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Characterization of stress-responsive genes, *hrcA-grpE-dnaK-dnaJ*, from phytopathogenic *Xanthomonas campestris*

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Abstract Sequencing of a 6.4-kb DNA fragment, cloned from the plant pathogenic bacterium *Xanthomonas campestris* pv. campestris 17 revealed five ORFs whose deduced amino acid sequences show strong similarities to the bacterial HrcA, GrpE, DnaK, DnaJ, and PdxK. The four heat shock genes are organized in the order *hrcA-grpE-dnaK-dnaJ*, a genome organization found in many gram-positive bacteria, but only in one gram-negative species (*Xylella fastidiosa*). These observations suggest that the HrcA-CIRCE system, comprising at least four genes arranged in this order, already existed for the regulation of stress responses before bacteria diverged into gram-negative and gram-positive groups. Primer-extension results suggested the presence of promoters at the regions upstream of *grpE* and *dnaK*. In the presence of stress, heat or ethanol (4%), the *X. campestris* pv. campestris 17 *grpE* and *dnaK* promoters were induced two- to three-fold over controls. Since the *grpE* and *dnaK* promoters possess *E. coli* σ^{32} promoter-like sequences, they are functional in *E. coli*, although at levels much lower than in *X. campestris* pv. campestris 17. Furthermore, expression of the *X. campestris* pv. campestris 17 *dnaK* promoter in *E. coli* was elevated by the cloned *X. campestris* σ^{32} gene, indicating that the cognate σ^{32} works more efficiently for the *X. campestris* promoters.

Keywords Stress-responsive genes · Promoter · σ^{32} · HrcA · Evolution

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Introduction

Xanthomonas campestris pv. campestris is a gram-negative plant pathogenic bacterium causing black rot in crucifers (Williams 1980). Little information is available regarding the mechanisms involved in pathogenicity and gene regulation of this organism, although some genes related to pathogenicity have been cloned and characterized (Barber et al. 1997; Chou et al. 1997; de Crecy-Lagard et al. 1990; Dow et al. 1995). Previous work from this laboratory showed that *X. campestris* recognizes most *Escherichia coli* σ^{70}-type promoters tested; but, *E. coli* recognizes only a small portion of *X. campestris* promoters and expresses these promoters at low levels (Weng et al. 1996). It appears that the σ^{70}-type promoters of *X. campestris* are structurally different from those of *E. coli*. Recently the *X. campestris rpoH* gene coding for the σ^{32} factor was cloned and the encoded protein shown to share a high degree of identity with the *E. coli* RpoH (Huang et al. 1998). Less is known about the structure of σ^{32}-type promoters of *X. campestris* and the expression of genes in response to stresses in this bacterium.

Heat shock proteins are highly conserved across prokaryotes and eukaryotes. Early studies on the regulation of heat shock proteins in *E. coli* identified a transcriptional mechanism in which RNA polymerase uses a different σ factor, σ^{32}, which recognizes sequences different from those recognized by σ^{70}-specific promoters and which is subject to feedback inhibition by products of the heat shock genes (Bukau 1993). Some of these heat shock proteins have been studied in great detail. The products of *dnaK*, *dnaJ* and *grpE*, for example, are known as molecular chaperones, playing an essential role in repair, folding, degradation and assembly of proteins under physiological conditions as well as following heat stress (Georgopoulos and Welch 1993; Hartl et al. 1994).

Recent studies of wide groups of eubacteria have revealed a variety of heat shock regulatory mechanisms, such as positive regulation by alternative σ factors and negative regulation by specific repressor-operator sys-

