlled via the LuxR homologue VirR (Burr *et al.*, 2006), while Car production is activated by the third LuxR homologue, CarR (McGowan *et al.*, 1995, 2005). In addition to these QS inputs, Car and exoenzyme production are coordinately regulated by the Hor global activator, which is a member of the SlyA family of transcriptional regulators (Thomson *et al.*, 1997; McGowan *et al.*, 2005).

While carrying out a screen for transposon-induced *Ecc* mutants showing defects in carbapenem production, we isolated two streptomycin-resistant derivatives of strain ATTN10 that showed reduced Car production, but did not appear to carry the transposon. The streptomycin resistance appeared to be a spontaneous mutation in these isolates, and we hypothesized that the impact on Car production may have been due to the same spontaneous mutation. We therefore carried out further experiments to isolate more spontaneous streptomycin-resistant mutants, without using transposon mutagenesis. Here, we describe

Abbreviations: Car, 1-carbapen-2-em-3 carboxylic acid; DiGE, difference gel electrophoresis; *Eca*, *Erwinia carotovora* subsp. *atroseptica*; *Ecc*, *Erwinia carotovora* subsp. *carotovora*; 3-oxo-C6-HSL, *N*-(3-oxohexanoyl)-L-homoserine lactone; QS, quorum sensing.

the nature of the corresponding mutations and their pleiotropic effects on Car antibiotic production, plant cell wall degrading enzymes, virulence and antibiotic resistance.

METHODS

Bacterial strains and culture conditions. All bacterial strains (Table 1) were cultured in LB medium (10 g tryptone, 5 g yeast extract, 5 g NaCl per litre) unless otherwise stated. For agar plates, Bacto agar (1.6 %, w/v) was added (or 0.7 %, w/v, for top agar layers). Growth temperatures were 30 °C for *Ecc* and 37 °C for *Escherichia coli* (*E. coli*) strains, unless otherwise stated. For isolation of streptomycin-resistant mutants, overnight cultures of strains ATTN10 or MS1 (a Lac⁻ derivative of ATTN10) were used to inoculate 50 ml LB to a starting OD₆₀₀ of 0.05. Cultures were incubated for 24 h (at 30 °C in a shaking incubator set at 250 r.p.m.), before resuspension in 1 ml LB and plating in 100 µl aliquots onto LB agar containing 100 µg streptomycin ml⁻¹.

DNA manipulations. Molecular biology techniques were performed as described by Sambrook *et al.* (1989). Enzymes were purchased from NEB or Invitrogen and used according to manufacturers' instructions. The Expand High Fidelity PCR system (Roche) was used in PCR. Oligonucleotides (Table 3) were purchased from Sigma-Genosys. DNA sequencing was carried out by the DNA Sequencing Facility, Department of Biochemistry, University of Cambridge, UK.

φKP-mediated generalized transduction. The *Ecc*-specific generalized transducing bacteriophage φKP was used to move chromosomal markers between *Ecc* strains, as described by Toth *et al.* (1993).

Antibiotic bioassay. Carbapenem antibiotic production was assayed via the inhibition of growth of a β-lactam-sensitive *E. coli* strain (ESS). A top agar layer seeded with ESS (100 µl overnight culture was added to 100 ml LB medium containing 0.7 %, w/v, agar) was poured onto standard LB agar plates. Aliquots (3 µl) of overnight *Ecc* cultures (normalized to an OD₆₀₀ of 1.0 by dilution with LB medium) were spotted onto the ESS lawn. Alternatively, cup wells were made in the ESS lawn using a sterilized cork borer, and the wells filled with 80 µl filter-sterilized (through a 0.2 µm filter) *Ecc* culture supernatant. Car production was indicated by a zone of antibiosis of the ESS lawn around the test site, following overnight incubation at 30 °C.

N-Acylhomoserine lactone bioassay. Bioassay plates were prepared as described for the antibiotic bioassay, except that the top agar layer was seeded with the *N*-acylhomoserine lactone sensor strain of *Chromobacterium violaceum* CV026 (1 ml of an overnight culture was added to 100 ml LB medium containing 0.7 %, w/v, agar). Aliquots (3 µl) of overnight *Ecc* cultures (normalized to an OD₆₀₀ of 1.0 by dilution with LB medium) were spotted onto the CV026 lawn. *N*-Acylhomoserine lactone production was indicated by a purple halo in the CV026 lawn around the test site, following overnight incubation at 30 °C.

