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# *dnaK* and the Heat Stress Response of *Pseudomonas syringae* pv. *glycinea*

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The *dnaK* gene from *Pseudomonas syringae* pv. *glycinea* PG4180 was cloned and sequenced. The *dnaK* coding region was 1,917 bp and contained a putative  $\sigma^{32}$  heat shock promoter 86 bp upstream of the translational start site. *grpE*, another heat shock gene, was found immediately upstream of the putative *dnaK* promoter. The predicted amino acid sequence of *dnaK* showed relatedness to the ATPase and substrate binding domains commonly found in heat shock proteins, as well as the highly conserved signature sequence motifs belonging to the Hsp70 protein family. Furthermore, the PG4180 *dnaK* gene complemented an *Escherichia coli dnaK* mutant for growth at temperatures above 37°C, indicating that a fully functional *dnaK* homologue had been cloned from *P. syringae* pv. *glycinea*. All attempts to eliminate *dnaK* function by insertion mutagenesis failed, possibly because DnaK performs essential functions in *P. syringae* pv. *glycinea*. Expression of *dnaK* in *P. syringae* pv. *glycinea* PG4180 was investigated by constructing *dnaK::uidA* transcriptional fusions; expression of *dnaK* increased markedly when cells were preincubated at 18°C and then shifted to 35°C. An anti-DnaK monoclonal antibody was used to detect DnaK; in *P. syringae* pv. *glycinea* race 4, DnaK levels followed cell density during a 6-h incubation at 26°C. When cells were shifted from 26°C to either 32 or 38°C, DnaK levels increased transiently, and then decreased rapidly. Although the cells continued to grow when incubated at 32°C, growth was not supported at 38°C. Our results indicate that *P. syringae* pv. *glycinea* responds to heat shock by producing DnaK, but DnaK does not aid in acclimation to sustained elevated temperatures.

Virulence factors in bacteria are often coordinately regulated by various environmental factors including temperature, osmolarity, and pH (Mekalanos 1992). In contrast to human and animal bacterial pathogens, little is known about how temperature impacts the virulence of phytopathogenic bacteria. A generalized response to increased temperatures in most organisms is the synthesis of heat shock proteins, which are

also called molecular chaperones. These proteins mediate the correct assembly of other polypeptides without being part of the functional assembled structure and promote the disassembly of proteins that have been damaged by stress (Ellis and Hemmingsen 1989; Mager and Kruijff 1995). The temperature at which cells reach the maximum level of heat shock protein synthesis depends on the optimal growth temperature for the organism. In general, a 10 to 12°C temperature increase beyond the optimal growth temperature is required to fully induce the synthesis of heat shock proteins (Key et al. 1981). During the temperature induction period, prokaryotes shift transcription from  $\sigma^{70}$  to  $\sigma^{32}$ , which enables RNA polymerase to recognize the promoters of heat shock genes (Segal and Ron 1995; Straus et al. 1990). In bacteria,  $\sigma^{32}$  activation is autoregulated by DnaK, which controls both the synthesis and stability of  $\sigma^{32}$  (Straus et al. 1990).

In several phytopathogenic bacteria, temperature is known to modulate virulence. For example, tumor induction by *Agrobacterium tumefaciens* is optimal at 22°C and does not occur at temperatures at or above 32°C (Braun 1947; Riker 1926). The lack of virulence at temperatures above 32°C has been attributed to the synthesis of nonfunctional VirA and VirB10 proteins, which are required for *vir* gene activation and T-DNA transfer, respectively (Banta et al. 1998; Jin et al. 1993). However, it is also important to note that heat shock proteins were observed in *A. tumefaciens* at temperatures only slightly higher than 32°C (Mantis and Winans 1992), suggesting that normal cellular activities were diverted to the heat shock response, a process that could affect virulence.

*Pseudomonas syringae* pv. *glycinea*, causal agent of bacterial blight of soybeans, is a cool-weather pathogen that causes significantly more damage at 22 to 26°C and relatively little disease at 30°C (Coerper 1919; Park and Lim 1986). The phytotoxin coronatine (COR) is a virulence factor in *P. syringae* pv. *glycinea* and contributes significantly to the chlorosis and stunting associated with bacterial blight of soybean (Gnanamaickam et al. 1982). In vitro studies indicated that the *P. syringae* pv. *glycinea* strain PG4180 produced COR at 18 and 24°C but not at 30°C (Palmer and Bender 1993). In subsequent studies, elevated temperatures (28 to 30°C) were shown to interfere with optimal functioning of the COR regulatory proteins by affecting protein stability or conformation (Budde et al. 1998; L. Wang, C. Bender, and M. Ullrich, unpublished).

The heat shock response of *P. syringae* has not been previously studied. Since the pathogenicity of *P. syringae* pv. *gly-*

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Nucleotide and amino acid sequence data are to be found at GenBank as accession number AF135163.

*cinea* is compromised at elevated temperatures, we investigated the expression and synthesis of *dnaK* in this bacterium before, during, and after heat shock.

## RESULTS

### Cloning *dnaK* from PG4180.

*P. syringae* pv. *glycinea* PG4180 was used in all cloning and complementation studies because it was easier to manipulate genetically than *P. syringae* pv. *glycinea* race 4. Furthermore, the use of PG4180 facilitated studies in which *dnaK* and COR gene expression were measured simultaneously. Primers complementary to conserved portions of *dnaK* were used to amplify and clone a 721-bp region of this gene from PG4180 (Fig. 1E). The polymerase chain reaction (PCR) product in pLK.721 was sequenced to verify that it contained *dnaK*, and our results indicated that the PG4180 *dnaK* fragment shared >75% amino acid identity to *dnaK* from *Escherichia coli*. Since the nucleotide sequence of *dnaK* in *E. coli* is 1,917 bp (Bardwell and Craig 1984), a cosmid library of PG4180 genomic DNA was constructed in pRK7813, and the 721-bp insert in pLK.721 was used to screen the library for clones containing the complete *dnaK* coding region. Two cosmid clones that hybridized to the 721-bp *dnaK* probe, pLH3 and pDF5, were chosen for further analysis. A 5-kb *EcoRI* fragment from pDF5 was subcloned in pZErO2.1, creating pLK1, and a 7-kb *HindIII* fragment from pLH3 was subcloned in pBluescript SK<sup>+</sup>, resulting in pLK2 (Fig. 1A, B). Both strands of the PG4180 *dnaK* gene were sequenced with pLK1 and pLK2 as templates.