tems, including the HrcA-CIRCE repressor-operator system (for review, see Narberhaus 1999). HrcA is encoded by the *hrcA* gene, which has been found in many gram-positive bacteria but only in a few gram-negative bacteria (Ahmad et al. 1999; Minder et al. 2000). CIRCE elements, with a consensus nucleotide sequence of TTAGCACTC-N9-GAGTGCTAA for HrcA repressor to bind, are present in front of some major heat shock genes such as *dnaK* and *groE* (Zuber and Schumann 1994). Three types of genome organization of *hrcA* with the heat shock genes *grpE*, *dnaK*, and *dnaJ* have been found in gram-positive bacteria (Ahmad et al. 1999). The first type, organized into the *dnaK* operon with the gene order *hrcA-grpE-dnaK-dnaJ*, is present in most gram-positive bacteria studied to date, including several species of *Bacillus*, *Clostridium acetobutylicum*, and *Staphylococcus aureus*. In the other two types of organization, either *hrcA* or *dnaJ* is detached from the other genes of the operon. In gram-negative bacteria many *grpE*, *dnaK*, and *dnaJ* genes have been characterized, but only the *Agrobacterium tumefaciens*, *Bradyrhizobium japonicum* and *Caulobacter crescentus hrcA* genes have been cloned and identified (Minder et al. 2000; Nakahigashi et al. 1999; Roberts et al. 1996), and the *Xylella fastidiosa hrcA* was recently revealed by genome sequencing (Simpson et al. 2000). These four genes are also organized differently in these gram-negative bacteria. In *X. fastidiosa*, the organization of these genes is the same as described above for most gram-positive bacteria. *A. tumefaciens* and *C. crescentus* possess separated *hrcA-grpE* and *dnaK-dnaJ* clusters (Nakahigashi et al. 1999; Roberts et al. 1996), while in *B. japonicum hrcA* and *grpE* are clustered but not *dnaK* and *dnaJ* (Minder et al. 2000). Note that no *hrcA* homologue has been detected in *E. coli* (Blattner et al. 1997; Roberts et al. 1996). Therefore, it is believed that expression of the *dnaK* genes identified in gram-negative bacteria is mostly regulated by the σ^{32} homologue, but additional mechanisms of heat shock regulation that regulate expression of *dnaKJ* mRNA are likely to exist in some gram-negative organisms (Nakahigashi et al. 1998).

Here we report the molecular cloning, sequencing, and analysis of the *dnaK* locus of *X. campestris* pv. campestris. Four stress-responsive genes were identified that had the same genome organization (*hrcA-grpE-dnaK-dnaJ*) as the corresponding genes in *X. fastidiosa* and in many gram-positive bacteria. Putative σ^{32} promoters upstream of *grpE* and *dnaK* were cloned, identified, and compared for their ability to cause expression following heat shock in *X. campestris* and *E. coli*.

Materials and methods

Bacterial strains and growth conditions

Luria-Bertani broth and L agar were used as the general-purpose media (Miller 1972). Ampicillin was added at a final concentration of 50 μg/ml. *E. coli* was grown at 37 °C and *X. campestris* (Yang and Tseng 1988) at 28 °C. Heat shock was achieved by transferring the exponentially growing cells from 28 °C to 35 °C and then growing the cells further for various lengths of time as required for the different experiments. Ethanol stress was created by including 4% (v/v) ethanol in the LB medium.

PCR amplification of an internal fragment of *dnaK*

A 0.7-kb internal fragment of the *dnaK* gene was amplified by PCR using the *X. campestris* pv. campestris 17 chromosome as the template and two degenerate oligonucleotide primers, according to procedures described previously (Galley et al. 1992; Gupta and Singh 1992). The upstream primer 5′-GCYGATATCACNGTNCCGGCRTACTTYAAC-3′ and the downstream primer 5′-CCNATCGATACNRCTTCRTCCGGGTTNAC-3′ were degenerated based on amino acids 138–147 (EAVITVPAYF) and 360–369 (FGKEPRKDVN) of the *E. coli* DnaK (Bardwell and Craig 1989), respectively. They represent two of the highly conserved regions in proteins of the Hsp70 family. The degenerate bases were: R, G or A; Y, T or C; N, G, A, T, or C. The underlined bases in the upstream and the downstream primers indicate the *Eco*RV and *Cla*I recognition sites, respectively.

DNA techniques

The methods described in Sambrook et al. (1989) were used for the preparation of bacterial chromosome, plasmid DNA, ^{32}P-labeled DNA probe, Southern hybridization, and agarose gel electrophoresis. DNA sequence was determined by the dideoxy chain-termination method (Sanger et al. 1977) with the Sequenase II sequencing kit from United States Biochemicals. All the sequences obtained were aligned and analyzed to identify putative ORFs and potential homologous genes from other organisms (in the PIR and SWISSPROT databases) using the Genetics Computer Group sequence analysis program package.

