Elevated Temperature Enhances Virulence of *Erwinia carotovora* subsp. *carotovora* Strain EC153 to Plants and Stimulates Production of the Quorum Sensing Signal, N-Acyl Homoserine Lactone, and Extracellular Proteins

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*Erwinia carotovora* subsp. *atroseptica*, *E. carotovora* subsp. *betavasculorum*, and *E. carotovora* subsp. *carotovora* produce high levels of extracellular enzymes, such as pectate lyase (Pel), polygalacturonase (Peh), cellulase (Cel), and protease (Prt), and the quorum-sensing signal N-acetylhomoserine lactone (AHL) at 28°C. However, the production of these enzymes and AHL by these bacteria is severely inhibited during growth at elevated temperatures (31.2°C for *E. carotovora* subsp. *atroseptica* and 34.5°C for *E. carotovora* subsp. *betavasculorum* and most *E. carotovora* subsp. *carotovora* strains). At elevated temperatures these bacteria produce high levels of RsmA, an RNA binding protein that promotes RNA decay. *E. carotovora* subsp. *carotovora* strain EC153 is an exception in that it produces higher levels of Pel, Peh, Cel, and Prt at 34.5°C than at 28°C. EC153 also causes extensive maceration of celery petioles and Chinese cabbage leaves at 34.5°C, which correlates with a higher growth rate and higher levels of rRNA and AHL. The lack of pectinase production by *E. carotovora* subsp. *carotovora* strain Ecc71 at 34.5°C limits the growth of this organism in plant tissues and consequently impairs its ability to cause tissue maceration. Comparative studies with *ahlI* (the gene encoding a putative AHL synthase), *pel-1*, and *peh-1* transcripts documented that at 34.5°C the RNAs are more stable in EC153 than in Ecc71. Our data reveal that overall metabolic activity, AHL levels, and mRNA stability are responsible for the higher levels of extracellular protein production and the enhanced virulence of EC153 at 34.5°C compared to 28°C.

Genes for virulence and pathogenicity in bacteria are subject to regulation by transcriptional factors, posttranscriptional mechanisms, indigenous signals, and signals of host origin, as well as environmental factors. One of the latter factors, temperature, has been found to play a critical role in numerous instances. For example, virulence genes of human-pathogenic *Yersinia* species are activated at 37°C (44). Toxin production by several plant-pathogenic bacteria (for example, production of phaseolotoxin by *Pseudomonas syringae* pv. phaseolicola and production of coronatine by *P. syringae* pv. coronafascians), on the other hand, is highly susceptible to high temperatures since little or no toxin production results from growth at 20°C or a higher temperature (4, 35, 36, 40). Recently, Weingart et al. (51) reported that in *P. syringae* pv. glycinea, coronatine production in minimal medium and the expression of a *cmr::egfp* fusion in minimal medium and in plant tissue were much higher at 18°C than at 28°C.

Many gram-negative bacteria produce the diffusible metabolite N-acetylhomoserine lactone (AHL) or analogs of this compound, which serve as quorum-sensing signals (reviewed in references 14, 27, 37, 46, 49, and 52). AHL controls secondary metabolite production, bacterial interactions with eukaryotic hosts, and diverse phenotypes that are regulated in a cell density-dependent manner. AHL production appears to be favored at lower temperatures (i.e., 25 to 30°C), and in vitro reactions of AHL synthases have been shown to be inhibited at high temperatures (ca. 35 to 37°C) (2, 53). Extrapolating from these observations, we predicted that at elevated temperatures (ca. 34 to 35°C) AHL production would be limiting, thereby causing suppression of the phenotypes that it controls. The data presented here for *Erwinia carotovora* subspecies support this hypothesis; the only exception is *E. carotovora* subsp. *carotovora* strain EC153, which produces higher levels of AHL during growth at 34.5°C than during growth at 28°C.

