JOURNAL OF BACTERIOLOGY, Nov. 2007, p. 7643–7652 Vol. 189, No. 21
0021-9193/07/$08.00+0 doi:10.1128/JB.00920-07

RelA-Dependent (p)ppGpp Production Controls Exoenzyme Synthesis in *Erwinia carotovora* subsp. *atroseptica*▽

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Received 12 June 2007/Accepted 22 August 2007

In this report, we investigate the link between nutrient limitation, RelA-mediated (p)ppGpp production, and virulence in the phytopathogen *Erwinia carotovora* subsp. *atroseptica*. A *relA* null mutant (JWC7) was constructed by allelic exchange, and we confirmed that, unlike the wild-type progenitor, this mutant did not produce elevated levels of (p)ppGpp upon nutrient downshift. However, (p)ppGpp production could be restored in strain JWC7 during nutrient limitation by supplying *relA* in *trans*. During growth on exoenzyme-inducing minimal medium, the *relA* mutant showed a diminution in secreted pectate lyase and protease activities and a severe defect in motility. The *relA* mutant was also impaired in its ability to cause rot in potato tubers. In the presence of serine hydroxamate (a competitive inhibitor of seryl tRNA synthase and a potent inducer of the stringent response in wild-type *E. carotovora* subsp. *atroseptica*), exoenzyme production was essentially abolished in JWC7 but could be restored in the presence of plasmid-borne *relA*. The inhibition of exoenzyme production in JWC7 caused by serine hydroxamate could not be overcome by addition of the quorum-sensing signal molecule, *N*-3-oxohexanoyl-L-homoserine lactone. Quantitative reverse transcription-PCR analysis of selected RNA species confirmed that the effects of *relA* on secreted pectate lyase activity and motility could be attributed to a reduction in transcription of the corresponding genes. We conclude that nutrient limitation is a potent environmental cue that triggers (p)ppGpp-dependent exoenzyme production in *E. carotovora* subsp. *atroseptica*. Furthermore, our data suggest that nutrient limitation [or rather, (p)ppGpp accumulation] is a prerequisite for effective quorum-sensing-dependent activation of exoenzyme production.

Erwinia carotovora subsp. *atroseptica* is a motile gram-negative opportunistic phytopathogen. Although it does not easily penetrate the outer surface of healthy roots or the aerial portions of plants, it readily infects tissue that has been damaged, e.g., through mechanical abrasion. In temperate regions, the species is best known for causing potato blackleg, a form of stem rot in growing plants, and soft rot in stored tubers. Due to the significant economic impact of *E. carotovora* subsp. *atroseptica*, the genome of *E. carotovora* subsp. *atroseptica* SCRI1043 has been sequenced (5). *E. carotovora* subsp. *atroseptica* produces numerous secreted enzymes that macerate plant tissues, releasing nutrients that enable rapid bacterial growth. The exoenzymes produced by *E. carotovora* subsp. *atroseptica* are primarily involved in degrading plant cell walls and include a range of pectate lyases (Pels), polygalacturonases, pectin methylesterases, proteases (Prts), and cellulases (Cels) (4). Over the last decade, it has become increasingly clear that production of many of these secreted virulence factors are under the control of an *N*-acyl-homoserine lactone (AHL)-dependent quorum-sensing system in *E. carotovora* subsp. *atroseptica* (28, 42). The main AHLs produced by strain SCRI1043 appear to be *N*-(3-oxohexanoyl)-L-homoserine lactone (OHHL) and *N*-(hexanoyl)-L-homoserine lactone. Both of these signaling molecules are made by the ExpI protein. Virulence factor production is greatly reduced in *expI* mutants as a consequence of inability to make AHLs (7).

Like most microbial genomes, the *E. carotovora* subsp. *atroseptica* genome is small, containing around 4,500 genes (5), many of which are nonessential and involved in responding to specific environmental challenges. One of the most frequently encountered environmental challenges is nutrient limitation—particularly amino acid starvation—which initiates a pleiotropic set of cellular changes collectively known as the stringent response (SR). During the SR, the cell diverts its metabolic resources away from macromolecular biosynthesis towards the de novo production of amino acids. Consequently, among the first manifestations of the SR is the shutting down of stable RNA (comprised of rRNA and tRNA) synthesis (9). This, in turn, leads to a reduction in the growth rate of the organism (transcription of the *rrn* operons is tightly coupled to the growth rate) (10, 11). The stringent response has been implicated in controlling virulence factor production in a number of pathogens (17, 20, 23, 31, 50).

The SR is brought about as a result of the rapid accumulation of two small molecule effectors (known as alarmones), guanosine 3′-diphosphate 5′-triphosphate (pppGpp) and guanosine 3′5′-bispyrophosphate (ppGpp) (34). The former metabolite is rapidly converted into the latter by a specific phosphatase. These compounds are synthesized through ATP-dependent phosphorylation of GTP and GDP (respectively). In enteric bacteria, RelA is the primary pppGpp biosynthetic enzyme. RelA forms a physical association with the ribosome and produces pppGpp when the ribosome stalls due to the

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▽ Published ahead of print on 31 August 2007.

TABLE 1. Bacterial strains, bacteriophage, and plasmids used in this study

Bacterial strain, bacteriophage, or plasmid	Relevant genotype or characteristics[a]	Reference or source
Erwinia carotovora subsp. *atroseptica* strains		
SCRI1043 (ATCC BAA-672)	Wild-type *E. carotovora* subsp. *atroseptica*	25
JWC7	SCRI1043 *relA*::Cm^r	This study
JWC8	SCRI1043 *rumA*::Tn Cm^r	This study
TB6	SCRI1043 *expI*::*lacZ*	7
Escherichia coli strains		
BW20767	Host strain for pDS1028	47
CC118λ*pir*	Host for suicide plasmid, pKNG101	24
HH26	Mobilizing strain for conjugal transfer	13
Bacteriophage ϕM1	*E. carotovora* subsp. *atroseptica* generalized transducing phage	51
Plasmids		
pCL1920	Low-copy-number cloning vector; Str^r	32
p*relA*	pCL1920 containing cloned *E. carotovora* subsp. *atroseptica relA*	This study
pDS1028	Plasmid (Tet^r) carrying Tn Cm^r plasposon	47
pNJ5000	Mobilizing plasmid used in marker exchange (Tet^r)	21
pKNG101	Allelic exchange vector; *sacB* Str^r	29
pKNGΔ*relA*	pKNG101 containing *relA* ORF disrupted with Cm^r cassette from pACYC184	This study
pACYC184	Cloning vector; Tet^r Cm^r	NEB
pBluescript II KS	Cloning vector; Amp^r	NEB
pBSΔ*relA*	pBluescript II KS containing *relA* ORF disrupted with Cm^r cassette from pACYC184	This study

[a] Cm^r, chloramphenicol resistance; Str^r, streptomycin resistance; Tet^r, tetracycline resistance; Amp^r, ampicillin resistance.

binding of uncharged tRNA in the A site, as happens during amino acid limitation (34). In many bacteria, a second (p)ppGpp synthase, SpoT, synthesizes the alarmone in response to carbon, phosphate, or iron limitation (36, 40, 45, 53). SpoT is bifunctional and can also break down ppGpp through a hydrolytic mechanism. All bacteria studied so far contain at least one RelA or SpoT ortholog, indicating that the SR plays a highly conserved role in prokaryotic stress survival (36).