Primer extension

Oligonucleotide primers with sequences complementary to positions 66–89 downstream of the *grpE* start codon (5′-AAGGACTCGATCTCGGCCTTGAGC-3′, designated primer #792) and positions 48–71 downstream of the *dnaK* start codon (5′-gccttgccgccgtccatgatcgcc-3′, designated primer #1434) were radioactively labeled as previously described (Weng et al. 1997). *Xanthomonas campestris* pv. campestris 17cellular RNA was isolated as previously described except that the cells were either grown at 28 °C or further grown at the heat-shock temperature (35 °C) for 15 min (Lin et al. 1999). Reverse transcription was then carried out using labeled oligonucleotides as the primers and the extracted RNA (30 μg) as the template. The same primers were used for DNA sequencing at the same time to identify the position of the transcriptional start sites.

Promoter localization

Putative promoter sequences upstream of *grpE* and *dnaK* were cloned by PCR amplification of the *X. campestris* pv. campestris 17 chromosome as template. To amplify the *grpE* upstream sequence, the primers used were the forward primer 5′-GAAGATCTTCCATGGAGCCGCCCGGCAC-3′ and the reverse primer 5′-GCTCTAGAGCGTCTTGGTTCATGTGCAG-3′ corresponding to nucleotides –125 to –107 and –6 to +12 relative to the *grpE* initiation codon, respectively. The PCR product was treated with Klenow enzyme and cloned into the *Eco*RV site among the multiple cloning sites of pBluescript II KS(+) (Stragene). Two plasmids with inserts in different orientations were obtained and confirmed by sequencing. The clone with the correct orientation was named pKSFe. The 233-bp *Not*I-*Xho*I fragment from plasmid pKSFe containing the putative *grpE* promoter was then cloned upstream of the reporter gene *lacZ* of the promoter-testing vector pFY13–9 (Yang 1997), forming plasmid pEZ+. To amplify the *dnaK* upstream sequence, the primers used were the forward

primer (5′-GAAGATCTTCACTGAGCACTGCTGCCGG-3′) and the reverse primer (5′-GCTCTAGAGCTCCTGATGAGTCTTTAGC-3′) located at nucleotides –146 to –129 and –26 to –7 relative to the *dnaK* initiation codon, respectively. The PCR product was treated with Klenow enzyme and cloned into the *Eco*RV-digested pBluescript II KS(+). Two plasmids with inserts in different orientations were obtained and confirmed by sequencing. The clone with the correct orientation was named pKSFk. The 235-bp *Not*I-*Xho*I fragment from plasmid pKSFk containing the putative *dnaK* promoter was then cloned upstream of the reporter gene *lacZ* of pFY13–9, forming plasmid pKZ+. Promoter activity was determined with the standard assay for the *lacZ* gene product β-galactosidase (Miller 1972). Results, presented in Miller units, were the average of at least three independent assays per construct.

Promoter expression during heat shock

Xanthomonas campestris and *E. coli* containing plasmids pEZ+ and pKZ+ were separately grown at their respective normal growth temperatures to mid-exponential phase in LB medium, then *X. campestris* cultures were shifted to 35 °C and *E. coli* cultures to 42 °C to initiate heat shock. At various time points after heat shock, 1-ml samples were removed from the cultures and assayed for β-galactosidase activity.

Nucleotide sequence accession number

The nucleotide sequence reported here has been registered in GenBank under accession number AF302775.

Results and discussion

Cloning, sequencing and genetic organization of the *dnaK* locus of *X. campestris* pv. campestris 17

A DNA fragment of approximately 0.7 kb was obtained by PCR amplification using the degenerate oligonucleotides as the primers and the *X. campestris* pv. campestris 17 chromosome as template. Sequence analysis of the fragment (696 bp) showed 82% similarity to the corresponding region of the *E. coli dnaK*. To isolate the *dnaK* gene, the genomic DNA of *X. campestris* pv. campestris 17 was digested with various restriction enzymes and hybridized with the ^{32}P-labeled probe prepared from the PCR fragment. Two *Cla*I fragments (2.1 and 2.3 kb) and two *Eco*RI fragments (1.4 and 3.6 kb) from the *X. campestris* pv. campestris 17 chromosome hybridized to the probe. These fragments were recovered separately from agarose gels and cloned into the *Eco*RI site of pBK-CMV or the *Cla*I site of pBluescript SK+ (pSK+). The four plasmids thus generated were designated as pRK1.4 (with an inserted 1.4-kb *Eco*RI fragment), pRK3.6 (with an inserted 3.6-kb *Eco*RI fragment), pBKC2.1 (with an inserted 2.1-kb *Cla*I fragment), and pBKC2.3 (with an inserted 2.3-kb *Cla*I fragment). Restriction mapping, Southern hybridization and sequencing data indicated that the 2.1-kb *Cla*I fragment (the pBKC2.1 insert, C2.1) and the 3.6-kb *Eco*RI fragment (the pRK3.6 insert, R3.6) overlapped by 15 nucleotides (Fig. 1).