### Southern blot hybridizations and sequence analysis.

PG4180 genomic DNA was digested with *HindIII*, *BamHI*, or *EcoRI* and analyzed by Southern blotting with the 721-bp *dnaK* probe. Only one fragment hybridized to the probe in each digest regardless of stringency, indicating that *dnaK* occurs as a single copy in PG4180 (data not shown). Identical hybridization results were obtained with a race 4 isolate of *P. syringae* pv. *glycinea* supplied by A. K. Vidaver.

The nucleotide sequence of the PG4180 *dnaK* gene is shown in Figure 2. The coding region was 1,917 bp and the upstream region contained the consensus sequence commonly found in *E. coli* heat shock promoters (single underscored sequences upstream of the translational start site in Figure 2). The PG4180 *dnaK* was highly related to *dnaK* from *E. coli* (70% nucleotide identity, 72% amino acid identity, 92% amino acid similarity). The translated product of the PG4180 *dnaK* gene contained two separate domains, a high-affinity ATPase domain (amino acids 1 to 385; 94% amino acid similarity to *E. coli*), and a substrate binding domain (amino acids 393 to 537; 92% similarity to *E. coli*; Fig. 1D). Conserved amino acids involved in the interaction of DnaK with Mg-ADP (D, K, and E at positions 8, 70, and 171, respectively; McKay et al. 1994), GrpE (N, G, and R at positions 29, 32, and 34, respectively; Buchberger et al. 1994), or target substrates (P at positions 464 and 466; Zhu et al. 1996) were also identified (Fig. 2). Conserved heat shock protein signature sequences (IDLGTTNS, VYDLGGGTFDVSVI, VILVGGQT RMPVQK) were found in the translated sequence of *dnaK* from PG4180 (Fig. 2) with the MOTIFS program included in the UWGCG (University of Wisconsin Genetics Computer

Group) sequence analysis software package (version 9.1). These conserved amino acids are presumably involved in the interaction of DnaK with other molecular chaperones or nascent polypeptides (Minder et al. 1997).

Additional sequencing of pLK2 indicated that *grpE*, which encodes another heat shock protein, was located 500 bp upstream of the *dnaK* translational start site (Fig. 1B, shaded rectangle). Comparison of the deduced amino acid sequence of the PG4180 *grpE* and the *E. coli* homologue indicated 48% identity and 66% similarity (data not shown).

### Complementation of an *E. coli dnaK* mutant.

The *dnaK* mutant, *E. coli* GW4813, lacks 800 bp upstream of the promoter and 933 bp of the *dnaK* coding sequence and is unable to grow at temperatures above 37°C. When pLK2 was introduced into *E. coli* GW4813, growth at 42°C was restored, indicating that the PG4180 *dnaK* homologue could complement the mutation in *E. coli* GW4813. Neither pLK.721 nor pBluescript SK<sup>+</sup> complemented GW4813 for growth at 42°C.

Several attempts were made to create a *dnaK* insertion mutant of PG4180. However, all efforts to insert a Km<sup>r</sup> or Cm<sup>r</sup> cassette into the central *EcoRI* site of *dnaK* were unsuccessful, even when the matings and screening procedures were performed at 18°C to facilitate the growth of temperature-sensitive *dnaK* mutants. Although it was possible to mobilize the construct containing a mutated *dnaK* into PG4180, Tc<sup>s</sup> colonies were not obtained via double homologous recombination. When plasmid isolation procedures indicated the disappearance of the plasmid construct, Southern blot hybridizations and PCR analysis indicated that the clones used for marker exchange had integrated into the chromosome via a single crossover event, leaving the *dnaK* gene fully intact.

### Growth of *P. syringae* pv. *glycinea* at various temperatures.

There was little difference in growth when *P. syringae* pv. *glycinea* was incubated at 26 to 33.5°C (Fig. 3A). The optimal temperature was 30.7°C for multiplication of *P. syringae* pv. *glycinea* race 4, whereas no doubling was observed when the culture was incubated at 38.5°C (Fig. 3B). All growth curves were repeated with similar results. The relationship between CFUs and OD was consistent and uniform for the temperatures studied, and similar results were obtained for PG4180 (data not shown), which is also a race 4 isolate.

### GUS assays.

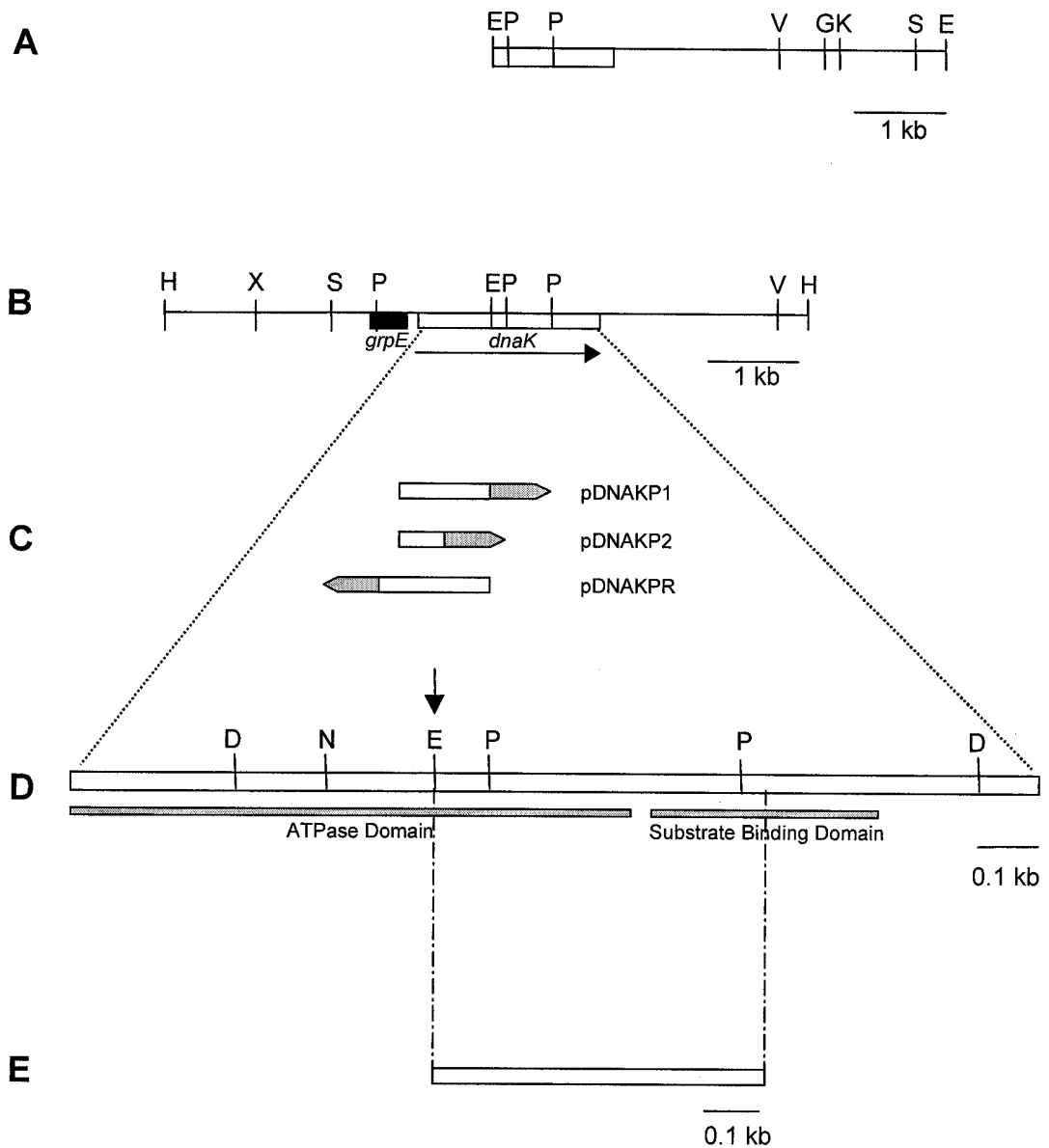
pDNAKP1, pDNAKP2, pDNAKPR (Fig. 1C), and pBBR.GUS were mobilized into *P. syringae* pv. *glycinea* PG4180 and assayed for β-glucuronidase (GUS) activity. Colonies of *E. coli* or *P. syringae* pv. *glycinea* PG4180 containing pDNAKP1 or pDNAKP2 developed a light blue color on media containing 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc), indicating a basal level of *dnaK* transcription at physiological temperatures (37 and 28°C, respectively). Cells containing pDNAKPR (*dnaK* in the transcriptionally inactive orientation) or pBBR.GUS (vector control) showed no color development on media containing X-Gluc.