Although perhaps best known for its role in controlling rRNA and tRNA biosynthesis, (p)ppGpp also plays a key role in regulating a large number of mRNA transcripts. It does so by binding to the β subunit of the RNA polymerase (RNAP), causing the latter to alter its promoter selectivity. This leads to the activation or repression of gene expression. The ppGpp/RNAP complex may affect global gene expression in three ways. First, the ppGpp/RNAP complex may stimulate the expression of other global regulators, which in *Escherichia coli* include the stationary-phase factor (RpoS), the leucine-responsive regulatory protein (Lrp), and integration host factor (26). However, in the absence of (p)ppGpp, expression of RpoS alone is insufficient to activate expression of the *rpoS* regulon (30). Therefore, it seems that the ppGpp-dependent conformational change in RNAP is a prerequisite for tight binding of RpoS to RNAP (and consequently, stimulation of the *rpoS* regulon). Second, the binding of ppGpp to RNAP may lower its affinity for certain promoters, e.g., the powerful P1 promoter of the *rrn* operons (11). This probably primarily explains the reduction in stable RNA associated with the SR. Finally, it is becoming increasingly clear that the packaging of DNA alters the topological state of SR-sensitive promoters, thereby altering their sensitivity to ppGpp (11).

We reasoned that nutrient limitation may be one of the triggers that facilitate production of virulence factors by *E. carotovora* subsp. *atroseptica*. Therefore, in the current study, we examined how disruption of the *relA* gene affects the production of secreted virulence factors in this organism. Our data show that the *relA* mutation has pleiotropic effects and that these appear to be largely independent of the quorum-sensing system. We conclude that RelA-mediated (p)ppGpp production in *E. carotovora* subsp. *atroseptica* constitutes a distinct layer of control over virulence and may function to tie exoenzyme production in with the nutritional requirements of the cell.

MATERIALS AND METHODS

Bacterial strains, bacteriophage, plasmids, and growth conditions. The bacterial strains, bacteriophage, and plasmids employed in this study are listed in Table 1. Unless otherwise indicated, the *E. carotovora* subsp. *atroseptica* strains were grown in baffled flasks at 25°C in Pel minimal medium (PMM) as described previously (12). When required, antibiotics were used at the following concentrations: kanamycin (Kn) at 50 μg/ml, ampicillin (Ap) at 100 μg/ml, streptomycin (Sm) at 100 μg/ml, tetracycline (Tet) at 10 μg/ml, and chloramphenicol (Cm) at 50 μg/ml.

Construction of the *relA* null mutant. A null mutant of *relA* gene (Δ*relA*) was constructed in an *E. carotovora* subsp. *atroseptica* SCRI1043 wild-type background by allelic exchange. The upstream and downstream regions of the *relA* gene were amplified by PCR using the oligonucleotide primers JW01Xb, JW02Ba, JW03Hi, and JW04Sa (Table 2). The chloramphenicol resistance (*cat*) cassette was PCR amplified from pACYC184 (New England Biolabs [NEB], Ipswich, MA) using primers JW05Ba and JW06Hi. The three amplified DNA fragments were digested with appropriate restriction enzymes (Table 2), then joined in a three-way ligation, and cloned into pBluescript II KS(+) (NEB) to generate pBSΔ*relA*. The *relA*::Cm^r fragment was excised from pBSΔ*relA* and cloned into the suicide vector pKNG101 (29), generating the marker exchange plasmid pKNGΔ*relA*. Marker exchange mutagenesis with pKNGΔ*relA* was car-

TABLE 2. Oligonucleotide primers used in this study

Primer[a]	Sequence of primer (5′ → 3′)[b]
JW01Xb	GCTCTAGATCAGTTCCCCGAAAATATGC (XbaI)
JW02Ba	CGGGATCCTGGTTTTGTGTTTTGCCTCA (BamHI)
JW03Hi	CCCAAGCTTGTCGCGACTTTGCTGCTTAT (HindIII)
JW04Sa	ACGCGTCGACGCAGGGTGTCTCGGTTTATC (SalI)
JW05Ba	CGGGATCCTGTGACGGAAGATCACTTCG (BamHI)
JW06Hi	CCCAAGCTTCTGCCATTCATCCGCTTATT (HindIII)
JW07Xb	GCTCTAGAATACCGGACACCTTGAGTCG (XbaI)
JW08Hi	CCCAAGCTTGACAAGCTGGAGAGGTTTCG (HindIII)
expI(f)	GGCCGTGCAATGTACTG
expI(r)	GTAATCATGTTAGGGTATTTTGTTTC
expR(f)	CTCCCTTTGCAAGGAGATGA
expR(r)	CACAACGTTGCCGATATGAA
fliC(f)	GAACGATCCTAACGGCAAAAC
fliC(r)	AGTGTTAGCGCCAACCTGAAT
pelC(f)	CGCTGGGCTGTTATTACTTG
pelC(r)	TCGATAATATCAATGATATCTTGCATAG
relA(f)	AACGATGTTTTTCCCAAACG
relA(r)	GCATCGAAATGGTGGAAATC
rpoS(f)	CGTCGGGCAAAATAAACTTC
rpoS(r)	GATAATGACTCGGCGGAAGA
spoT(f)	GCCATCAGCGTTTCGTAATC
spoT(r)	GCCCGAAGATCAGATCAAAC
virR(f)	CCCGTCTCAGGTTGTGATC
virR(r)	CGATGTTGTCTTCCCAAGAG
fur(f)	TGCTGCGTCAATTCAAAGAC
fur(r)	AGGATCCTGTCAGCCATCAC
rsmA(f)	GATCGGCGATGAGGTAAC
rsmA(r)	GGCCTGAATACGCTGATAG
celV(f)	TGACGTTAGGTGTGGTAACAAC
celV(r)	CTCTCAGTTGCAC TCTTTTTCC

[a] The f and r letters in parentheses after the gene indicate forward and reverse orientation, respectively.