β-Galactosidase assay. Samples (1 ml) were taken at hourly intervals throughout growth of strains in LB medium. Cells were permeabilized by the addition of 50 µl toluene followed by vortexing for 30 s. The organic and aqueous phases were allowed to separate and β-galactosidase assays were performed using the (aqueous) lysate. The rate of colour development at 37 °C, following the addition of ONPG, was measured using a Unicam He4ios α spectrophotometer set to measure absorbance at 420 nm. Activities are expressed as arbitrary units and are proportional to the increase in A₄₂₀ per minute

per constant cell density. Three replicates of each strain to be tested were cultured and assayed for β-galactosidase activity.

Pectate lyase, cellulase and protease agar plate assays.

Aliquots (5 µl) of overnight *Ecc* cultures (normalized to an OD₆₀₀ of 1.0 by dilution with LB medium) were spotted onto pectate lyase assay plates [1.6 % (w/v) Bacto agar, 0.1 % (w/v) yeast extract, 0.1 % (w/v) (NH₄)₂SO₄, 1 mM MgSO₄, 0.5 % (v/v) glycerol, 0.5 % (w/v) polygalacturonic acid, (sodium salt), 20 % (v/v) pel phosphate buffer (15 g Na₂HPO₄, 0.7 g NaH₂PO₄·H₂O per litre, pH 8.0)], cellulase assay plates [1.6 % (w/v) Bacto agar, 1 % (w/v) carboxymethylcellulose, 0.5 % (w/v) yeast extract, 0.2 % (v/v) glycerol, 2 % (v/v) 50 × phosphate buffer (350 g K₂HPO₄, 100 g KH₂PO₄ per litre, pH 6.9–7.1), 0.1 % (w/v) (NH₄)₂SO₄, 0.01 % (w/v) MgSO₄] or protease assay plates [Oxoid nutrient agar, supplemented with 0.03 % (w/v) gelatin]. Exoenzyme production was visualized following overnight incubation at 30 °C. Pectate lyase plates were flooded with 7.5 % (w/v) copper acetate for 1 h; positive colonies were identified by double, cream haloes on a translucent blue-green background. Cellulase plates were stained with 0.2 % (w/v) Congo red solution for 20 min, bleached with 1 M NaCl for 15 min, then stained with 1 M HCl for 5 min; positive colonies were identified by orange-red haloes on a dark blue background. Protease plates were flooded with 4 M (NH₄)₂SO₄; positive colonies were identified by clear haloes on an opaque white background.

Potato tuber virulence assays.

Potato tubers (*Solanum tuberosum* 'Maris Piper') were washed and surface-sterilized by immersion in 5 % hypochlorite solution for 10 min, followed by washing in running water. Overnight cultures of test strains were diluted in LB and approximately 10⁴ c.f.u. were injected into uniform holes which had been bored into the potato using a pipette tip. Each potato contained an inoculation site for the wild-type, in addition to those for the various mutants tested. The inoculation sites were sealed using silicon grease. Inoculated potatoes were wrapped in alternating layers of damp tissue paper and cling-film and incubated at 25 °C. The mass of soft rot was determined at 24 h intervals.

Western blot analysis.

Culture samples were harvested and resuspended in 100 mM Tris, pH 8.0, to an OD₆₀₀ of 2.0. Following sonication, cracking buffer was added to 1 × final concentration [5 × cracking buffer was made up as follows: 1 ml 0.5 M Tris/HCl (pH 6.8), 0.8 ml glycerol, 1.6 ml 10 % (w/v) SDS, 0.4 ml β-mercaptoethanol, 0.4 ml 1 % (w/v) bromophenol blue, 3.8 ml H₂O]. Samples were boiled for 5 min prior to loading equivalent amounts of total protein on a 12 % SDS-polyacrylamide gel. SDS-PAGE was performed as described by Laemmli (1970). After electrotransfer to a PVDF filter, CarA protein was immunodetected with CarA-specific polyclonal antibodies and chemiluminescent reagents (ECL + Plus, Amersham).

2D-difference gel electrophoresis (2D-DiGE).