The soft-rotting *E. carotovora* subspecies are characterized by their ability to produce high levels of extracellular enzymes, such as pectate lyase (Pel), polygalacturonase (Peh), cellulase (Cel), and protease (Prt). These enzymes, particularly the pectinases, are primarily responsible for the degradation of plant cell wall components, which leads to tissue maceration and cell death (3, 7). In addition, *E. carotovora* subsp. *carotovora* uses the type III secretion system to produce effector proteins, including harpin, that determine the outcomes of bacterial interactions with certain plant hosts (1, 5, 19, 28, 39). Genetic and physiological data indicate that production of these enzymes and effector proteins is coregulated by the quorum-sensing signal AHL (reference 52 and references therein), plant signals (7, 33), and various transcriptional factors (9, 11, 12, 13, 16, 24, 25, 30, 31, 42, 47). In addition, extracellular protein/enzyme production is also subject to posttranscriptional regulation by RsmA, an RNA binding protein, and *rsmB* RNA (Rsm is the regulator of secondary metabolites) (6, 10,
E. carotovora in Ecc71 and most other carotovora extremely low at 34.5°C; and (v) than at 28°C, and, in contrast, the AHL levels in Ecc71 are stability; (iv) EC153 produces higher levels of AHL at 34.5°C higher levels of transcripts in EC153 are due in part to RNA decay. The lack of extracellular Pel production at elevated temperatures (e.g., 31.2°C) by E. carotovora subsp. atroseptica has been documented previously (22). However, the basis for this temperature-sensitive exoenzyme production was not determined. In this report we document that E. carotovora subsp. atroseptica, E. carotovora subsp. betavasculorum, and most strains of E. carotovora subsp. carotovora, including Ecc71, do not produce significant levels of Pel or AHL at elevated temperatures, although they are capable of growing under these conditions. In addition, we identified two E. carotovora subsp. carotovora strains, including EC153, that produce elevated levels of extracellular enzymes or AHL at 34.5°C. Since EC153 and Ecc71 have previously been studied in various contexts (8, 26, 32, 43), we selected these two strains for a comparative analysis of their responses to elevated temperatures. These two strains were isolated from very different environments; Ecc71 was isolated in a temperate region (The Netherlands) (50), and EC153 was isolated from Mexican pepper in a tropical region (Mexico) (21).

Our data reveal that (i) EC153 grows faster and exhibits higher metabolic activity at 34.5°C than at 28°C and that the converse is true for Ecc71; (ii) EC153 has higher levels of transcripts of exoprotein genes at 34.5°C than at 28°C; (iii) the higher levels of transcripts in EC153 are due in part to RNA stability; (iv) EC153 produces higher levels of AHL at 34.5°C than at 28°C, and, in contrast, the AHL levels in Ecc71 are extremely low at 34.5°C; and (v) rsmA expression is stimulated in Ecc71 and most other E. carotovora subspecies at elevated temperatures (an exception is EC153). We concluded that the inhibition of exoenzyme production at elevated temperatures in most E. carotovora subspecies is at least partially due to mRNA decay promoted by elevated levels of RsmA.

### MATERIALS AND METHODS

**Bacterial strains, plasmids, and media.** The bacterial strains and plasmids used in this study are described in Table 1. The strains carrying drug markers were maintained on Luria-Bertani (LB) agar containing appropriate antibiotics. The wild-type strains were maintained on LB agar. The compositions of LB medium and minimal salts medium supplemented with sucrose and celery extract have been described previously (6, 33). When required, antibiotics were added to media as follows: ampicillin, 100 mg/liter; kanamycin, 50 mg/liter; spectinomycin, 50 mg/liter; and tetracycline, 10 mg/liter. Media were solidified using 1.5% agar.

**Extracellular enzyme assays.** The growth conditions, methods for preparation of culture supernatants, quantitative assay conditions for Pel, and semiquantitative agarose plate assay conditions for Pel, Peh, Prt, and Cei have been described previously (6, 33).

**DNA techniques.** Standard procedures were used for isolation of plasmids and chromosomal DNAs, electroporation, restriction endonuclease digestion, gel electrophoresis, and DNA ligation (41). PCR was performed as described by Liu et al. (24). Restriction and modifying enzymes were obtained from Promega Biotec (Madison, WI). The Primer-a-Gene DNA labeling system of Promega was used for labeling DNA probes.