[b] Where appropriate, the restriction enzyme recognition sites incorporated into the primers are underlined and listed in parentheses after the sequence.

ried out as described previously by McGowan et al. (35). Chromosomal disruption of the *relA* gene was confirmed by PCR analysis using primers complementary to the *cat* cassette and the *relA* gene outside of the region used in marker exchange. The resulting *relA* mutation was then reintroduced into wild-type *E. carotovora* subsp. *atroseptica* SCRI1043 by generalized transduction, using bacteriophage ϕM1 (51) as a vehicle. The resulting *relA* mutant was denoted JWC7.

Construction of plasmid for the expression of *relA* in *trans*. The promoterless *relA* and flanking sequence (2,636 bp in total) with its native ribosome binding site was PCR amplified from SCRI1043 chromosomal DNA using oligonucleotide primers JW07Xb and JW08Hi (Table 2). The PCR fragment was digested with the appropriate restriction enzymes (XbaI and HindIII) and cloned into the low-copy-number plasmid pCL1920 (32) to generate p*relA*. In this plasmid, *relA* expression is driven from the plasmid-borne *lac* promoter.

Construction of an arrayed *E. carotovora* subsp. *atroseptica* mutant library. The mobilizable plasmid, pDS1028 (47), carries a Cm^r plasposon, and to ensure proper, high-efficiency maintenance in the host strain *E. coli* BW20767 (47), it carries a tetracycline (Tet^r) gene in the plasmid backbone. Transposition in pDS1028 is driven by a hyperactive transposase (encoded in the plasmid backbone), and the plasposon is flanked by optimized inverted repeats, ensuring very high mutagenesis frequencies ($>10^{-4}$ per cell). The plasposon in pDS1028 also carries an omega (transcriptional termination) fragment, which prevents readthrough from the Cm^r gene, making this vector a useful vehicle for the creation of large numbers of "clean" knockout mutations. Plasmid pDS1028 was introduced into the wild-type strain, SCRI1043, by conjugation, and transconjugants were selected on minimal agar medium (12) containing Cm. The colonies were then patched onto Cm-containing plates, pooled for DNA preparation, and screened, essentially as described by Holeva et al. (27).

Assay of secreted exoenzymes. Pel assays were performed using a method adapted from the procedure of Starr et al. (49). The rates of polygalacturonic acid breakdown were measured spectrophotometrically at 37°C and are expressed as A_{235}/ml/min. Prt and exoenzyme plate assays were performed essentially as previously described by Pemberton et al. (41).

Siderophore assay. To assay siderophore production on plates, we used chrome azurol S plates, as described by Schwyn et al. (46). For liquid assays, we followed the procedure described in reference 46, except that we used piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES) buffer (pH 6.8) instead of piperazine buffer.

Tuber virulence assay. Potato tubers (cultivar Maris Piper) were used to perform the tuber virulence assay with the *E. carotovora* subsp. *atroseptica* wild-type and mutant strains as previously described by Burr et al. (7). Briefly, tubers were immersed in 1% Virkon, then washed in deionized water, and air dried. The dried tubers were pierced in two places with a plastic pipette tip to a depth of ca. 2 cm (one piercing for each strain assayed). The wounds were inoculated with 10 μl of a suspension of bacteria containing 300 CFU and then sealed with silicon grease. The tubers were wrapped in damp paper and cellophane and placed in a sealed bag at 25°C. Each tuber was inoculated (at separate sites) with the wild-type strain and with JWC7. Tubers were removed to assess the extent of rotting after 48 h, 72 h, or 96 h of incubation. The rotted matter was scooped out of each tuber and weighed. Rotted matter from seven tubers were measured for each time point.

OHHL assay. OHHL was assayed in liquid cultures as previously described (8). Briefly, 100-μl aliquots of culture supernatant, diluted 1/100, were dispensed into black optiplate 96-well plates. The *E. coli* sensor strain was grown in LB containing 10 μg/ml Tet to an optical density at 600 nm of 1. Following this, 100-μl aliquots were mixed with the *E. carotovora* subsp. *atroseptica* culture supernatants, and the plates were incubated at 37°C for 2 h. Luminescence was measured using an Anthos Lucy luminometer. Dilutions of OHHL were used to generate a standard curve.

Assay of (p)ppGpp production. Thin-layer chromatography (TLC) was used for the (p)ppGpp accumulation analysis. *E. carotovora* subsp. *atroseptica* cells were grown overnight in planktonic culture in LB medium containing Sm. These cells were consecutively subcultured two times into fresh LB medium containing Sm to ensure that the cells were uniformly in log-phase growth. Aliquots (10 ml) of log-phase (optical density at 600 nm, ~0.15 to 0.2) cells were sedimented (4,000 × *g*, 10 min, 20°C) and washed twice in 20 ml of sterile water. After the second wash, the bacteria were resuspended in 1 ml of water. Aliquots (50-μl volume) of the washed bacteria were diluted into an equal volume of 2× LB medium or 2× low-phosphate Pel minimal medium (LP-PMM) containing $^{32}P_i$ (specific activity, 100 μCi/ml) and in the presence or absence of 400 μM serine hydroxamate (SHX), as indicated. The samples were agitated at room temperature for 30 min, then mixed with formic acid (2 M final concentration), and left on ice for 15 min. The samples were then passed through three consecutive cycles of freeze-thawing. Cell debris was removed by sedimentation (8,000 × *g*, 10 min, 20°C), and aliquots (6 μl) of the cleared supernatant were spotted onto polyethyleneimine-cellulose TLC plates. The TLC plates were developed in 1.5 M KH_2PO_4 (pH 3.4) until the solvent front reached 17 cm above the origin. The plates were air dried and exposed to film to generate autoradiographic images. The (p)ppGpp spots migrated in the expected R_f ranges.

RNA extraction and transcription analysis. Samples of culture were harvested for RNA extraction during the late exponential phase of growth. Total RNA was prepared using the QIAGEN RNeasy kit and RNase-free DNase (QIAGEN, Hilden, Germany), according to the manufacturer's instructions. The RNA integrity was evaluated by agarose gel electrophoresis. cDNA was synthesized from 200 ng of RNA template in a reaction mixture containing 10 units of SuperScript II reverse transcriptase (Invitrogen), 1× strand buffer, 10 mM dithiothreitol, 10 pmol random hexamer (Amersham), 1 mM deoxynucleoside triphosphates, and one unit of RNAguard (GE Healthcare). The reactions were allowed to proceed for 10 min at 25°C, followed by 60 min at 42°C and finally, 15 min at 70°C. Quantitative PCR amplification was performed in 25-μl reactions containing 0.2 μl cDNA template, 0.5 μM of each respective primer, 12.5 μl of SYBR green master mix (Applied Biosystems), and 9 μl of water. The sequences of the primers used are shown in Table 2. The PCR amplifications all yield products of approximately 100 bp in length. All amplifications were carried out in optical-grade 96-well plates on an ABI Prism 7000 sequence detection system (Applied Biosystems) with a first step at 50°C for 2 min, a second step at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The fluorescence data were processed using the SDS software provided by ABI and yielded threshold cycle values for each sample. All samples were analyzed in triplicate. To obtain the relative gene dosage, 16S rRNA was used as a control, and the corrected gene dosage was derived on the assumption that the concentration of mRNA of the gene of interest/concentration of 16S rRNA equals 1 in the wild type.