Four overnight cultures of each of strains MS1 and M3 were used to inoculate 50 ml LB in 500 ml capacity flasks, to a starting OD₆₀₀ of 0.05. Cultures were grown at 30 °C, 250 r.p.m., for 16 h. Intracellular protein was harvested from 20 ml of culture by centrifuging at 2739 g at 4 °C for 10 min and resuspending in CHAPS/urea lysis buffer containing 1 × protease inhibitor. Each sample was sonicated (3 × 10 s bursts, keeping samples on ice). The cell debris was pelleted by centrifugation (2220 g, 15 min, 4 °C). The supernatant was taken off into a fresh tube and centrifuged again in a benchtop microfuge (max speed, 10 min, 4 °C). The supernatant was removed into a fresh tube and stored in aliquots at -20 °C. Protein concentration was estimated using the Bio-Rad DC kit. Protein samples were reciprocally labelled with fluorescent cyanine dyes, Cy3 and Cy5 (GE Healthcare), as described by Coulthurst *et al.* (2006). A pool of all samples was labelled with Cy2 to use as a standard for each gel. 2D-PAGE separation of the proteins was also carried out as described by

Table 1. Bacterial strains, plasmids and bacteriophage

Strain	Genotype/phenotype	Reference
<i>Ecc</i>		
ATTn10	Wild-type	McGowan <i>et al.</i> (1996)
MS1	Lac ⁻ derivative of ATTn10	Holden <i>et al.</i> (1998)
AB1	<i>rpsL</i> K43T, <i>Stm</i> ^R derivative of ATTn10	This study
AB2	<i>rpsL</i> K43T, <i>Stm</i> ^R derivative of ATTn10	This study
M1	<i>rpsL</i> K43N, <i>Stm</i> ^R derivative of MS1	This study
M2	<i>rpsL</i> K43R, <i>Stm</i> ^R derivative of MS1	This study
M3	<i>rpsL</i> K43T, <i>Stm</i> ^R derivative of MS1	This study
M4	<i>rpsL</i> K43T, <i>Stm</i> ^R derivative of MS1	This study
M6	<i>rpsL</i> K43T, <i>Stm</i> ^R derivative of MS1	This study
M7	<i>rpsL</i> K43R, <i>Stm</i> ^R derivative of MS1	This study
M8	<i>rpsL</i> K43R, <i>Stm</i> ^R derivative of MS1	This study
M9	<i>rpsL</i> K43N, <i>Stm</i> ^R derivative of MS1	This study
M10	<i>rpsL</i> K43R, <i>Stm</i> ^R derivative of MS1	This study
M11	<i>rpsL</i> K43T, <i>Stm</i> ^R derivative of MS1	This study
M12	<i>rpsL</i> K43T, <i>Stm</i> ^R derivative of MS1	This study
M13	<i>rpsL</i> K43R, <i>Stm</i> ^R derivative of MS1	This study
M14	<i>rpsL</i> K43R, <i>Stm</i> ^R derivative of MS1	This study
M15	<i>rpsL</i> K43R, <i>Stm</i> ^R derivative of MS1	This study
M16	<i>rpsL</i> K43R, <i>Stm</i> ^R derivative of MS1	This study
GB7	<i>carA</i> :: <i>TnphoA</i> -2' derivative of MS1, Tet ^R	Thomson <i>et al.</i> (1997)
7M1	<i>carA</i> :: <i>TnphoA</i> -2', <i>rpsL</i> K43N derivative of strain M1, generated by transduction using ϕ KP grown on GB7	This study
7M3	<i>carA</i> :: <i>TnphoA</i> -2', <i>rpsL</i> K43T derivative of strain M3, generated by transduction using ϕ KP grown on GB7	This study
7M7	<i>carA</i> :: <i>TnphoA</i> -2', <i>rpsL</i> K43R derivative of strain M7, generated by transduction using ϕ KP grown on GB7	This study
GB3	<i>carR</i> :: <i>TnphoA</i> -2' derivative of MS1, Tet ^R	Thomson <i>et al.</i> (1997)
M1GB3	<i>carR</i> :: <i>TnphoA</i> -2', <i>rpsL</i> K43N derivative of strain M1, generated by transduction using ϕ KP grown on GB3	This study
M3GB3	<i>carR</i> :: <i>TnphoA</i> -2', <i>rpsL</i> K43T derivative of strain M3, generated by transduction using ϕ KP grown on GB3	This study
M7GB7	<i>carR</i> :: <i>TnphoA</i> -2', <i>rpsL</i> K43R derivative of strain M7, generated by transduction using ϕ KP grown on GB3	This study
DET107	<i>hor</i> :: <i>lacZ</i> derivative of MS1	McGowan <i>et al.</i> (2005)
DETM1	<i>hor</i> :: <i>lacZ</i> , <i>rpsL</i> K43N derivative of DET107	This study
DETM3	<i>hor</i> :: <i>lacZ</i> , <i>rpsL</i> K43T derivative of DET107	This study
DETM7	<i>hor</i> :: <i>lacZ</i> , <i>rpsL</i> K43R derivative of DET107	This study
PRTX1	<i>carI</i> :: <i>TnphoA</i> -2' derivative of MS1, Tet ^R	Cox <i>et al.</i> (1998)
PRTM1	<i>rpsL</i> K43N, <i>carI</i> :: <i>TnphoA</i> -2' derivative of strain M1, generated by transduction using ϕ KP grown on strain PRTX1	This study
PRTM3	<i>rpsL</i> K43T, <i>carI</i> :: <i>TnphoA</i> -2' derivative of strain M3, generated by transduction using ϕ KP grown on strain PRTX1	This study
PRTM7	<i>rpsL</i> K43R, <i>carI</i> :: <i>TnphoA</i> -2' derivative of strain M7, generated by transduction using ϕ KP grown on strain PRTX1	This study
SM1	Δ <i>carA</i>	McGowan <i>et al.</i> (1997)
<i>E. coli</i>		
ESS	β -Lactam sensitive	McGowan <i>et al.</i> (1997)
Plasmids		
pQE-80L	Qiagen expression vector; <i>lacI</i> ^q , Amp ^R	Qiagen
pAB536	Derivative of pQE-80L with Amp ^R gene disrupted by insertion of a Kan ^R cassette (PCR-amplified from pACYC177) into the unique <i>Xmn</i> I site	This study
pAB539	Derivative of pAB536 carrying <i>rpsL</i> from <i>Ecc</i> strain MS1	This study
pACYC177	Medium-copy cloning vector, Amp ^R Kan ^R	NEB
Bacteriophage		
ϕ KP	Generalized transducing phage for <i>Ecc</i> strain ATCC 39048 and its derivatives	Toth <i>et al.</i> (1993)