**Northern blot and Western blot analyses.** Bacterial cultures were grown at 28°C and at 31.2°C or 34.5°C in minimal salts medium supplemented with sucrose and celery extract. Cells were collected when the cultures reached a Klett value of ca. 200. RNA isolation and Northern blot analysis were performed by using previously described procedures (24). The probes used were the 183-bp NdeI-DNA fragment of cell’ from Ecc71 in pGEM-T Easy (41). The probes were determined with a bicinchoninic acid protein assay kit (Pierce, Rockford, IL) used according to the manufacturer's specifications. Western blot analysis of total bacterial protein concentrations, equal amounts of cultures from each sample were precipitated with trichloroacetic acid. The protein concentrations were determined with a bicinchoninic acid protein assay kit (Pierce, Rockford, IL) used according to the manufacturer's specifications. Western blot analysis of the total bacterial protein was performed as described by Mukherjee et al. (28).

**Antisera raised against RsmA of strain Ecc71 (11) were used as probes.**

**RNA stability assays.** Cultures were grown at 28°C and 34.5°C in minimal salts medium supplemented with sucrose and celery extract to a turbidity of ca. 160 Klett units, and rifampin (Fisher Scientific Inc., Pittsburgh, PA) was added to a final concentration of 200 μg/ml. Aliquots (10 ml) were collected at 0, 2.5, 5, 7.5, and 10 h (24). RsmA functions as a global negative regulator by promoting RNA decay.

**TABLE 1. Bacterial strains and plasmids used in this study**

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
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<tr>
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<td>Erwinia carotovora subsp. betavasculorum Ecc11129</td>
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<tr>
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<td>pAKC856</td>
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<tr>
<td>pHV200</td>
<td>Ap+, 8.8-kb SalI fragment containing the lux operon</td>
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and 10 min in tubes containing 5 ml of diethyl pyrocarbonate-treated ice-cold water. Extraction of total RNA and Northern blot analysis were performed using previously described procedures (24). After blots were washed, they were exposed to an imaging plate for a bioimaging analyzer (Fujifilm Medical System USA, Inc. Stamford, CT). The Metamorph imaging system (Universal Imaging Corp., Downington, PA) was used for the densitometric analysis.

Bioluminescence assays for AHL production. 

E. carotovora strains were grown in minimal salts medium supplemented with sucrose and celery extract to a Klett value of ca. 200. Supernatants from cultures grown at 28°C and at 31.2°C or 34.5°C were collected and assayed using an Escherichia coli-based bioassay system described by Chatterjee et al. (6). There is a liner relationship between the level of AHL production and the emission of bioluminescence expressed in relative light units per minute per milliliter.

Plant tissue maceration. 

Tissue maceration tests with celery petioles and Chinese cabbage leaves were performed by using previously described procedures (33). Host plants were inoculated with 2 × 10⁸ cells per inoculation site and incubated at 28°C and 34.5°C for 24 h. The extent of tissue maceration was estimated visually.

The size of the bacterial population in a celery petiole was calculated based on the numbers of colonies that survived from the petiole at 28°C and 34.5°C. The initial inoculum was ca. 2.5 × 10⁷ cells per inoculation site. At different times after inoculation three petioles for each treatment were used for analysis. About 2 cm² tissue from each petiole around the inoculation site was ground and diluted in 55 mM phosphate buffer (pH 7.0), and serial dilutions were plated on LB agar. The plates were incubated at 28°C, and the number of colonies was calculated to determine the total population in each 2 cm² of petiole.

RESULTS

Effect of temperature on production of extracellular enzymes and AHL. 

In a preliminary trial we found that in minimal salts medium supplemented with sucrose and celery extract, E. carotovora subsp. carotovora strains Ecc71, AH2, DB26, Ecc193, EC153, and DB21 and E. carotovora subsp. betavasculorum strain Ecb11129 grew poorly at temperatures higher than 36°C but grew well at 34.5°C. E. carotovora subsp. atroseptica strain Eca12 failed to grow at temperatures higher than 33°C but grew well at 31.2°C. To examine the effects of temperature on production of Pel, we grew Ecb11129 and several E. carotovora subsp. carotovora strains at 28°C and 34.5°C and Eca12 at 28°C and 31.2°C in minimal salts medium supplemented with sucrose and celery extract, which is known to activate extracellular protein production. Figure 1A shows that EC153 and DB21 produced higher levels of Pel at 34.5°C than at 28°C. Ecc71, AH2, DB26, Ecc193, EC153, and DB21 produced higher levels of Pel at 28°C than at the elevated temperatures. DB21 and EC153 also produced higher levels of Peh, Cel, and Prt at 34.5°C than at 28°C, in contrast to...
other *E. carotovora* strains tested, including Ecc71; Fig. 1B shows the enzyme levels in EC153 and Ecc71.