RESULTS

Genetic context of the *E. carotovora* subsp. *atroseptica relA* gene and in silico analysis. The *relA* gene (ECA3569; 2,238 bp in length) is located between nucleotides 4004843 and 4007080

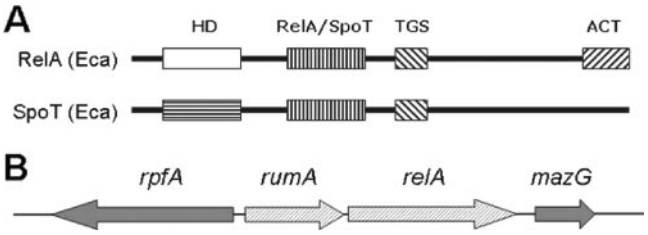

FIG. 1. (A) Domain structure of the *Erwinia* RelA and SpoT proteins. "HD" is the phosphohydrolase domain. A putatively functional copy of this domain is present in SpoT but is mutated in RelA, making this protein unable to hydrolyze ppGpp (see text for details). Following a region of conserved sequence in common between many RelA and SpoT orthologs, the TGS domain is named after its occurrence in ThrRS, GTPase, and SpoT, and it probably binds a regulatory (nucleotide) ligand. The ACT domain is presumed to have some regulatory role. Eca, *E. carotovora* subsp. *atroseptica*. (B) Genetic context of the *relA* gene in strain SCRI1043.

on the *E. carotovora* subsp. *atroseptica* SCRI1043 chromosome. The protein predicted to be encoded by ECA3569 is 84.2 kDa in size and shares 84.9% identity with the *E. coli* homolog over its entire length (745 residues). Like the *E. coli* protein, the *E. carotovora* subsp. *atroseptica* homolog lacks a catalytically active N-terminal phosphohydrolase (HD) domain (3). Instead, the H and D residues required for phosphohydrolase activity are replaced with F and P (respectively), suggesting that the *E. carotovora* subsp. *atroseptica* protein functions exclusively as a (p)ppGpp synthase. However, the *E. carotovora* subsp. *atroseptica* RelA protein retains the ACT and TGS domains that are associated with RelA and SpoT from most other organisms (Fig. 1A). The ACT domain is predicted to function in regulation, while the TGS domain is thought to play a role in nucleotide binding (18, 54, 55). *E. carotovora* subsp. *atroseptica* SCRI1043 also contains a *spoT* homolog (ECA038).

In *E. carotovora* subsp. *atroseptica* SCRI1043, *relA* is flanked by *mazG* and *rumA* (Fig. 1B). The *mazG* gene encodes a nucleotide phosphohydrolase and appears to constitute an independent transcriptional unit, while *rumA* encodes a 23S rRNA (uracil-5-)-methyltransferase. Transcription of *relA* is probably driven from a conserved putative σ^{70} promoter located between nucleotides 950 and 980 of the *rumA* gene. In *E. coli*, RumA methylates U1939 on the 23S rRNA molecule (33). Agarwalla et al. (1) have suggested that U1939 may be strategically positioned for sensing the presence of uncharged tRNA in the A site, thereby potentially linking the function of RumA with that of RelA. To investigate whether *rumA* had any possible role in virulence, we isolated a *rumA* mutant by plasposon (pDS1028) (12, 47) mutagenesis of *E. carotovora* subsp. *atroseptica* 1043. However, this mutant, carrying an insertion at position 648 of the *rumA* gene, had no obvious virulence-related phenotypes (data not shown).

A *relA* mutant does not respond to nutrient downshift by producing (p)ppGpp. The *relA*-mediated SR to nutrient limitation has been shown to affect virulence and quorum-sensing-related phenotypes in several gram-negative bacteria (14, 16, 17, 23). PCR screening of the arrayed SCRI1043 mutant bank failed to identify any mutants carrying insertions in the *relA* gene. Therefore, to investigate whether *relA* affects virulence in *E. carotovora* subsp. *atroseptica*, a *relA* null mutant was generated by using a marker exchange strategy to replace the chromosomal copy of the gene with a *cat* cassette as described in Materials and Methods. Following this, the mutation was reintroduced back into the wild-type strain by generalized transduction using bacteriophage ϕM1 (51) as a vehicle, essentially recreating the *relA* mutation in a "clean" genetic background. This was done to reduce the probability of unlinked, secondary mutations being responsible for any observed phenotype associated with the *relA* mutation. The resulting chromosomal insertion was confirmed by PCR and sequencing, and the strain was named JWC7. The *relA* mutant grew at the same rate as the wild type when cultured in LB and reached the same final optical density (data not shown), indicating that it did not suffer any obvious growth impairment.

Next, we investigated whether the disruption of RelA function in strain JWC7 affected (p)ppGpp levels in the cell. As shown in Fig. 2A, upon nutrient downshift from growth in LB medium to phosphate-limiting Pel minimal medium (PL-PMM), exponentially growing wild-type *E. carotovora* subsp. *atroseptica* (containing a control plasmid, pCL1920) rapidly produces both pppGpp and ppGpp. In contrast, very little (p)ppGpp was detectable in wild-type cells that were diluted into rich medium (LB). No (p)ppGpp accumulation was seen in JWC7 upon nutrient downshift, confirming that this strain is not responsive to amino acid limitation. To determine whether (p)ppGpp production in JWC7 could be restored in the presence of functional *relA*, we cloned the *relA* open reading frame (ORF) and its associated ribosome binding site into pCL1920 (32), creating plasmid p*relA*. The copy number of pCL1920 in an *E. coli* host is one to five copies per cell (32). In the presence of p*relA*, (p)ppGpp production was restored upon nutrient restriction in JWC7 (Fig. 2A), thus confirming that functional RelA was necessary for (p)ppGpp production.