Sequencing of the C2.1 and R3.6 fragments revealed five ORFs . The polypeptide encoded by the first ORF exhibited significant identity with the C-terminus of HrcA from bacteria. ORFs2, 3, and 4 encoded proteins sharing similarities with bacterial GrpE, DnaK, and DnaJ, respectively. ORF 5 encoded a polypeptide similar to the N-terminus of *E. coli* PdxK (pyridoxine kinase) (Yang et al. 1996). To isolate the complete *hrcA* gene, the leftmost 0.4-kb *Cla*I-*Stu*I fragment from the C2.1 region (Fig. 1) carrying the *hrcA* C-terminus was used as a probe for hybridization with the *X. campestris* pv. campestris 17 genomic library. Sequencing of a positive clone (pHrcES1), carrying an *Eco*RI-*Stu*I fragment (ES1), revealed 967 bp, of which 364 bp overlapped with the C2.1 fragment. Summing up the sequences from ES1, C2.1 and R3.6 fragments, a total of 6,400 bp were obtained.

The *hrcA* gene (nucleotides 178–1,230) could encode a protein of 350 amino acids with a calculated molecular mass of 38.3 kDa and 80% sequence identity with *X. fastidiosa* HrcA (Table 1, Simpson et al. 2000). Downstream of *hrcA*, with an intergenic region of 99 nucleotides, was *grpE* (nucleotides 1,330–1,848), which could encode a polypeptide of 172 amino acids with a calculated molecular mass of 18.9 kDa and similarity to GrpE proteins from other bacteria (Table 1), especially *X. fastidiosa* (81%). *dnaK*, located 141 nucleotides downstream from *grpE*, was 1,929 nucleotides long (nucleotides 1,990–3,918) and could encode a polypeptide of 642 amino acids (68.8 kDa) with a high degree of identity to the DnaK of *X. fastidiosa* (86%) and other bacteria (Table 1). Separated by an intergenic region of 138 nucleotides from *dnaK*, *dnaJ* (nucleotides 4,057–5,187) could encode a

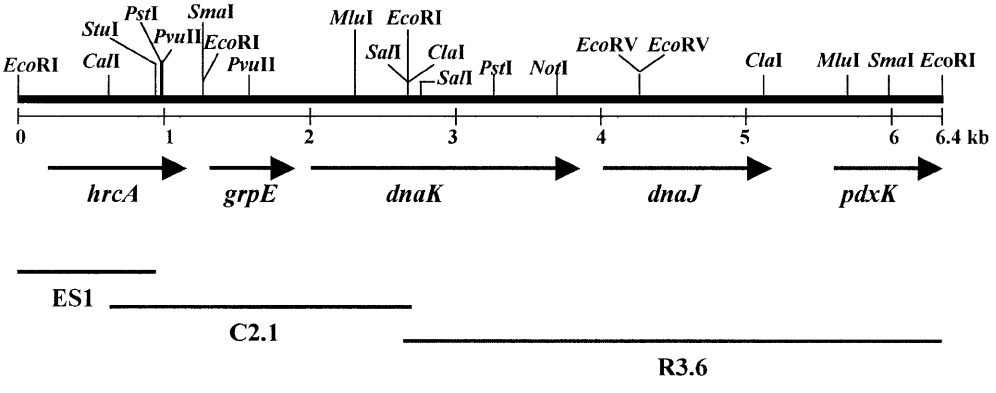

Fig. 1 Genetic diagram of the *Xanthomonas campestris* pv. campestris 17 *dnaK* region. Significant restriction enzyme sites are indicated. *Arrows* indicate the direction of gene transcription. The locations of the 1.0-kb *Eco*RI-*Stu*I fragment of pHrcES1, the 2.1-kb *Cla*I fragment of pBKC2.1 and the 3.6-kb *Eco*RI fragment of the pRK3.6 are shown below the diagram