When PG4180 transconjugants containing pDNAKP1, pDNAKP2, pDNAKPR, or pBBR.GUS were grown in KMB broth at 28°C, growth curves were similar, indicating

that the transcriptional fusions had no significant effect on growth (data not shown). A time course experiment at 28°C was conducted in which GUS activity in the PG4180 transconjugants was measured every 2 h for 10 h; all PG4180 derivatives were grown in KMB broth and the experiment was performed twice. GUS activity in PG4180(pDNAKP1) and PG4180 (pDNAKP2) increased with cell growth and was similar for both transconjugants (data not shown); therefore, pDNAKP2 was used in all further experiments since it contained less of the *dnaK* coding region than pDNAKP1 (Fig. 1C).

Transcriptional activity of PG4180 containing pDNAKP2, pCFLP, or pBBR.GUS was evaluated at 18°C, a temperature

that is optimal for COR production in PG4180 (Palmer and Bender 1993). pCFLP contains the *cfl::uidA* transcriptional fusion; the *cfl* gene is required for COR synthesis, and the *cfl* promoter was shown to be optimally transcribed at 18°C (Liyanage et al. 1995). Growth of PG4180 containing pDNAKP2, pCFLP, or pBBR.GUS was similar when incubated at 18°C for 72 h (Fig. 4). However, *cfl::uidA* (pCFLP) and *dnaK::uidA* (pDNAKP2) transcriptional fusions were differentially expressed; *cfl* transcription increased rapidly after 10 h and GUS activity exceeded 30,000 U at 72 h (Fig. 4). However, expression of the *dnaK::uidA* transcriptional fusion remained low throughout the sampling period, even though growth continued to increase (Fig. 4).



**Fig. 1.** Restriction maps of (A) pLK1, (B) pLK2, (C) *dnaK::uidA* transcriptional fusions, (D) *Pseudomonas syringae* pv. *glycinea* PG4180 *dnaK*, and (E) pLK.721. B, *Hind*III site at the right border of pLK2 was located in the vector polylinker. Locations of *grpE* and *dnaK* are indicated by shaded and open rectangles, respectively. C, Solid and open bars represent the *uidA* gene and the *dnaK* promoter region, respectively. D, Arrow indicates location used for insertion of the antibiotic resistance cassettes and shaded rectangles indicate location of the conserved heat shock domains. Abbreviations: E, *Eco*RI; P, *Pst*I; V, *Eco*RV; G, *Bgl*II; K, *Kpn*I; S, *Sma*I; H, *Hind*III; X, *Xho*I; D, *Dde*I; N, *Not*I.

1	GAGCAGGCTTGAATCAGCTGTAAGGCCCCCATTTAAAGGTCAAGCGTTTAAGAGCTACC	60
61	GCACGGTGTAAACGCACTGGCGGCAACCAAATCCAAGTTTTGCAAGTTTCGGGAGAGTCA	120
121	AGAT <b>GGACAGAATTATCGGTATCGACCTGGGGACTACCAACTCCTGCGTCTCCATTCTGG</b>	180
1	<b>M D R I I G I <u>D L G T T N S</u> C V S I L E</b>	20
181	AAAACGGTAACGTCAAGGTTATCGAGAACGCAGAAGGTACTCGTACTACACCTTCGATCA	240
21	N G N V K V I E <u>N A E G T R</u> T T P S I I	40
241	TTGCTTATGCCAATGACGGTGAGATCCTGGTTCGGTTCAGTCCGCCAAGCGTCAGGCCGTTA	300
41	A Y A N D G E I L V G Q S A K R Q A V T	60
301	CCAATCCGCACAACACGCTGTACGCGGTAAAGCGTCTGATTGGTCGCAAGTTCGAAGAGG	360
61	N P H N T L Y A V <u>K</u> R L I G R K F E E D	80
361	ATGTCGTTTCAGAAAGACATCCAGATGGTTCCTTACAAGATCGTCAAGGCTGACAACGGTG	420
81	V V Q K D I Q M V P Y K I V K A D N G D	100
421	ATGCCTGGGTTGAAGTGAATGGCCAGAAAATGGCACCTCCTCAGATTTCCGCTGAAATCC	480
101	A W V E V N G Q K M A P P Q I S A E I L	120
481	TGAAGAAAATGAAAAGACTGCCGAAGACTACCTCGGTGAAGCGGTTACCGAAGCGGTCA	540
121	K K M K K T A E D Y L G E A V T E A V I	140
541	TCACCGTTCCGGCCTATTTCAACGACAGTCAGCGTCAGGCAACCAAGGACGCCGGCCGTA	600
141	T V P A Y F N D S Q R Q A T K D A G R I	160
601	TTGCCAGTGTGGACGTCAAGCGCATCATCAACGAACCAACCGCGCCGCTCTGGCTTACG	660
161	A S V D V K R I I N <u>E</u> P T A A A L A Y G	180
661	GTATGGATAAGGCCAAGGGCGATCACACTGTCATCGTTTATGACCTGGGTGGCGGTACGT	720
181	M D K A K G D H T V I <u>V Y D L G G G T F</u>	200
721	TCGACGTCTCGGTTCATCGAGATTGCCGAAGTTGATGGCGAGCACCAGTTCGAAGTGTGG	780
201	<u>D V S V I E I</u> A E V D G E H Q F E V L A	220
781	CCACCAACGGCGACACGTTCCCTGGGCGGTGAAGACTTTGACATTCGTCTGATCGACTACT	840
221	T N G D T F L G G E D F D I R L I D Y F	240
841	TCGTCCACGAATTCAAGAAAGAAAGCGGCATGAACCTCAAGGGTGACCCGCTGGCCATGC	900
241	V H E F K K E S G M N L K G D P L A M Q	260
901	AGCGTCTGAAAGAAGCTGCCGAGAAAGCCAAGATCGAGCTTTCTTCCAGCACGCAGACCG	960
261	R L K E A A E K A K I E L S S S T Q T E	280
961	AAGTCAACCTGCCGTACATCACTGCAGATGCAACCGGTCCAAAGCACCTGGTCGTGAAAA	1020
281	V N L P Y I T A D A T G P K H L V V K I	300
1021	TTTCGCGCTCCAAGCTGGAATCGCTGGTTGAAGACCTGGTTCAGCGCACCATCGCTCCTT	1080
301	S R S K L E S L V E D L V Q R T I A P C	320