A *relA* null mutant of *E. carotovora* subsp. *atroseptica* exhibits virulence-related phenotypes. The major phytopathogenic exoenzymes made by *E. carotovora* subsp. *atroseptica* are the Pels, which are produced only in small quantities when the cells are grown in amino acid-replete rich media such as LB (M. Welch and N. Gardiol, unpublished observations). Therefore, and to accurately gauge the impact of the SR on exoenzyme production throughout growth, we assayed Pel production in PMM. This medium is amino acid limiting, but it strongly induces secreted Pel activity (12). Pel production in the wild type rose sharply in the late exponential phase of growth (Fig. 3A) as previously reported (7, 12). No Pel production could be detected in an *expI* mutant, TB6, confirming the central role played by quorum sensing in controlling this phenotype (7). In the *relA* mutant, Pel production was delayed and reached only about half of the maximal activity observed for the wild type, even after an extended incubation period of 49 h. A similar pattern of results were obtained when we assayed another known *E. carotovora* subsp. *atroseptica* virulence factor, secreted Prt, throughout growth (Fig. 3B). We also found that the motility of the *relA* mutant was impaired compared with the wild type (Fig. 4A). Strain JWC7 also had a diminished ability to rot potato tubers; 48 h postinoculation, JWC7 generated just 0.77 ± 0.57 g of rot compared with 2.27 ± 0.83 g of rot caused by the wild type (seven tubers). This may be related to the establishment of infection at the early stages, since at later time points (72 h and 96 h), the amount of rot generated

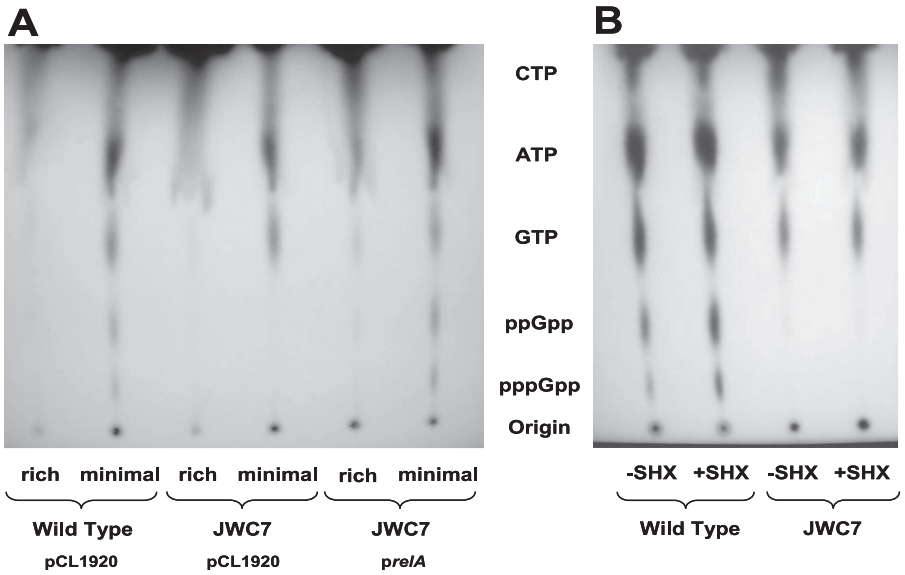

FIG. 2. (A) Effect of nutrient downshift on (p)ppGpp production in *E. carotovora* subsp. *atroseptica*. (B) Serine hydroxamate (SHX) increases (p)ppGpp production in wild-type *E. carotovora* subsp. *atroseptica*. The figures show autoradiographic images of TLC plates, developed as described in Materials and Methods.

by both strains was similar (data not shown). However, not all virulence factors were affected by the *relA* mutation; siderophores play a central role in the virulence of *Erwinia* species (15), yet production of these molecules by JWC7 was essentially normal (Fig. 4B).

The *relA* mutation can be complemented by *relA* in *trans*. The ability of plasmid-borne *relA* to complement a range of virulence-related phenotypes (Prt, Pel, secreted Cel activities, and motility) in the wild-type and JWC7 strains was examined (Fig. 5, left plates). Although p*relA* showed some ability to complement these phenotypes in JWC7, in no case did it fully restore them to wild-type levels. This may indicate that the precise timing of *relA* expression or the expression level of this gene affects virulence. With the exception of the motility phenotype, which was pronounced, indicating that motility is exquisitely sensitive to (p)ppGpp levels, the phenotypic changes associated with the *relA* mutation were rather modest. Presumably, this is because (i) over the time taken for the colonies to grow in these plate assays, residual SpoT-derived (p)ppGpp can compensate for the absence of RelA-derived (p)ppGpp,

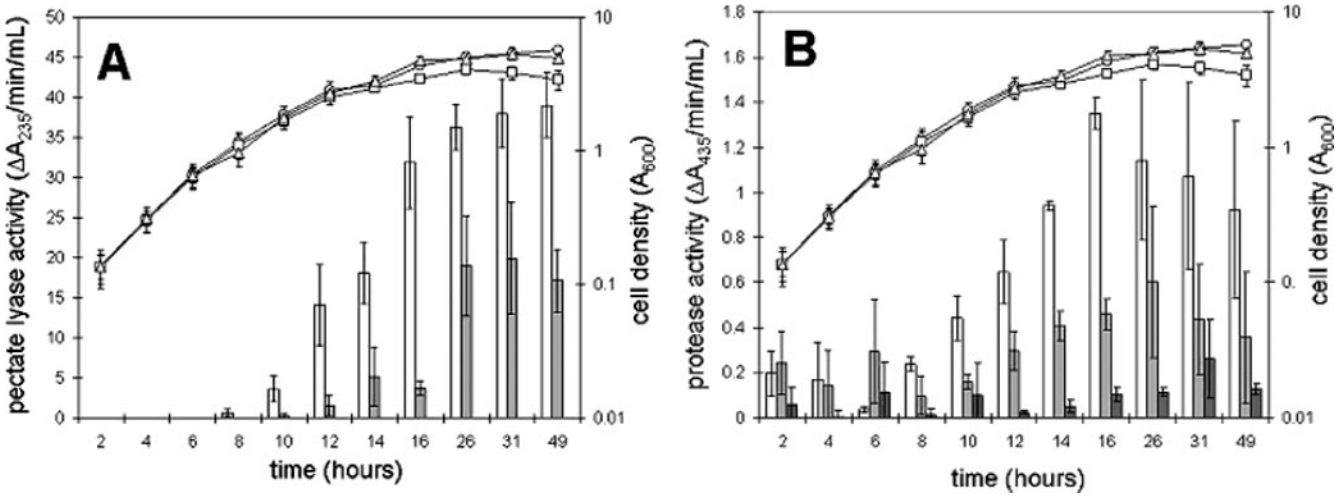

FIG. 3. Effects of *relA* and quorum sensing on growth and production of secreted pectate lyase (Pel) and protease (Prt) activities. (A) Effects of *relA* and *expI* on secreted Pel activity. (B) Effects of *relA* and *expI* on secreted Prt activity. The indicated strains were grown in PMM. Growth was recorded as optical density or absorbance at 600 nm. Pel activity was measured spectrophotometrically as the rate of breakdown of polygalacturonic acid at 235 nm. Prt activity was measured spectrophotometrically as the rate of breakdown of azocasein at 435 nm. Key to symbols: for growth curves, ○, strain SCRI1043; □, JWC7; △, TB6; for exoenzyme activities, white bars, SCRI1043; light gray bars, JWC7; dark gray bars, TB6. The *expI* mutant, produced essentially no secreted Pel activity. Each error bar represents 1 standard error of the mean. Note that the time axis is not linear for the last three time points.