AHL, the quorum-sensing signal, is required for exoenzyme production and virulence in *E. carotovora* subspecies (6, 18, 38). Therefore, the elevated levels of extracellular enzymes in EC153 were predicted to result from higher levels of AHL production. The results of a bioluminescence assay (Fig. 2A) showed that AHL levels were indeed higher in a spent culture of EC153 grown at 34.5°C than in a spent culture of EC153 grown at 28°C. The results of a Northern blot analysis of *E. coli* bioluminescence indicator strain. The bars indicate the means of three experiments, and the error bars indicate the standard deviations. For Northern blot analysis, 10 µg of total RNA was used for each lane.

Effects of temperature on primary metabolism and production of transcripts of exoenzyme genes. In a subsequent analysis of the temperature responses we used two representative *E. carotovora* subs. *carotovora* strains, strains EC153 and Ecc71, both of which grow at 34.5°C. Growth curves of these strains in minimal salts medium supplemented with sucrose and celery extract, however, revealed that the doubling time of EC153 at 34.5°C (1.2 h) was much shorter than the doubling time at 28°C (2.2 h), while the doubling time of EC153 at 34.5°C (2.3 h) was longer than the doubling time at 28°C (1.8 h) (Fig. 4A). In addition, the maximum levels of growth of Ecc71 and EC153 were similar at 28°C (Klett values, ca. 360 to 380). On the other hand, at 34.5°C the growth of EC153 reached a Klett value of ca. 380, whereas Ecc71 stopped growing at a Klett value of ca. 280. To determine if the elevated temperature had any adverse effect on the primary metabolism of these bacteria, we compared the levels of expression of 16S rRNA genes (at a Klett value of ca. 200) at 34.5°C and 28°C by Northern analysis. We chose rRNA because as part of the protein synthetic apparatus, rRNA is made at all stages of growth. Total RNAs from EC153 and Ecc71 grown at 28°C and 34.5°C were hybridized with the [α-32P]dATP-labeled 16S rRNA gene of Ecc71. As shown in Fig. 4B, EC153 contained slightly higher levels of 16S rRNA at 34.5°C than at 28°C at a Klett value of 200. By contrast, Ecc71 produced a lower level of 16S rRNA at 34.5°C than at 28°C.

To determine if the transcript levels of exoenzyme genes were affected upon growth at elevated temperatures, we determined the levels of *pel-1*, *pel-1*, and *celV* mRNAs in EC153 and Ecc71. Figure 4C shows that the levels of these transcripts in EC153 were higher at 34.5°C than at 28°C. By contrast, the levels of these transcripts in Ecc71 were much lower at 34.5°C than at 28°C (Fig. 4C).

mRNA stability and evidence for a role of RsmA. Our observations on the levels of *rsmA* transcripts and RsmA proteins in response to an elevated temperature prompted the hypothesis that at elevated temperatures most *E. carotovora* subspecies contain higher levels of RsmA, triggering RNA decay and thereby causing inhibition of AHL and exoenzyme production. To test this hypothesis, we compared the RNA stabilities of the levels of the *rsmA* transcript and RsmA protein by Northern and Western blot analyses (Fig. 3). In EC153 the levels of the *rsmA* RNA and RsmA protein were slightly lower at 34.5°C than at 28°C, in contrast to Ecc71, Eca12, and Ecb11129, in which these levels were higher at the elevated temperature than at 28°C (Fig. 3).
and exoenzyme genes of EC153 and Ecc71 grown at 28°C and 34.5°C. The results of a Northern blot analysis (Fig. 5A) revealed that in EC153 pel-1, peh-1, and ahlI RNAs were more stable at 34.5°C than at 28°C. By contrast, the mRNAs of these genes in Ecc71 were less stable at 34.5°C than at 28°C (Fig. 5B).