Table 2. Car phenotypes and genotypes of *rpsL* mutants

Strain	Car phenotype	Codon 43 sequence	Amino acid at codon 43	Amino acid change
MS1, ATTn10	Wild-type	AAA	Lysine	K43
AB1, AB2, M3, M4, M6, M11, M12	Car ⁻	ACA	Threonine	K43T
M1, M9	Car ⁻	AAC	Asparagine	K43N
M2, M7, M8, M10, M13, M14, M15, M16	Car reduced	AGA	Arginine	K43R

Coulthurst *et al.* (2006). Proteins were separated over pH 4–7 in the first dimension followed by SDS-PAGE on a 12 % gel for the second dimension. Labelled proteins were visualized using a Typhoon 9410 imager (Amersham Biosciences). Gels were then fixed and stained using colloidal Coomassie blue. The images were subjected to a biological variance analysis (BVA) using DeCyder BVA software (GE Healthcare). Protein spots which were increased in abundance by more than 1.5-fold and with a corresponding *P*-value <0.01 from univariate statistical analysis using a Student's *t*-test, in the wild-type or in the mutant, were identified. For mass spectrometry identification of protein spots, the spots were cut out from Coomassie-stained gels, digested with trypsin and then analysed by LC-MS/MS using a Q-TOF mass spectrometer (Waters) coupled to an Acquity nano-chromatography system (Waters). The resulting output files were used to search against a protein database derived from the *Erwinia carotovora* subsp. *atroseptica* (*Eca*) SCRI1043 genome sequence (<http://www.sanger.ac.uk>; Bell *et al.*, 2004) using MASCOT version 2.1.6 (Matrix Science), to allow identification of the protein species present in each spot.

RESULTS

Isolation and characterization of spontaneous streptomycin-resistant *Ecc* mutants

During a screen for *Ecc* transposon mutants exhibiting either increased or decreased levels of Car antibiotic production, two streptomycin-resistant derivatives of the wild-type (ATTn10) were isolated which showed reduced Car production (AB1 and AB2; Tables 1 and 2). However, further analysis suggested that they did not carry a transposon insertion (data not shown). We hypothesized that these isolates were spontaneous streptomycin-resistant mutants, and we considered the possibility that the effect on Car production may be related to the streptomycin resistance phenotype. To investigate this further, 15 more streptomycin-resistant derivatives of MS1 (itself a Lac⁻ derivative of ATTn10), strains M1–M4 and M6–M16 (Tables 1 and 2) were isolated, by plating onto medium containing streptomycin. Spontaneous streptomycin-resistant mutants were very rare, but could be isolated at a frequency of approximately 10⁻¹³ (data not shown).