Table 1 Sequence identity of the deduced stress-responsive proteins of *X. campestris* pv. campestris 17 to the corresponding proteins of other bacteria. NA, gene mentioned but sequence not available in GenBank; Inc, sequence from GenBank is incomplete; – no homologous gene has been reported

Bacterium	Protein			
	HrcA	GrpE	DnaK	DnaJ
X. fastidiosa	80%	81%	86%	71%
C. crescentus	39%	37%	60%	NA
A. tumefaciens	31%	31%	61%	Inc
B. japonicum	30%	36%	Inc	NA
P. aeruginosa	–	43%	69%	52%
E. coli	–	36%	70%	46%

polypeptide of 376 amino acids with a calculated molecular mass of 40.5 kDa and sharing similarity with DnaJ of other bacteria (Table 1). A large intergenic region of 373 nucleotides separated *dnaJ* from *pdxK* (nucleotides 5,561–6,400), which remained open until the end of the available sequence.

From the results presented above, it is apparent that the four putative stress-responsive genes of *X. campestris* are clustered and organized in the order *hrcA-grpE-dnaK-dnaJ* (Fig. 1). This type of genome organization resembles that found in many gram-positive bacteria (Ahmad et al. 1999) and in the gram-negative *X. fastidiosa*, but is dissimilar to the type of organization in the gram-negative *A. tumefaciens*, *B. japonicum* and *C. crescentus* (Fig. 2, Minder et al. 2000; Nakahigashi et al. 1999; Roberts et al. 1996). These comparisons suggest that the HrcA-CIRCE system containing at least four genes arranged in the order *hrcA-grpE-dnaK-dnaJ* already existed for regulation of stress responses before the microorganisms diverged into gram-negative and gram-positive bacteria. However, a CIRCE-like element has not been detected in *E. coli* and some other gram-negative bacteria, suggesting that the HrcA-CIRCE system in these bacteria was lost during evolution.

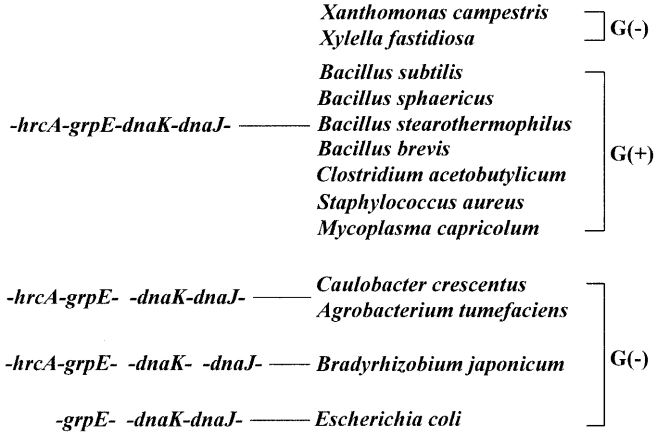

Fig. 2 Genome organization of the heat shock genes *hrcA*, *grpE*, *dnaK*, and *dnaJ* in various gram-positive bacteria (type 1 organization) and five gram-negative bacteria with known *hrcA* genes. The organization of the corresponding genes of *E. coli*, which has no *hrcA* gene, is also included. Note that organization of the gram-negative genes is not as conserved as in the gram-positive counterparts

Regulatory region of the *X. campestris* stress-responsive genes

In *E. coli* and several other bacteria, heat shock response is mediated by a positive regulator protein, the σ^{32} factor, which recognizes promoters with the consensus sequence 5′-TCTCNCCCTTGAA-13–17 nt-CCCCATNTA-3′; this sequence is different from the promoter sequences for the vegetative sigma factor σ^{70} (Cowing et al. 1985). In the *X. campestris* pv. campestris 17 stress-responsive genes, the σ^{32} type promoter was only found in the upstream regions of *grpE* (a –35 box 5′-GAATTTGCTTGAA-3′ and a –10 box 5′-GCCCACATA-3′ with a spacer of 12 nucleotides) and *dnaK* (a –35 box 5′-CAGGTGGCTTGAA-3′ and a –10 box 5′-CCCCACATC-3′ separated by 14 nucleotides) (Table 2). In both cases, the –35 region had a 7/13 match and the –10 region had a 7/9 match. No sequences resembling the σ^{32}-type –35/–10 transcription signals were observed in the *dnaJ* upstream region. Only an *E. coli* σ^{70}-type promoter was present in the *X. campestris* pv. campestris 17 *hrcA* upstream region, and neither σ^{32}type promoter nor CIRCE-like sequence was found. These data suggest that the *X. campestris* pv. campestris 17 *hrcA* may not be autoregulated via CIRCE or regulated by the positive regulator σ^{32}, and therefore other types of mechanisms may be involved in its regulation.