(continued on next page)

**Fig. 2.** Nucleotide sequence of *Pseudomonas syringae* pv. *glycinea* PG4180 *dnaK* including the putative promoter region. Translational start and stop sites are indicated in bold. Putative ribosome binding site is double-underscored. Conserved nucleotides found in the promoter regions of heat shock genes are underscored. Deduced amino acid sequence of *dnaK* is indicated below the DNA sequence. Amino acids presumably involved in interaction of DnaK with Mg-ADP, the co-chaperone GrpE, or target substrates are underlined and printed in bold italics. Hsp70 signature sequences are shaded.

In temperature shift experiments, PG4180 derivatives containing pDNAKP2, pCFLP, or pBBR.GUS were initially grown at 18°C for 24 h, then shifted to either 24 or 35°C for an 8-h incubation. Growth continued to increase when cells were shifted to 24°C; however, 35°C was unfavorable for growth, and cell doubling did not occur (Fig. 5A). When cells were shifted to 24°C, transcriptional activity of the *dnaK::uidA* fusion in PG4180(pPDNAKP2) increased slightly and then remained constant (Fig. 5B). In contrast, the shift to 35°C resulted in a

threefold increase in activity of the *dnaK::uidA* fusion, and GUS levels remained high throughout the sampling period (Fig. 5B).

*cfl* gene expression was also monitored after cells were shifted from 18 to 24 or 35°C. Although *cfl::uidA* activity in PG4180(pCFLP) increased with time at 24°C (Fig. 5B), expression never reached the levels observed at 18°C (Fig. 4). At 35°C, *cfl::uidA* transcriptional activity decreased beginning at 2 h, and GUS levels remained low throughout the sampling period (Fig. 5B).

Fig. 2. (continued from preceding page)

1081	GCGAAATGGCGCTTAAAGATGCTGGCATCGACAGAAGCAAGATCAACGACGTGATTCTGG	1140
321	E M A L K D A G I D R S K I N D V I L V	340
1141	TCGGTGGTCAGACCCGTATGCCGCTGGTGCAGAAGCTGGTAACCGAGTTCTTCGGTAAAG	1200
341	G G Q T R M P L V Q K L V T E F F G K E	360
1201	AAGCACGTAAAGACGTCAACCCTGACGAAGCCGTTGCAATGGGTGCTGCCATCCAGGGCG	1260
361	A R K D V N P D E A V A M G A A I Q G A	380
1261	CTGTATTGGCCGGTGTGTCAAAGACGTTCTGCTGCTCGACGTCAGCCCGCTGACTCTGG	1320
381	V L A G D V K D V L L L D V S P L T L G	400
1321	GTATCGAAGCCATGGGCGGGGTGATGACTGCGCTGATCAAAAAACACCACGATTCCTAC	1380
401	I E A M G G V M T A L I K K T P R F L P	420
1381	CAAGAAATCCCAGTGTTCTCACTGCCGATGACAACCAAGAGAACGCGGTAGCCATCCACG	1440
421	R N P S V L T A D D N Q E N A V A I H V	440
1441	TACTGCAGGGTGAGCGCAAGCAAGCCGGTCAGAACAAGTCCTTGGGCAAGTTCGACCTGG	1500
441	L Q G E R K Q A G Q N K S L G K F D L A	460
1501	CCGAGATTCCACCTGCTCCACGTGGCGTGCCACAGATCGAAGTAACCTTCGACATCGACG	1560
461	E I P <u>P</u> A <u>P</u> R G V P Q I E V T F D I D A	480
1561	CAAACGGCATTCTGCATGTTGGCGCGAAAGACAAGGCGACCGGCAAGCAGCAGTCCATCG	1620
481	N G I L H V G A K D K A T G K Q Q S I V	500
1621	TGATCAAGGCCAACTCCGGTCTGTCCGAGGAAGAAATTCAGCAAATGGTCCGTGATGCTG	1680
501	I K A N S G L S E E E I Q Q M V R D A E	520
1681	AAGTCAACTCCGAAGAAGATCGCAAGTTCGAAGAGCTGGCCTCTGCCCGTAACCAGGGTG	1740
521	V N S E E D R K F E E L A S A R N Q G D	540
1741	ATGCGCTGGTCCACTCGACTCGAAAATGATCGCTGATGCGGGTGACAAGGTCCTGCTG	1800
541	A L V H S T R K M I A D A G D K V T A E	560
1801	AACAGAAAACGGCTGTTCGAGGCTGCACTGGTTGCTCTGGAAGCTGCTATCAAGGGCGATG	1860
561	Q K T A V E A A L V A L E A A I K G D D	580
1861	ACAAGGCTGCTATCGAAGCCAAGGTTGAAGAGCTGTGCAAGGTTTCCGCGCCAATCGCTC	1920
581	K A A I E A K V E E L S K V S A P I A Q	600
1921	AGAAGATGTACGCCGAGCAGGCCGAAAACCTGAAGCAGCGGCCAAGCCTGCTGAAGAAA	1980
601	K M Y A E Q A E N P E A A A K P A E E N	620
1981	ATGCCAAGGCTGATGACGTTGTTCGATGCCGAGTTCGAAGAAGTGAAAGACCACAAGTAA	2039
621	A K A D D V V D A E F E E V K D H K *	639