Streptomycin is an aminoglycoside antibiotic that acts by binding to the bacterial ribosome and causing mistranslation of proteins (reviewed by Kornder, 2002). Spontaneous streptomycin resistance is therefore often associated with mutations in ribosomal protein subunits or rRNA which prevent the antibiotic from binding to the ribosome. Common examples of these mutations include substitutions of lysine-43 or lysine-88 of the S12 ribosomal protein subunit, which is encoded by the *rpsL* gene (e.g. Nair *et al.*, 1993; Shima *et al.*, 1996; Sreevatsan *et al.*, 1996; Björkman *et al.*, 1998; Gregory *et al.*, 2001). In addition, a recent crystal structure study by Carter *et al.* (2000) showed that streptomycin makes contact with the ribosomal protein S12 when bound to the 30S ribosome of *Thermus thermophilus*.

To investigate the basis of streptomycin resistance in the *Ecc* isolates, the *rpsL* loci were PCR amplified and sequenced using primers AB454 and AB455 (Table 3). Each of the streptomycin-resistant isolates carried a single, point mutation in the *rpsL* gene which would result in an amino acid change at codon 43, from the *Ecc* wild-type lysine to either threonine, asparagine or arginine (Table 2). The MICs of streptomycin for mutant strains harbouring the K43N, K43T or K43R mutations (strains M1, M3 and M7 respectively) were greater than 512 µg streptomycin ml⁻¹, whereas the MIC of streptomycin for the wild-type MS1 is 16 µg ml⁻¹ (data not shown). However, as expected, these strains exhibited little or no cross-resistance to spectinomycin, gentamicin or kanamycin (data not shown).

Antibiotic production by *rpsL* mutants

The effects of the *rpsL* mutations on Car production in overnight cultures were assessed and two phenotypic groups of *rpsL* mutants were identified: group 1 mutants did not produce any detectable Car, while group 2 mutants produced reduced amounts of Car compared to the wild-type (Fig. 1a). These Car phenotypes could generally be correlated with the nature of the ribosomal protein S12

Table 3. Oligonucleotide primers used in this study

Primer	Sequence (5'–3')	Annealing temp.	Description
AB454	CTAGAATTCTGCGTCCTC	47 °C	Designed to anneal approx. 100 bp upstream of <i>Eca rpsL</i> ORF
AB455	CATGGAAATACTCCGTTG	47 °C	Designed to anneal approx. 100 bp downstream of <i>Eca rpsL</i> ORF

mutation: group 1 mutants generally carried K43N or K43T mutations, while group 2 strains carried the K43R mutation (Fig. 1a, Table 2). The exception was strain M14, which did not produce Car despite having the group 2 K43R mutation. It is possible that this strain carries another uncharacterized mutation that affects Car production. Similar results were also seen when filter-sterilized

culture supernatant samples, taken throughout growth of representative K43N, K43T and K43R mutants, were assayed for the presence of the Car antibiotic (Fig. 1b).

Since *rpsL* encodes the ribosomal protein S12, it is likely that any phenotypes associated with *rpsL* mutations are due to altered translation of one or more cellular proteins. To

Fig. 1. Car antibiotic production and *carA* transcription by *rpsL* mutants. (a) Cultures of the strains to be tested were spotted as indicated onto a top agar layer seeded with the *E. coli* β -lactam-sensitive strain *ESS*. Car antibiotic production is indicated by a zone of antibiosis of the *E. coli* lawn. (b) Car antibiotic present in filter-sterilized culture supernatants was assayed at hourly intervals throughout growth of *Ecc rpsL* mutants K43N, K43T and K43R, compared to the wild-type, K43 (strains M1, M3, M7 and MS1 respectively). The culture supernatants were placed in cup wells cut into a top agar layer seeded with *E. coli* strain *ESS*. The radii of the antibiosis haloes were measured and the data shown are the means of three independent experiments; error bars represent one standard deviation. (c) Transcription of the first gene in the *car* biosynthetic operon, *carA*, was followed using a *carA::lacZ* transcriptional reporter in the K43N, K43T, K43R and K43 (WT) *rpsL* genetic backgrounds. Samples were taken hourly throughout growth and β -galactosidase activity determined. Data shown are the means of three independent experiments; error bars represent one standard deviation.