The genome of *X. fastidiosa*, a plant pathogenic bacterium closely related to *X. campestris* (Wells et al. 1987), has recently been sequenced (Simpson et al. 2000). A genome-wide search revealed that: (1) only one CIRCE-like sequence was found, located upstream of *groESL*, (2) neither an obvious σ^{32}-type promoter nor a CIRCE-like sequence was present upstream of the *hrcA*, (3) a putative σ^{32}-type promoter with a –35 box 5′-CAGCCGCTTGAG-3′ and a –10 box 5′-CCCCACATT-3′ separated by 14 nucleotides was found 57 nucleotides upstream of the *dnaK* initiation codon, and (4) a putative σ^{32}-type promoter with a –35 box 5′-TGGGTGGCTTGAA-3′ and a –10 box 5′-GCCCACATA-3′ separated by 12 nucleotides was located 3 nucleotides downstream of the assigned *grpE* initiation codon, which had no preceding Shine-Dalgarno sequence. Because the positioning was unusual, we searched for a possible downstream ATG. A second ATG was found 82 nucleotides downstream of the originally assigned *grpE* initiation codon, which was more likely the real initiation codon since it was preceded by a well-matched Shine-Dalgarno sequence (AGGA). In summary, *X. campestris* and *X. fastidiosa* possess the same type of regulatory sequences in the upstream regions of the corresponding stress-responsive genes and analogous genes may use the same mechanisms for regulation.

In addition to regulation by heat shock that is mediated by a σ^{32}-type promoter, some bacteria use the σ^{70}-type

Table 2 Comparison of DNA sequence between σ^{32}-like promoters of *X. campestris and E. coli*

Promoter	–35 region	Spacing (bp)	–10 region	Reference
X. campestris				
dnaK	CAGGTGGCTTGAA	14	CCCCACATC	This study
grpE	GAATTTGCTTGAA	12	GCCCACATA	This study
σ^{32} consensus	NANNTNGCTTGAA	12–14	NCCCACATN	This study
E. coli				
σ^{32} consensus	TCTCNCCCTTGAA	13–17	CCCCATNTA	Wu and Newton (1996)

promoter for transcription of the *dnaKJ* operon at physiological temperatures. Our previous study suggested that many of the *X. campestris* σ^{70}-type promoters differ in sequence from the *E. coli*-type –35/–10 region. Therefore we cannot predict whether there are two distinct promoters regulating the expression of the *X. campestris dnaKJ* operon.

Four regions having the potential to form stem-loop structures that resemble transcriptional terminators were identified. The first (T*hrcA*), spanning nucleotides 1,337–1,353, with a calculated ΔG of –34.6 kcal/mol was located downstream of the *hrcA* termination codon. The second (T*grpE*), spanning nucleotides 1,852–1,879, with a calculated ΔG of –11.9 kcal/mol was located immediately downstream of the *grpE* termination codon. The third (T*dnaK*), spanning nucleotides 3,942–3,973, with a calculated ΔG of –9.7 kcal/mol was located immediately after the termination codon of *dnaK*. There was no string of T residues characteristic of typical Rho-independent transcription terminators following these three stem-loop structures. In contrast, the fourth stem-loop structure (T*dnaJ*), immediately after the termination codon of *dnaJ* and spanning nucleotides 5,226–5,251, with a calculated ΔG of –20.4 kcal/mol was followed by a string of seven T residues. The presence of a stem-loop structure after the *hrcA*, *grpE*, *dnaK* and *dnaJ* genes suggested that these genes might each be transcribed independently.