### Detection of DnaK.

The anti-DnaK monoclonal antibody specifically cross-reacted with a 69-kDa protein in *P. syringae* pv. *glycinea* race 4 (data not shown), which corresponds to the predicted size of DnaK based on sequence analysis of the gene from both *E. coli* (Bardwell and Craig 1984) and *P. syringae* pv. *glycinea*. Figure 6A, B, and C depict the OD<sub>600</sub> of *P. syringae* pv. *glycinea* race 4 and the amount of DnaK detected during a 6-h incubation period at 26, 32, and 38.5°C, respectively. At 26°C, a basal level of DnaK was produced and detection was correlated with cell growth (Fig. 6A). When *P. syringae* pv. *glycinea* cells were initially grown at 26°C and then shifted to 32 or 38°C, there was a rapid, transient increase in DnaK production beginning at 2 h that decreased rapidly at 3 to 4 h (Fig. 6B, C). Although cells continued to grow at 32°C, 38.5°C was unfavorable for growth of *P. syringae* pv. *glycinea*, and the OD<sub>600</sub> remained low (<0.2).

### DISCUSSION

Southern blot hybridizations at low and high stringencies suggested that *dnaK* exists as a single copy in *P. syringae* pv. *glycinea* PG4180. In contrast, some prokaryotes and many eukaryotes harbor multiple forms of DnaK (Seaton and Vickery 1994; Werner-Washburne et al. 1987). The translational product of the PG4180 *dnaK* contained highly conserved mo-

tifs belonging to the Hsp70 protein family, including ATPase and substrate binding domains (Fig. 1C). The amino terminus of the PG4180 DnaK was more homologous to other DnaK proteins than the carboxyl terminus, a feature common to all members of the Hsp70 family (Miernyk 1997).

In *E. coli*, the *rpoH* gene product,  $\sigma^{32}$ , positively regulates the heat shock genes and directs the core RNA polymerase to the *dnaK* promoter. In *E. coli*, the *dnaK* promoter recognized by  $\sigma^{32}$  is located 121 bp upstream of the *dnaK* translational start site (Cowing et al. 1985). In *P. syringae* pv. *glycinea* PG4180, a potential promoter sequence for *dnaK* was located 86 bp upstream of the translational start site (Fig. 2). The location and sequence of the PG4180 *dnaK* promoter are similar to those of  $\sigma^{32}$ -dependent promoters of *E. coli*, which are among the strongest promoters in *E. coli* (Mager and Kruijff 1995). The *E. coli*  $\sigma^{32}$  consensus sequence is TCTC-CCCTTGAA (-35) and CCCCAT-TA (-10); these two regions are separated by 13 to 17 bp (Table 1). In PG4180, the putative -35 and -10 regions are 7/12 and 8/8 nucleotides identical to the *E. coli* consensus and are separated by 14 bp (Table 1).

In many organisms, *dnaK* and *dnaJ* are organized as an operon, and there is usually a third member associated with the *dnaKJ* genes, the co-chaperone *grpE*. Additional sequencing localized *grpE* immediately upstream of the *dnaK* promoter (Fig. 1B), an organization that is conserved in *Clostridium acetobutylicum* (Naberhaus et al. 1992) and *Staphylococcus*

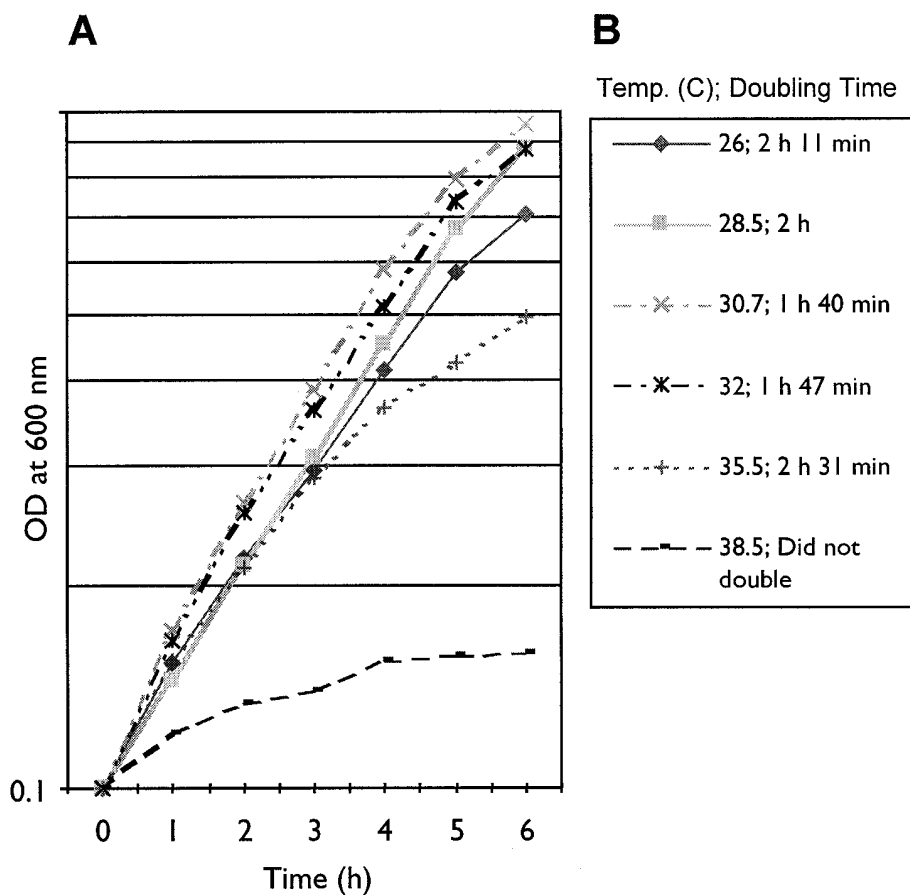


Fig. 3. A, Growth curves and (B) doubling times of *Pseudomonas syringae* pv. *glycinea* race 4 at various temperatures (°C). All cultures were grown in Luria-Bertani broth with aeration. The experiment was repeated with similar results.