test whether a Car regulator was involved in mediating the effect of the *rpsL* mutations on Car antibiotic production, the effect of the *rpsL* mutations on transcription of the first gene in the *car* biosynthetic operon, *carA*, was investigated. The chromosomal *carA::lacZ* transcriptional reporter fusion from strain GB7 (Table 1) was moved into the K43N, K43T and K43R genetic backgrounds (strains M1, M3 and M7 respectively) by generalized transduction. The β -galactosidase activities of the resulting strains (7M1, 7M3 and 7M7; Table 1), measured throughout growth, showed that *carA* transcription was reduced in each *rpsL* mutant background (Fig. 1c). Transcription of *carA* was affected most in the K43N and K43T backgrounds, which correlates well with the effects of the *rpsL* mutations on Car antibiotic production (Fig. 1a, b, c).

Effect of *rpsL* mutations on Car regulators

The effect of the *rpsL* mutations on transcription of the known Car transcriptional regulators (*carI*, *carR* and *hor*) was assessed. The chromosomal *carI::lacZ* and *carR::lacZ* transcriptional fusions were introduced into strains M1, M3 and M7 by generalized transduction, using the markers from strains PRTX1 and GB3 respectively (Table 1). Spontaneous streptomycin-resistant derivatives of the *hor::lacZ* fusion strain (DET107) were isolated after plating DET107 onto medium containing streptomycin, and the natures of the *rpsL* mutations were checked by PCR amplification and sequencing (Table 1). Transcription of *carI*, *carR* and *hor* was assessed by assaying β -galactosidase activity throughout growth. However, no significant differences in transcription of *carI*, *carR* or *hor* were observed in any of the *rpsL* mutants when compared with strains harbouring the wild-type copy of *rpsL* (data not shown).

The concentration of the QS signalling molecule 3-oxo-C6-HSL was also measured in samples of culture supernatants taken from representatives of each type of *rpsL* mutant; however, no significant differences were observed in the *rpsL* mutants compared to the wild-type (data not shown). This suggests that translation of CarI (the 3-oxo-C6-HSL synthase) is unaffected in the *rpsL* mutant backgrounds.

Addition of the QS signal, 3-oxo-C6-HSL, bypasses the *rpsL* phenotype

Interestingly, although *rpsL* mutants produced apparently wild-type levels of the QS signal 3-oxo-C6-HSL, Car production by these mutants was found to increase when an excess of 3-oxo-C6-HSL was provided (Fig. 2a). In addition, a Western blot analysis showed that CarA protein was undetectable in a K43T mutant in the absence of exogenously added 3-oxo-C6-HSL, but was detectable when an excess of 3-oxo-C6-HSL was present (Fig. 2b). Thus the effects of the K43N and K43T mutations on CarA protein levels and antibiotic production could be partially overcome by provision of an excess of 3-oxo-C6-HSL.

Ecc rpsL mutations have a pleiotropic effect on exoenzyme production and on virulence *in planta*

The effects of *rpsL* mutations on other phenotypes were tested. The K43T and K43N mutants were pleiotropic, exhibiting reduced production of the major exoenzymes of *Ecc* (pectate lyase, cellulase and protease; Fig. 3a) and reduced virulence in potato tuber assays (Fig. 3b). By contrast, the K43R mutant was not significantly affected in either exoenzyme production or virulence (Fig. 3a, b).

Proteomic analysis of the K43N ribosomal protein S12 mutant

To determine whether an *rpsL* mutation simply affects levels of all proteins within the cell, or affects a specific subset of proteins, a quantitative proteomic analysis was carried out. Intracellular proteins were prepared from

Fig. 2. The *rpsL* phenotype can be bypassed by the addition of excess 3-oxo-C6-HSL. (a) Car production by *Ecc* strains MS1 (K43), M1 (K43N), M3 (K43T) and M7 (K43R) was tested by spotting cultures onto a top agar layer seeded with the β -lactam-sensitive *E. coli* strain ESS. Drops (1 μ l) of a 1 mg ml⁻¹ solution of synthetic 3-oxo-C6-HSL were spotted on top of the dried-in culture as indicated. Car production was indicated by antibiosis of the *E. coli* lawn. (b) Immunodetection of CarA protein. Strains ATn10 or AB1 (K43T) were cultured in LB medium with or without 1 μ g ml⁻¹ synthetic 3-oxo-C6-HSL, to the late-exponential phase of growth before harvest. Equivalent amounts of total protein from each strain were run on a 12% SDS-PAGE gel and then electro-transferred to a PVDF membrane. CarA protein was detected using an anti-CarA antibody. As a control, the anti-CarA antibody failed to hybridize to proteins extracted from a *carA* deletion strain, SM1 (data not presented).