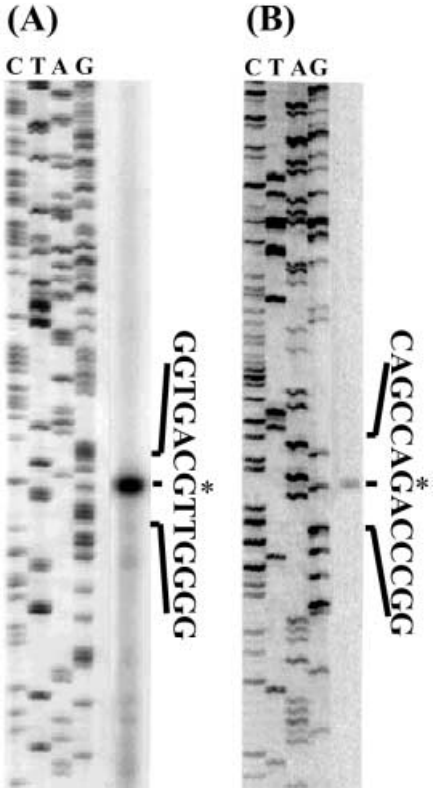

Fig. 3A, B Transcriptional start site mapping by primer extension. Primer extension reactions were carried out with the oligonucleotide primers described in Materials and methods using RNA isolated from *X. campestris* pv. campestris 17 cells treated by heat shock at 35 °C for 15 min. Products of the reactions were electrophoresed along with DNA sequence ladders generated with the same primers. The DNA sequences surrounding the transcriptional start sites of *grp*E (**A**) and *dnaK* (**B**) are shown. The identified transcriptional start site is indicated by an *asterisk*

Determination of the transcription start sites of the *dnaKJ* operon

To determine the transcriptional start sites of *grpE* and *dnaK*, primer extension was carried out on RNA extracted from *X. campestris* pv. campestris 17 cultured at 28 °C and further treated by heat shock at 35 °C for 15 min. Extension reaction using primer #792, complementary to nucleotides 66–89 relative to the *grpE* initiation codon, generated a product initiating with a G base that was 35 nucleotides upstream from the *grpE* initiation codon and 10 nucleotides downstream from the predicted –10 box (Fig. 3A). Primer extension reaction utilizing primer #1434, complementary to nucleotides 48–71 relative to the *dnaK* initiation codon, yielded one extension product initiating with a G base that was 47 nucleotides upstream from the *dnaK* initiation codon and 8 nucleotides downstream from the predicted –10 box (Fig. 3B). In parallel experiments using the RNA samples prepared from cells without heat shock, signals of the extension products were very weak for both genes (data not shown). Therefore, these primer extension results support the notion that promoters are present upstream of *grpE* and *dnaK*.

Comparison of levels of activation of the *grpE* and *dnaK* promoter activities by heat stress

The upstream regions of *grpE* and *dnaK* were separately amplified by PCR and cloned into the promoter-testing vector pFY13–9, generating plasmids pEZ+ and pKZ+,

Table 3 Induction of β-galactosidase activity driven by *dnaK* or *grpE* promoter in *X. campestris* pv. campestris 17 and *E. coli* by heat shock. Enzyme activity is expressed as Miller units. pKZ$^+$ and pEZ$^+$ are the *dnaK* and *grpE* promoter regions, respectively, cloned in the promoter-proving vector pFY13–9. pET21b-*rpoH* is the *rpoH* gene from Xc11 cloned in pET21b whose expression can be induced by IPTG. The normal temperature for *X. campestris* pv. campestris was 28 °C, and for *E. coli* 37 °C. The heat shock temperature for *X. campestris* pv. campestris was 35 °C, and 42 °C for *E. coli*

Strain	Normal temperature	Heat shock	Enzyme induction (-fold compared to normal growth temperature)
X. campestris pv. campestris 17 (pKZ+)	365	708	1.9
X. campestris pv. campestris 17 (pEZ+)	45	128	2.8
E. coli(pKZ+)	46	91	2.0
E. coli(pEZ+)	24	55	2.3
E. coli(pKZ+, pET21b-*rpoH*)	47	93	2.0
E. coli(pKZ+, pET21b-*rpoH*) +IPTG for 2 h	150	158	1.1

respectively, as described in Materials and methods. In these two plasmids, the putative promoters were aligned in the same direction as the reporter *lacZ*. Promoter activities were determined by measuring the β-galactosidase levels in strains *X. campestris* pv. campestris 17 (pEZ+) and *X. campestris* pv. campestris 17 (pKZ+) grown at 28 °C or after further growth at 35 °C. At the normal growth temperature, about 45 and 365 units of the enzyme were detected for the *grpE* promoter and *dnaK* promoter, respectively (Table 3). After heat shock, the β-galactosidase levels increased gradually, reached their maxima at 60 min (about 128 units for the *grpE* promoter and about 708 units for the *dnaK* promoter; Table 3), and then declined. The increase was about 2.8-fold for the *grpE* promoter and 1.9-fold for the *dnaK* promoter after heat shock.