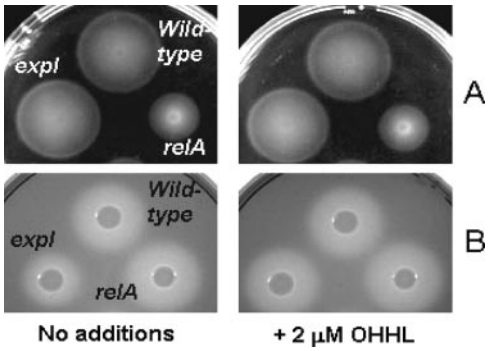

FIG. 4. Effects of the *relA* mutation and quorum sensing on motility and siderophore production in *E. carotovora* subsp. *atroseptica*. (A) Motility of the SCRI1043 (wild-type), TB6 (*expI*), and JWC7 (*relA*) strains in the presence and absence of exogenous OHHL (2 μM concentration). (B) Siderophore production by each strain. To assay motility, similar numbers of cells from each of the three indicated strains were spotted into soft (0.3% agar) LB agar in the absence (left) or presence (right) of 2 μM OHHL. Swarms were allowed to develop for 24 h before the plates were photographed. Siderophore production was assayed on chrome azurol S plates. The size of the halo around each colony is proportional to the amount of secreted siderophore present (7).

and (ii) the colonies do not encounter severe amino acid limitation. To investigate this further, we repeated the experiment in conditions expected to elicit severe amino acid starvation. This was done by supplementing the plates with SHX. This compound is a competitive inhibitor of seryl tRNA synthase and is a potent inducer of the SR in other gram-negative bacteria. As a first step and to confirm that SHX increases (p)ppGpp synthesis in *E. carotovora* subsp. *atroseptica*, we examined whether SHX affected the (p)ppGpp content of the cell. TLC analysis (Fig. 2B) revealed that SHX treatment did indeed increase the amount of (p)ppGpp in wild-type *E. carotovora* subsp. *atroseptica* compared with the untreated control and that SHX addition had no apparent effect on the very low level of (p)ppGpp production by JWC7. In the presence of SHX, the exoenzyme phenotypes associated with JWC7 were more pronounced (Fig. 5, right plates). Indeed, although the wild type continued to produce exoenzymes in these conditions, Prt production by JWC7 was essentially abolished and Pel activity was reduced to very low levels. This may be due in part to the fact that as an inhibitor of seryl tRNA synthase, SHX also inhibits translation, and in line with this, we found that this compound reduced the growth rates of both wild-type *E. carotovora* subsp. *atroseptica* and JWC7 (data not shown). However, in the presence of p*relA*, the exoenzyme phenotypes and motility were restored in JWC7, suggesting that the primary effect of SHX on these phenotypes was mediated through (p)ppGpp. We conclude that the virulence phenotype(s) of JWC7 is directly attributable to the *relA* mutation, rather than some secondary mutation elsewhere in the genome.

The effects of *relA* are not mediated via quorum sensing. We wondered whether the diminution in secreted exoenzyme activity in the *relA* mutant upon amino acid starvation might be a consequence of reduced quorum sensing. We investigated this by testing whether exogenous OHHL could restore secreted Prt production in the SHX-treated JWC7 strain (Fig. 6). In the absence of OHHL, the noncomplemented *relA* mutant and the *expI* mutant produced almost no secreted Prt activity. In contrast, the complemented *relA* mutant produced nearly wild-type levels of Prt. However, in the plate containing OHHL, the noncomplemented *relA* mutant continued to produce very little secreted Prt activity, even though Prt production in the *expI* mutant was restored to wild-type levels. This shows that OHHL cannot overcome the RelA-dependent reduction in exoenzyme production caused by SHX. We also

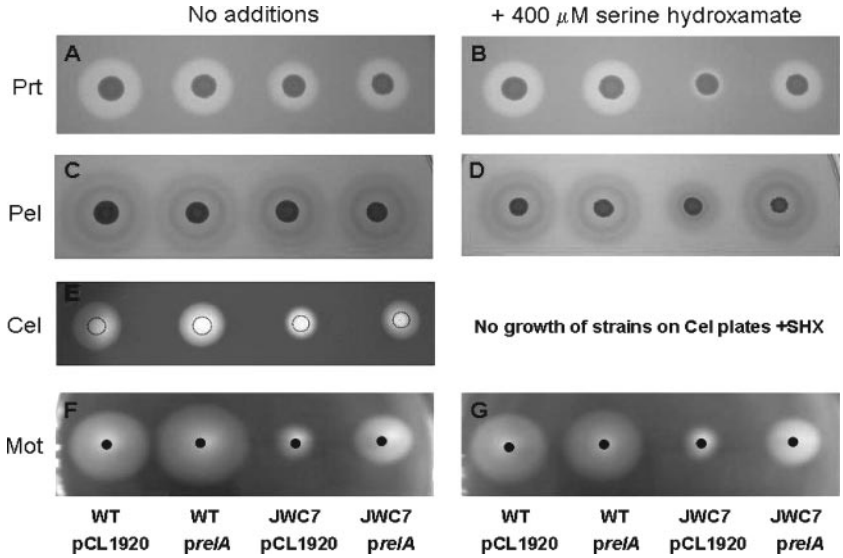

FIG. 5. Complementation of the exoenzyme and motility phenotypes associated with the *relA* mutant by plasmid-borne *relA*. Indicator plates show production of secreted protease (Prt) (A and B), pectate lyase (Pel) (C and D), cellulase (Cel) (plate E), and motility (Mot) (F and G). The plates in the panels on the left-hand side of the figure contained no other additives, while those on the right contained 400 μM SHX. Plates were spotted with the indicated strains of *E. carotovora* subsp. *atroseptica* carrying the plasmids shown and incubated for 48 h (A to E) or 20 h (F and G) before development/photography. WT, wild type.