Fig. 3. Effects of *rpsL* mutations on virulence. (a) Exoenzyme production by *rpsL* mutants. Aliquots (3 μ l) of overnight cultures, normalized to an OD₆₀₀ of 1.0 with fresh LB medium, were spotted as indicated on the relevant assay plates. Cellulase activity is indicated by an orange-red halo on a dark blue background; pectate lyase activity is shown by a double, cream halo on a blue-green background; protease activity is indicated by a clear halo on an opaque white background. (b) Virulence in potato tuber assays. Potato tubers were inoculated with approx 10^4 c.f.u. of the strains to be tested and incubated at 25 °C. Potatoes were harvested and the mass of soft-rotted tissue was determined at each time point. Results shown are the means of at least eight replicates for each time point; error bars represent one standard error of the mean.

stationary-phase cultures of the wild-type (MS1) and a K43T ribosomal protein S12 mutant (M3), and the intracellular proteomes were subjected to a biological variance analysis (BVA). Representative images are shown in Fig. 4. A total of 55 protein spots were identified that were more abundant in the *rpsL* mutant compared to the wild-type (circled in Fig. 4a; >1.5-fold difference, $P < 0.01$), while 45 protein spots were less abundant in the *rpsL* mutant compared to the wild-type (circled in Fig. 4b; >1.5-fold difference, $P < 0.01$). Of these 100 spots, 16 were sufficiently abundant for mass spectrometry analysis (numbered in Fig. 4a and b). Most of these spots contained more than one protein species, so it was not possible to determine unequivocally which species was affected in the *rpsL* mutant. However, it was possible to identify spot no. 1, which was highly similar to ATP phosphoribosyltransferase from *Eca* strain SCRI1043. This protein showed an increased abundance in the *rpsL* mutant. ATP phosphoribosyltransferase catalyses the first step in histidine biosynthesis, the formation of *N*-1-(5'-phosphoribosyl)-ATP from ATP and 5-phosphoribosyl 1-pyrophosphate (Ames *et al.*, 1961; Voll *et al.*, 1967). Spots 12 and 13 were identified as CarB from ATTN10, and spot 15 as CarC from ATTN10. These three protein species were all reduced in abundance in the *rpsL* mutant compared to the wild-type, which is consistent with the Car phenotype of the *rpsL* mutant.

DISCUSSION

In this study, 17 'spontaneous' streptomycin-resistant derivatives of *Ecc* strain ATTN10 were isolated, carrying a single point mutation within the *rpsL* gene. These point mutations were predicted to cause an amino acid substitution at codon 43, from the wild-type lysine, to threonine, asparagine or arginine. The MIC of streptomycin for representative K43T, K43N and K43R mutants was determined to be $>512 \mu\text{g ml}^{-1}$ in each case, compared to the wild-type MIC of $16 \mu\text{g streptomycin ml}^{-1}$.

The K43R mutants exhibited reduced Car production and slightly reduced virulence compared to the wild-type strains. By contrast, the K43T and K43N mutants produced no detectable Car, reduced amounts of the exoenzymes pectate lyase, cellulase and protease, and were reduced in virulence in potato tuber assays. The effects of the *rpsL* mutations on Car production were shown to be due to reduced transcription of the *car* biosynthetic genes. In contrast to our results, other studies involving several *Streptomyces* species have associated mutations in *rpsL* with increases in the amount of antibiotic produced (e.g. Shima *et al.*, 1996; Okamoto-Hosoya *et al.*, 2003a, b; Hu & Ochi, 2001). In *Streptomyces coelicolor* A3(2), a K88E *rpsL* mutant exhibited increased actinorhodin production (in addition to streptomycin resistance) due to an increase in protein synthesis caused by upregulation of expression of the