Since we have previously shown that most *X. campestris* promoters do not express well in *E. coli*, we were curious as to whether the *grpE* and *dnaK* promoters could express in *E. coli*. Interestingly, these two promoters were functional in *E. coli*, although the β-galactosidase levels were about 24 and 46 units by *E. coli*(pEZ+) and *E. coli*(pKZ+), respectively. After heat shock at 42 °C, the β-galactosidase levels increased gradually and reached their maxima at 60 min (about 55 units for the *grpE* promoter and about 91 units for the *dnaK* promoter), an approximately two-fold increase in both cases (Table 3). The levels then decreased. The activities of the *grpE* and *dnaK* promoters in *E. coli* were approximately two- and eight-fold lower than in *X. campestris* pv. campestris 17 , respectively, when the promoter activities were compared between the two bacteria at the corresponding growth temperatures. In other words, although the –10 and –35 sequences of the *X. campestris* σ^{32}-recognized promoters resemble the consensus sequence of *E. coli* σ^{32} promoters (Table 2), promoter activities expressed were still different in the two bacteria. One of the possible reasons for these differences could be the variation in recognition affinity between the promoter and σ^{32}.

To investigate the effect of RpoH on the expression of *X. campestris dnaK* promoter in *E. coli*, plasmid pET21bt-*rpoH* carrying the cloned *X. campestris rpoH* gene (Huang et al. 1998) was provided *in trans*. This cloned *rpoH* gene was under the control of *lac* promoter inducible by the addition of IPTG. After introduction of pET21bt-*rpoH* into *E. coli*(pKZ+), the resultant strain *E. coli*(pKZ+, pET21bt-*rpoH*) was used to assay β-galactosidase activity. Without IPTG induction, the β-galactosidase activity was only 47 units for *E. coli*(pKZ+, pET21bt-*rpoH*) (Table 3). In contrast, in the presence of IPTG to induce the *X. campestris rpoH* gene for 120 min, β-galactosidase levels were elevated three-fold in *E. coli*(pKZ+, pET21bt-*rpoH*) (Table 3). These results indicate that the *X. campestris dnaK* promoter requires the cognate RpoH for efficient transcription. However, the levels after elevation were still lower than those in *X. campestris*, indicating that other cognate cellular factors might also be required in addition to σ^{32} in the regulation of the transcriptional event. To test if expression from pKZ+ in the presence of pET21bt-*rpoH* was further stimulated by heat shock, *E. coli*(pKZ+, pET21bt-*rpoH*) was shifted to 42 °C after IPTG induction for 120 min. Samples were taken at an interval of 10 min and assayed for β-galactosidase activity until 30 min after heat shock. No further increase in enzyme activity was detectable (150 units at normal growth temperature vs 158 units after heat shock; Table 3). These results suggest that the *X. campestris* σ^{32} expressed from pET21bt-*rpoH* might have been sufficient for full transcription of the *dnaK* promoter in the heterologous background, and although production of the *E. coli* σ^{32} can be induced by heat shock, it could not further enhance the *X. campestris dnaK* promoter activity.

The effects of ethanol stress on the expression of *grpE* and *dnaK* promoters in *X. campestris* were also evaluated. β-Galactosidase activity assays using the transcriptional fusion constructs pEZ+ and pKZ+ showed that exposure of *X. campestris* pv. campestris 17 (pEZ+) and *X. campestris* pv. campestris 17 (pKZ+) to ethanol (final concentration 4%) for 45 min caused 2.0- and 1.5-fold increases in the activity of the *grpE* and *dnaK* promoters, respectively, compared to the control *X. campestris* pv. campestris 17 cells.

Conclusions

While completing preparation of this manuscript, the complete nucleotide sequence of the genome of the plant

pathogen *X. fastidiosa* was published (Simpson et al. 2000). It is interesting to find that *X. campestris* and *X. fastidiosa* heat shock genes have the same organization. This type of genome organization, while present in many gram-positive bacteria, has been the only cases found in gram-negative bacteria. In addition, high degrees of sequence identity shared between the corresponding proteins and the same structural elements in the corresponding regulatory regions are present. These findings further support the proposal of Wells et al. (1987) that *X. fastidiosa* is closely related to *X. campestris*.

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