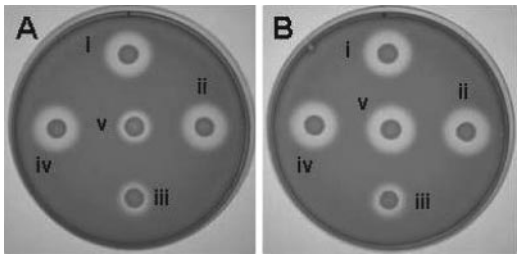

FIG. 6. Exogenous OHHL does not restore the Prt phenotype associated with the *relA* mutant. Protease plates were spotted with wild-type *E. carotovora* subsp. *atroseptica*(pCL1920) (i), wild-type *E. carotovora* subsp. *atroseptica*(p*relA*) (ii), JWC7(pCL1920) (iii), JWC7(p*relA*) (iv), and TB6 (v). The plate on the left in panel A contained 400 μM SHX, while the plate on the right in panel B contained 400 μM SHX plus 2 μM OHHL. Plates were developed after 48 h at 25°C.

assayed OHHL production (with or without SHX treatment in PMM) throughout the growth curve of the wild-type strain and JWC7. OHHL production by the mutant was similar to that of the wild type in both conditions (data not shown). Furthermore, not all of the phenotypes affected by the *relA* mutation were under quorum-sensing control. For example, the motility of the *expI* mutant was similar to that of the wild type and was unaffected by OHHL addition (Fig. 4A), indicating that this virulence phenotype is not regulated by quorum sensing.

Clearly, an alternative possibility is that the *relA* mutant is impaired in its ability to sense and respond to OHHL. However, it should be noted that not all quorum-sensing-controlled phenotypes were affected in strain JWC7, making this explanation unlikely. For example, although siderophore production is clearly controlled by quorum sensing and is lower in the *expI* mutant compared with the wild type (Fig. 4B), this phenotype was apparently unaffected by *relA*. Taken together, these results suggest that the exoenzyme phenotype associated with the *relA* mutation during an imposed stringent response is independent of quorum sensing.

Quantitative RT-PCR analysis of selected genes. To further investigate how the *relA* mutation affected exoenzyme and virulence factor production in *E. carotovora* subsp. *atroseptica*, we used quantitative reverse transcription-PCR (RT-PCR) analysis to measure the precise levels of 16S rRNA and the mRNA species encoding a selection of genes (*pelC*, *rpoS*, *virR*, *expI*, *expR*, *spoT*, *fliC*, and *relA*) in the wild-type strain and in strain JWC7 (Fig. 7). RNA samples were prepared from cells harvested in the late exponential phase of the growth curve. The absolute levels of the standard (16S RNA) in the JWC7 samples and in the wild-type samples were the same (data not shown), indicating that at this point in the growth curve at least, RT-PCR can provide an accurate assessment of gene expression. This analysis showed that transcription of the *spoT* and *rpoS* genes was altered little in the *relA* mutant. However, and consistent with the phenotypic assays, the expression of *pelC* and *fliC* were down-regulated in JWC7. Also, and as expected, no *relA* transcript could be detected in JWC7. Interestingly, three genes that are known to be involved in quorum sensing (*virR*, *expI*, and *expR*) were up-regulated in the *relA* mutant. VirR is a repressor, so up-regulation of this transcript might be expected to decrease exoenzyme production (7). As outlined in the introduction, ExpI generates OHHL, which

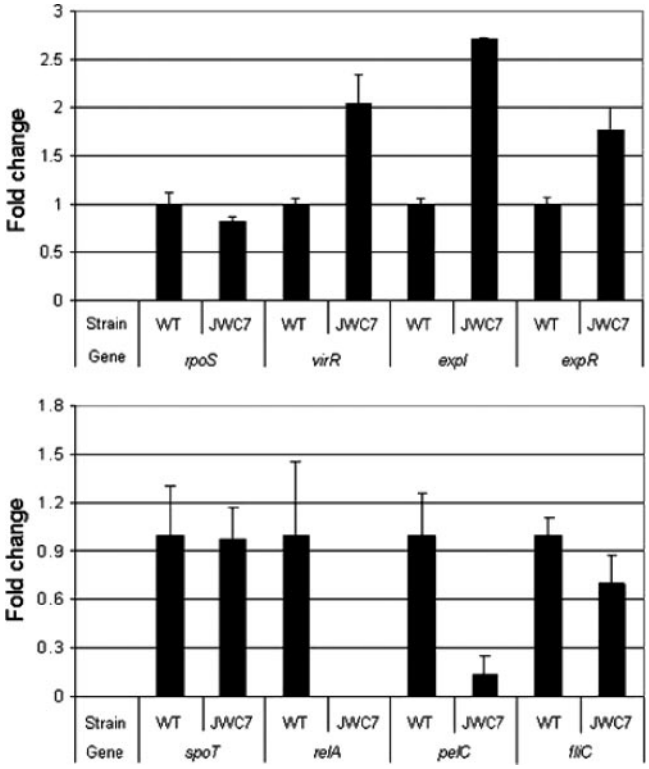

FIG. 7. Effect of *relA* on the transcription of selected genes involved in *E. carotovora* subsp. *atroseptica* virulence and adaptation. The figure shows quantitative RT-PCR results for the indicated *E. carotovora* subsp. *atroseptica* genes. Each value represents the average gene expression relative to 16S rRNA for each strain. The average values were determined from three independent RNA extractions/RT-PCR amplifications. Error bars represent standard errors of the means. Genes that were mostly up-regulated by the *relA* mutation are shown in the top graph, and genes that were mostly down-regulated by the *relA* mutation are shown in the bottom graph. WT, wild type (SCRI1043).

(according to current models [7]) should compensate for the repression brought about by VirR overexpression. The function of ExpR in strain SCRI1043 is not clear, although it is known to be convergently transcribed (and overlapping) with *expI* and can bind OHHL, albeit with low affinity (7). We also examined whether the levels of transcripts encoding a Cel gene (*celV*), the global iron uptake regulator (*fur*), and *rsmA* (a global regulator of exoenzyme production in some strains of *Erwinia* [38, 39]) were altered in JWC7, but they were not (data not shown).

DISCUSSION

In this study, we show that the *relA* gene product affects virulence factor production by the phytopathogen *E. carotovora* subsp. *atroseptica*. Our data confirm that RelA is the major (p)ppGpp-producing enzyme in *E. carotovora* subsp. *atroseptica* during nutrient downshift and that production of this alarmone is a prerequisite (during severe amino acid limitation) for quorum-sensing-dependent activation of exoenzyme production. From an anthropocentric viewpoint, this seems to make good economical sense; when the amino acid

supply for macromolecular biosynthesis becomes limiting, one obvious solution would be to increase the production of extracellular hydrolytic enzymes, which might, in the presence of a suitable host or substrate, generate an exploitable nutritional windfall.