Fig. 4. Intracellular proteomic analysis of an *Ecc* K43T ribosomal protein S12 mutant. Results are shown for a representative experiment in which proteins originating from the wild-type were labelled with Cy5 and proteins originating from the K43T mutant were labelled with Cy3. (a) Cy5 image of 2D-DiGE gel with protein spots which are decreased in abundance in the *rpsL* mutant circled in green. The circled and numbered protein spots (9–16) were excised from the gel and analysed by mass spectrometry. (b) Cy3 image of 2D-DiGE gel with protein spots which are increased in abundance in the *rpsL* mutant circled in red. The circled and numbered protein spots (1–8) were excised from the gel and analysed by mass spectrometry.

ribosome recycling factor (Okamoto-Hosoya *et al.*, 2003a; Hosaka *et al.*, 2006). K43N, K43T and K43R mutations resulted in streptomycin resistance, but had no impact on antibiotic production in *S. coelicolor* (Okamoto-Hosoya *et al.*, 2003a). A recent study by Kurosawa *et al.* (2006) showed that amylase and protease production in *Bacillus subtilis* strain 168 was enhanced in *rpsL* K56R mutants (equivalent to K43R of *E. coli* or *Ecc*), but production was unaffected in K56T and K56N mutants. In a study by Björkman *et al.* (1998), *Salmonella rpsL* mutations were shown to have an effect on virulence in a mouse model. K43T and K43N mutants were less able to compete with wild-type *Salmonella typhimurium* strain LT2 in the mouse model. However, K43R mutants were able to compete effectively with the wild-type LT2.

Since *rpsL* encodes the ribosomal protein S12, it is possible that translation of one or more proteins is directly affected in the *rpsL* mutants and the effect on *carA* transcription may be an indirect one, potentially due to a reduction in translation of a Car regulatory protein. Three Car regulators are known to date: CarI (the 3-oxo-C6-HSL synthase), CarR (the LuxR homologue which responds to the 3-oxo-C6-HSL signal) and Hor (a SlyA family global regulator). Transcription of the genes encoding each of

these three proteins appeared unaffected in the *rpsL* mutants, although it is formally possible that translation of the corresponding messages could be altered. We have shown that production of the 3-oxo-C6-HSL QS signalling molecule is not significantly affected in the *rpsL* mutant background under the conditions tested, which strongly suggests that the *rpsL* mutations do not affect CarI enzyme synthesis. It is interesting that the Car phenotypes of K43N and K43T *rpsL* mutants can be partially bypassed by the addition of 3-oxo-C6-HSL. The Car and exoenzyme phenotypes of *hor* mutants have also been shown to be bypassed by a physiological excess of 3-oxo-C6-HSL (McGowan *et al.*, 2005). Therefore, it is an attractive hypothesis that Hor protein levels may be altered in these *rpsL* mutants, as this could account for the observed Car, exoenzyme and virulence phenotypes. However, it has not been possible to verify this by Western blot analysis, due to a lack of an anti-Hor antibody with sufficient specificity for Hor (data not shown).

Another possibility was that *rpsL* mutations might simply result in a global reduction in translation of all cellular proteins. The *rpsL* mutation did not adversely affect growth rate under the conditions tested (for example, see Fig. 1), which argues against a widespread effect on the intracel-

lular proteome. However, in order to investigate the effects of the K43T mutation further, we performed a quantitative proteomic comparison of all intracellular proteins extracted from the K43T mutant compared to the wild-type. The results indicated that the abundance of a small subset of intracellular proteins is affected by the K43T mutation: 55 protein spots were increased in abundance in the *rpsL* mutant, while 45 protein spots were decreased in abundance (Fig. 4). The *rpsL* mutations therefore affect a subset of the intracellular proteome. It is possible that the protein species affected by these *rpsL* mutants may share common characteristics, such as their amino acid composition, or share similarities in the codon usage of the corresponding transcripts. This would be an interesting area for further investigation. Three of the protein species that were reduced in the *rpsL* mutant were identified as CarB or CarC, which is consistent with the effect of the *rpsL* mutation on carbapenem antibiotic production in *Ecc*. It is not known whether any of the other proteins that are modulated in the *rpsL* mutants play a regulatory role in the multiple phenotypes exhibited by the mutant. However, given that regulatory proteins are usually present only in low abundance, it is more likely that these up- or down-modulated proteins identified in the proteomic analysis of the *rpsL* mutant represent additional targets within the regulon influenced pleiotropically by ribosomal protein S12.

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