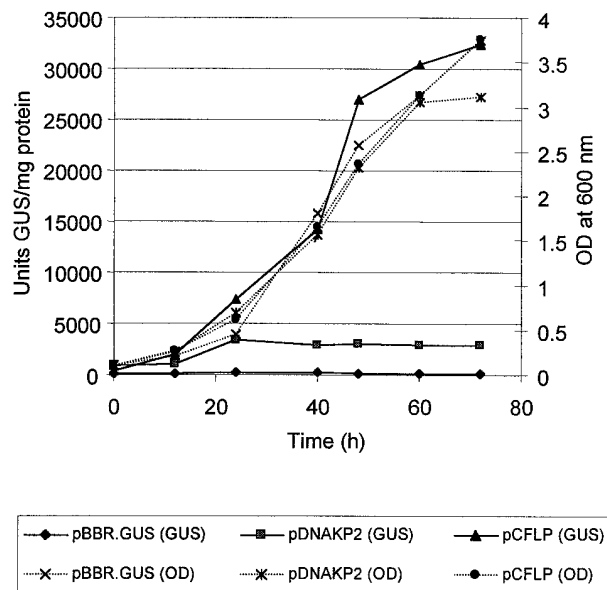
*aureus* (Ohta et al. 1994). Additional sequencing upstream of *grpE* and downstream of *dnaK* (1 kb in either direction) did not identify a potential *dnaJ* homologue in PG4180; however, it remains likely that *dnaJ* is located further downstream of *dnaK* or elsewhere on the PG4180 chromosome.

The activity of the PG4180 *dnaK* gene was analyzed in complementation experiments. When pLK2 was introduced into the *E. coli dnaK* mutant GW4813, growth at 42°C was restored. Growth of GW4813 at 42°C could not be restored by the introduction of pLK.721, which lacks the PG4180 *dnaK* promoter region and the first 700 bp of the *dnaK* coding region. Interestingly, *dnaK* from *Bacillus megaterium*, *Borrelia burgdorferi*, and *Mycobacterium tuberculosis* did not complement an *E. coli dnaK* mutant for growth at elevated temperatures (Mehlert and Young 1989; Sussman and Setlow 1987; Tilly et al. 1993).

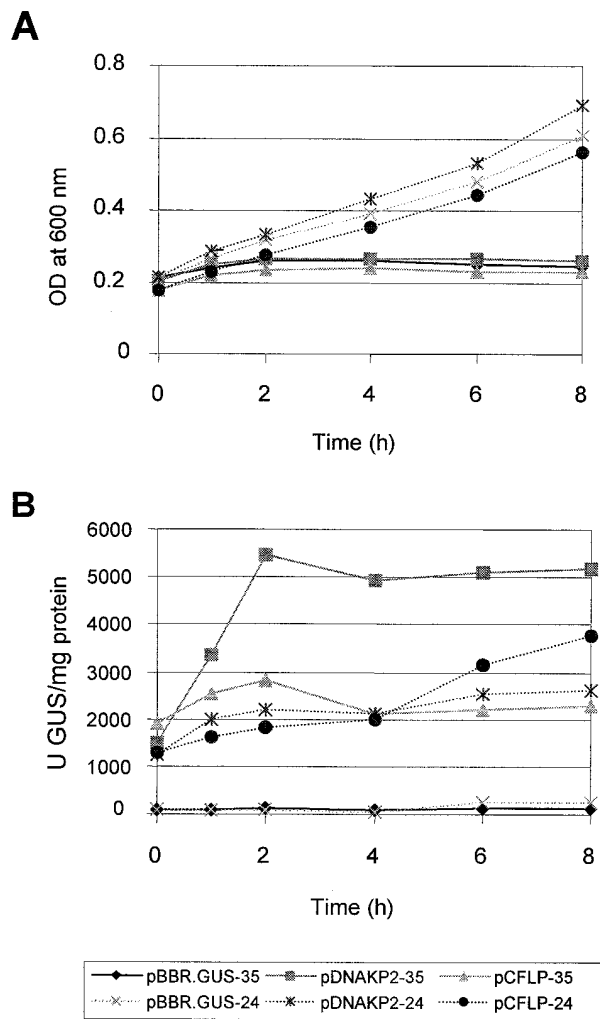
In the present study, we attempted to construct a PG4180 *dnaK* mutant by introducing antibiotic resistance cassettes into the *EcoRI* site located in the center of *dnaK* (Fig. 1C). All attempts to obtain a PG4180 *dnaK* mutant by homologous recombination were unsuccessful, even when selection proceeded at 18°C to encourage the survival of temperature-sensitive *dnaK* mutants. Since the antibiotic cassettes used to generate *dnaK* mutants could have polar effects, the possibility exists that DNA located downstream of *dnaK* might influence our attempts to isolate mutants. However, no distinct open reading frames were identified in a 1-kb region downstream of the *dnaK* coding sequence. Our inability to isolate a PG4180 *dnaK* mutant may indicate that DnaK serves an essential housekeeping function in this organism, and elimination of the gene results in a loss of viability. Although *dnaK*

mutants of *E. coli* have been recovered (Bukau and Walker 1989), many attempts at creating *dnaK* mutants have been unsuccessful (Brans et al. 1996; Minder et al. 1997). Since efforts to obtain a PG4180 *dnaK* mutant were unsuccessful, we also tried to obtain a PG4180 *grpE* mutant. These attempts were also unsuccessful, indicating that disruption of *grpE* may also be lethal for PG4180. The absence of GrpE may result in the accumulation of proteins that cannot be properly assembled, thus explaining why efforts to delete *grpE* in *E. coli* have been unsuccessful (Ang and Georgopoulos 1989).

Transcriptional fusions were constructed to monitor *dnaK* gene expression in vitro. At 28°C in KMB broth, *dnaK::uidA* transcriptional activity in PG4180 correlated with cell growth. Similar results were obtained for *dnaK* in *E. coli* cultures grown at physiological temperatures (Minder et al. 1997).



**Fig. 4.** Temporal expression of  $\beta$ -glucuronidase (GUS) activity in PG4180 containing *dnaK::uidA* (pDNAK2) and *cfl::uidA* (pCFLP) transcriptional fusions. pBBR.GUS contains a promoterless *uidA* gene. All cultures were grown in Hoitink-Sinden medium optimized for coronatine production (HSC) with antibiotic selection (chloramphenicol at 25  $\mu$ g/ml) at 18°C. Growth curves and GUS activity were based on mean values from two parallel cultures. The experiment was repeated with similar results.