Our data show that during growth in minimal medium, the *relA* mutant still produces exoenzymes, albeit in reduced quantity, and although virulence in the tuber is slightly impaired, the mutant strain is still capable of causing effective rot. This indicates that the basal level of (p)ppGpp in the cell, even in minimal medium, is sufficient to permit exoenzyme synthesis. Presumably, this basal level of (p)ppGpp is derived from SpoT activity. In contrast, during severe amino acid starvation (in this study, mimicked by treatment of the cells with SHX), exoenzyme synthesis was reduced to negligible levels and could not be elevated even in the presence of a high concentration of the quorum-sensing signal molecule, OHHL. This was not due to the translational inhibitory effect of SHX, since exoenzyme production could be restored in the presence of this compound by expression of *relA* in *trans*. The SR therefore imposes a distinct and very potent level of control over virulence in *E. carotovora* subsp. *atroseptica* and permits coordinated production of exoenzymes in response to an environmental constraint (nutrient availability) rather than to an intercellular signal. It may be no coincidence that maximal exoenzyme production is achieved in vitro during transition to the stationary phase, which corresponds not only to the point in the growth curve at which OHHL levels peak but also to the point when nutrient supplies become limiting. Consistent with this dual requirement for optimal virulence factor production, *E. carotovora* subsp. *atroseptica* produces very few exoenzymes in rich media, such as LB, even though the quorum-sensing signaling system is operational in these circumstances (J. Wang, N. Gardiol, and M. Welch, unpublished observations). Furthermore, exoenzyme production cannot be precociously induced early on in the growth curve through the provision of exogenous OHHL, suggesting that the timing of virulence factor production is linked to the transition into the stationary phase. However, not all virulence-related phenotypes were affected equally in the *relA* mutant, and some traits seem to be much more sensitive to variations in (p)ppGpp levels than others. For example, motility was substantially reduced in the *relA* mutant even in the absence of SHX, suggesting that this phenotype is exquisitely responsive to environmental cues.

Previous workers have shown that in other organisms, (p)ppGpp can exert effects on the physiology of the cell via modulation of quorum sensing. For example, van Delden et al. (52) reported that overexpression of *E. coli relA* in *Pseudomonas aeruginosa* advances the production of the quorum-sensing signaling molecules *N*-(3-oxododecanoyl)-L-homoserine lactone and *N*-(butanoyl)-L-homoserine lactone, although subsequently, Erickson et al. noted that overexpression of the endogenous *relA* gene in *P. aeruginosa* has little effect on these signaling molecules (14). The same team also showed that deletion of *relA* leads to a diminution in the virulence of *P. aeruginosa* towards *Drosophila melanogaster* (14). In the current work, we found that the phenotypic effects of *relA* seemed to be independent of the quorum-sensing system, and not all quorum-sensing-controlled phenotypes were affected in the *relA* mutant (e.g., siderophore production). However, on a note of caution, RT-PCR analysis showed that, of the transcripts we examined, *expI*, *expR*, and *virR* (all directly involved in quorum sensing) were significantly modulated, so the two signaling systems clearly do communicate, although more work needs to be done to elucidate the details of this.

In *E. coli* and other bacteria (6, 19, 26, 48), elevated (p)ppGpp levels lead to an increase in RpoS (sigma 38) production. This sigma factor controls the expression of a wide range of genes during stationary-phase growth and during adaptation to certain environmental stresses. Mukherjee et al. (38, 39) and later, Andersson et al. (2), independently examined how disruption of *rpoS* in *Erwinia carotovora* subsp. *carotovora* affects exoenzyme production and virulence. The former workers found that in *E. carotovora* subsp. *carotovora* strain 71, RpoS positively controls the expression of *rsmA*, whose protein product is involved in promoting the decay of selected transcripts encoding exoproducts, such as Pel, Cel, and Prt. Consistent with this, exoenzyme production was higher in an *rpoS* mutant of strain Ecc71. However, working in a different strain of *E. carotovora* subsp. *carotovora* (SCC3193), Andersson and colleagues found no elevation of exoenzyme production in an *rpoS* mutant, although the mutant did cause more severe symptoms in planta. These workers concluded that RpoS plays a more prominent role in protection and adaptation to the environmental challenges encountered during infection rather than in controlling virulence per se. In our growth conditions though, *rpoS* transcript levels in the *relA* mutant and the wild type were similar, indicating that (p)ppGpp does not increase *rpoS* transcription in strain SCRI1043, although it is of course possible that (p)ppGpp may have some posttranscriptional effect(s) on RpoS levels. Moreover, transcript levels of *rsmA* were unaffected by the *relA* mutation. Taken together, these results suggest that the effects of *relA* on exoenzyme production in SCRI1043 are more likely to be mediated directly by (p)ppGpp than via RpoS-controlled RsmA.

Our data (Fig. 2) show that in spite of the pronounced effects it has on virulence factor production, disruption of the *relA* gene in *E. carotovora* subsp. *atroseptica* does not completely eliminate (p)ppGpp synthesis in the cell. The residual low level of this alarmone in the cell is presumably derived from SpoT action, and experiments are currently under way to investigate this possibility and the role of SpoT (if any) in virulence. What is clear, however, is that the loss of *relA* in strain JWC7 is not compensated for by an up-regulation of *spoT* gene expression. This is consistent with the fact that although RelA and SpoT mediate their effects via a common effector molecule (ppGpp), these proteins respond to different environmental inputs.

The results of this study form part of a growing body of evidence which collectively indicates that virulence factor production by pathogens is tightly linked to their nutritional requirements and/or adaptation to the host environment. RelA has now been implicated in controlling the virulence of a waterborne human pathogen (*Vibrio cholerae*) (23, 40), an opportunistic human pathogen (*P. aeruginosa*) (14, 52), an enteric pathogen (*Salmonella enterica* serovar Typhimurium) (43, 45, 48), gram-positive and gram-negative intracellular pathogens (*Mycobacterium tuberculosis* [44], *Legionella pneumophila* [22], and *Listeria monocytogenes* [50]), a mucosal pathogen (*Helicobacter pylori* [37]), *Neisseria gonorrhoeae* (16), the principal

etiological agent of dental carries (*Streptococcus mutans*) (31), and a food-borne pathogen *Campylobacter jejuni* (17), to name but a few. This list can now be extended to include the soil-dwelling phytopathogen *Erwinia carotovora* subsp. *atroseptica*. Current work is aimed at elucidating in more detail the link(s) between expression of *E. carotovora* subsp. *atroseptica* virulence factors and nutritional status/growth phase.

ACKNOWLEDGMENTS

This work was funded by the United Kingdom BBSRC (grant P19162) and by the Royal Society. The research was carried out under project license PHL 177A/4627 (10/2003) from the United Kingdom Department for Environment, Food and Rural Affairs (DEFRA).

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