We next compared the levels of exoenzymes in the RsmA+/H11001 strain Ecc71 and an RsmA+/H11002 mutant of this strain. Our data showed that Ecc71 grown at 34.5°C produced a barely detectable level of Pel (Fig. 1A), and the levels of Peh, Cel, and Prt remained practically undetectable (Fig. 1B). By contrast, a readily detectable level of Pel and substantial levels of Peh, Prt, and Cel were produced by the RsmA− mutant grown at 34.5°C (Fig. 6). Since the levels of exoenzymes were lower in the RsmA− mutant at 34.5°C than at 28°C (Fig. 6), we concluded that the effects of the elevated temperature were partially relieved in the absence of RsmA and that some other factor(s) in addition to RsmA controls the temperature response.

Effect of temperature on colonization and maceration of plant tissue. The results of inoculation of celery petioles (Fig. 7A) and Chinese cabbage leaves (Fig. 7B) demonstrated that EC153 caused more extensive tissue maceration at 34.5°C than at 28°C. By contrast, Ecc71 produced much less severe symptoms at 34.5°C than at 28°C, although, as expected, it caused extensive tissue maceration at 28°C. To determine if Ecc71 is impaired in the ability to colonize celery petioles, we inoculated Ecc71 into celery petioles, incubated the petioles at 28°C and 34.5°C, and determined the sizes of the populations of bacteria in the tissue 12, 24, and 36 h postinoculation. Celery petioles similarly inoculated with EC153 served as controls. Figure 7C shows that EC153 multiplied faster at 34.5°C than at 28°C; the size of the final population at 28°C was about 60% of the size of the final population at 34.5°C. By contrast, Ecc71 multiplied faster at 28°C than at 34.5°C, although compared to the EC153 population the population was slightly smaller at 12 and 24 h postinoculation. The extremely poor growth of Ecc71 in celery petioles at 34.5°C was striking compared to the growth of Ecc71 in minimal salts medium supplemented with sucrose and celery extract at this temperature (Fig. 4A and 7C). We attributed the difference to the absence of enzyme production at 34.5°C. Since exoenzymes are presumably required to produce carbon sources for growth of the bacterium, in plant tissues enzyme-deficient bacteria could essentially face starvation. To test this hypothesis, we compared the growth of Ecc71 and the growth of EC153 in minimal salts medium supplemented with pectin as the sole carbon source at 28°C and 34.5°C. As a control, we included minimal salts medium supplemented with glucose, in which these strains should grow at both temperatures. At 34.5°C, Ecc71 barely grew in pectin medium, whereas EC153 grew well in this medium at both temperatures (Fig. 8). Both Ecc71 and EC153 grew in glucose medium at 28°C and 34.5°C. These results indicate that the inability of Ecc71 to colonize celery tissue at the elevated
temperature was at least partially due to the lack of exoenzymes, especially the pectinases.

DISCUSSION

Previous studies have documented that the level of pectinase production is reduced at elevated temperatures; this was true for Pel at 30.5°C in E. carotovora subsp. atroseptica (22), for Pel at 37°C in Erwinia chrysanthemi (17), and for pectin lyase (Pnl) at 34°C in E. carotovora subsp. carotovora (34). However, the basis for the reductions was unclear. In this study we extended these observations by documenting production of low levels of pectinases in E. carotovora subsp. atroseptica, E. carotovora subsp. betavasculorum, and E. carotovora subsp. carotovora strains. More importantly, we found that these effects correlated with the levels of quorum-sensing signals (AHLs) and the production of rsmA RNA and RsmA proteins. While most E. carotovora subsp. carotovora strains exhibited temperature-sensitive responses, we also identified exceptions. For example, E. carotovora subsp. carotovora strain EC153 grew better and produced higher levels of rRNA, AHLs, and extracellular enzymes at 34.5°C than at 28°C. Our findings, especially the higher levels of rRNA in EC153 and the shorter doubling time at 34.5°C than at 28°C, suggest that the overall metabolic activities of this strain are greater at the elevated temperature.

AHL is required for the production of exoenzymes in soft-rotting E. carotovora subspecies, including E. carotovora subsp. atroseptica, E. carotovora subsp. betavasculorum, and E. carotovora subsp. carotovora. The reduced levels of AHL in E. carotovora subsp. atroseptica, E. carotovora subsp. betavasculo-
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