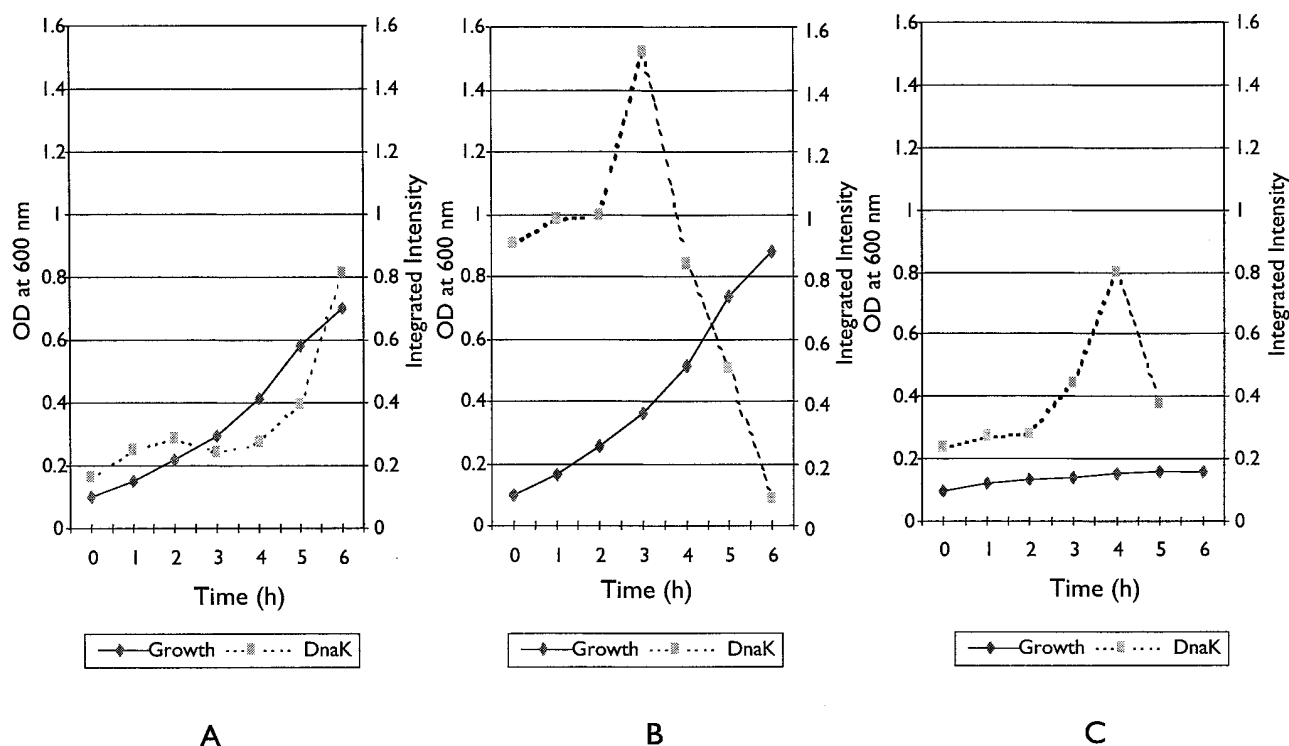
**Fig. 5.** Effect of heat shock on expression of *dnaK* and *cfl* promoter fusions in *Pseudomonas syringae* pv. *glycinea* PG4180. pBBR.GUS, pDNAK2, and pCFLP were the vector control, transcriptionally active *dnaK::uidA* fusion, and transcriptionally active *cfl::uidA* fusion, respectively. All cultures were grown at 18°C for 24 h in Hoitink-Sinden medium optimized for coronatine production (HSC) containing chloramphenicol at 25  $\mu$ g/ml, and then shifted to the new growth temperatures (24 or 35°C). A, Growth curves and (B)  $\beta$ -glucuronidase (GUS) activity were based on mean values from two parallel cultures. The experiment was repeated with similar results.



When PG4180 derivatives were grown at 18°C, a suboptimal temperature for growth, *cfl::uidA* transcriptional activity was extremely high, and *dnaK::uidA* expression remained low but detectable (Fig. 4). Expression of the *cfl::uidA* fusion increased slowly when bacteria were shifted from 18 to 24°C, a temperature that sustains both growth and COR production (Fig. 5B). However, *cfl::uidA* transcriptional activity remained low when cells were shifted from 18 to 35°C. These results are relevant to disease development in the field, where COR contributes to both symptom development and virulence. At temperatures above 30°C, COR production is negligible and symptom development decreases substantially (Palmer and Bender 1993). In contrast, increased *dnaK::uidA* transcriptional activity was detected 2 h after the temperature was shifted from 18 to 35°C. The increased transcriptional activity

after the temperature upshift was also observed at the protein level (Fig. 6B, C). However, elevated levels of *dnaK* transcription and translation did not result in the acclimation of PG4180 to growth at 35 or 38°C.

In order to assess the fate of DnaK in *P. syringae* pv. *glycinea*, Western blotting (immunoblotting) was used to detect DnaK in cultures grown at various temperatures. These experiments differed from typical heat shock experiments, which usually subject a bacterial culture to a short heat treatment (2 to 3 min) and then return the cells to the original growth temperature. In our experiments, cultures were maintained at the new temperature for the duration of the study. We were interested in mimicking conditions found in nature, where phytopathogenic bacteria encounter elevated temperatures for prolonged periods of time. The specific cross-reaction of an *E.*



**Fig. 6.** Growth of *Pseudomonas syringae* pv. *glycinea* race 4 and DnaK detection by Western blotting (immunoblotting) at (A) 26, (B) 32, and (C) 38.5°C. All cultures were grown in Luria-Bertani (LB) broth for 16 to 20 h, adjusted to  $OD_{600} = 0.1$ , and then shifted to 26, 32, or 38.5°C. Total cellular proteins from *P. syringae* pv. *glycinea* were isolated, separated on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gels, and transferred to nitrocellulose membranes. Individual Western blot bands representing DnaK were quantified and shown as integrated intensities. The experiment was repeated several times with similar results.

**Table 1.** *dnaK* promoter sequences

Promoter <sup>a</sup>	-35 region <sup>b</sup>	Spacer region	-10 region <sup>b</sup>
<i>Pseudomonas syringae</i> pv. <i>glycinea</i> <i>dnaK</i>	GAGCAGGCTTGAA	TCAGCTGTAAGGC	CCCCATTAAA
<i>Bradyrhizobium japonicum</i> <i>dnaK</i> P1	<u>TTCGGTCCTTGCG</u>	GGCCCCCCCCCCC	TCCTATATGAG
<i>Bradyrhizobium japonicum</i> <i>dnaK</i> P2	ACGGGGGCTTGCC	GAAGCCCGACCTGT	TAATAACTCCT
<i>Agrobacterium tumefaciens</i> <i>dnaK</i>	<u>CTTGCGCCTTGCA</u>	GCAGGAAAAATCGCT	<u>CCTTATATACG</u>
<i>Caulobacter crescentus</i> <i>dnaK</i> P1	<u>TATGGCCTTGCG</u>	GGCGGCCCTATC	<u>CCCCATATCCG</u>
<i>Escherichia coli</i> $\sigma^{32}$ consensus	TCTC-CCCTTGAA	13 to 17 bp	CCCCAT-TA
$\alpha$ -purple proteobacteria heat shock consensus <sup>c</sup>	CTTG	17 to 18 bp	CYTAT-T-G

<sup>a</sup> Comparison of the putative *P. syringae* pv. *glycinea* PG4180 *dnaK* promoter sequence with the *dnaK* promoters from *B. japonicum* (Minder et al. 1997), *A. tumefaciens* (Segal and Ron 1995), and *C. crescentus* (P1) (Gomes et al. 1990).

<sup>b</sup> The nucleotides identical to those in the consensus sequence for *E. coli*  $\sigma^{32}$  promoters (Yura et al. 1993) are underlined.

<sup>c</sup> The nucleotides found in the -35/-10 consensus sequence for heat shock promoters in  $\alpha$ -proteobacteria (Segal and Ron 1995) are also indicated. Abbreviation: Y, pyrimidine.

*coli* anti-DnaK monoclonal antibody to DnaK in *P. syringae* pv. *glycinea* enabled us to clearly detect the protein temporally at various temperatures. At 26°C, a basal level of DnaK was detected until cells reached the early phase of stationary growth (Fig. 6A). These results are similar to those reported for DnaK in *E. coli* (Rockabrand et al. 1995). When *P. syringae* pv. *glycinea* cells were initially grown at 26°C and then shifted to 32°C, there was an immediate increase in the detection of DnaK; this was followed by decreased detection at 3 h (Fig. 6B). Although a transient increase in DnaK was observed at 2 h when cells were shifted to 38.5°C, *P. syringae* pv. *glycinea* was unable to acclimate to this elevated temperature (Fig. 6C).

In *E. coli*, DnaK, DnaJ, and GrpE make up the Hsp70 chaperone system, which is involved in many metabolic processes. For example, *E. coli* cells that lacked functional copies of the Hsp70 family did not produce flagella, suggesting that DnaK, DnaJ, and GrpE might be required for the proper assembly of flagella (Shi et al. 1992). Other studies indicate that the DnaK chaperone system functions in the localization and regulation of membrane proteins, including those that belong to the two-component signal transduction family (Clarke et al. 1996; Kelley and Georgopoulos 1997). Furthermore, the Hsp70 chaperonins participate in nucleotide excision repair, thereby enhancing the resistance of bacteria to UV radiation (Zou et al. 1998). Consequently, DnaK is not only important in the heat shock phenomenon, but also in many other physiological processes necessary for bacterial survival.

This is the first report of heat shock genes in *P. syringae*, a ubiquitous plant pathogen found in both extreme and temperate climates. Since *P. syringae* pv. *glycinea* is predominantly a cool-weather pathogen, temperature is an important factor in the ability of this organism to incite disease in the soybean host. Our results indicate that the heat shock response in *P. syringae* pv. *glycinea* can be induced at moderate temperatures (32°C) that are only slightly higher than the optimal temperature for growth in vitro (30.7°C). One possible explanation for reduced virulence of *P. syringae* pv. *glycinea* at elevated temperatures is the onset of the heat shock response, which diverts energy and resources away from processes that increase virulence, such as COR synthesis.

## MATERIALS AND METHODS

### Bacterial strains and culture conditions.

The bacterial strains and plasmids used in this study are listed in Table 2. *E. coli* DH5 $\alpha$  was used in all cloning experiments, and pRK2013 was used as a mobilizer plasmid in triparental matings (Bender et al. 1991). All *E. coli* strains were maintained in Luria-Bertani (LB) medium or Terrific Broth (TB) (Sambrook et al. 1989). The *P. syringae* pv. *glycinea* strain PG4180 was grown in King's medium B (KMB) (King et al. 1954) or mannitol-glutamate (MG) medium (Keane et al. 1970) at 28°C. In some experiments, PG4180 was grown in Hoitink-Sinden medium optimized for coro-

**Table 2.** Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Reference or source
<i>Escherichia coli</i>		
DH5 $\alpha$		Sambrook et al. 1989
GW4813	<i>dnaK52::Cm<sup>r</sup></i>	Paek and Walker 1987
PBL500	<i>lacZ::Tn5laqI<sup>91</sup></i>	Rockabrand et al. 1995
<i>Pseudomonas syringae</i> pv. <i>glycinea</i>		
PG4180	Pathogenic on soybeans	Bender et al. 1993
race 4	Pathogenic on soybeans	A. K. Vidaver
Plasmids		
pBluescript II SK <sup>+</sup>	Ap <sup>r</sup> ; ColEI origin; 2.9-kb cloning vector	Stratagene, La Jolla, CA
pZErO2.1	Km <sup>r</sup> ; 3.3-kb cloning vector	Invitrogen, Carlsbad, CA
pRK7813	Tc <sup>r</sup> ; 10.5-kb cosmid vector	Jones and Gutterson 1987
pRK415	Tc <sup>r</sup> ; RK2-derived cloning vector	Keen et al. 1988
pBSL15	Ap <sup>r</sup> Km <sup>r</sup> ; contains 1.2-kb Km <sup>r</sup> cassette in pUC18	Alexeyev 1995
pMCm	Ap <sup>r</sup> Cm <sup>r</sup> ; contains 2.9-kb Cm <sup>r</sup> cassette in pMTL24	Murillo et al. 1994
pBBR1MCS	Cm <sup>r</sup> ; 4.7-kb broad-host-range cloning vector	Kovach et al. 1994
pBBR.GUS	Cm <sup>r</sup> ; 6.6-kb, contains <i>uidA</i> in pBBR1MCS	Peñaloza-Vázquez and Bender 1998
pCFLP	Cm <sup>r</sup> ; contains <i>cfl::uidA</i> in pBBR1MCS	Peñaloza-Vázquez and Bender 1998
pCR2.1	Ap <sup>r</sup> Km <sup>r</sup> ; 3.9-kb cloning vector	Invitrogen
pLK.721	Ap <sup>r</sup> ; 721-bp polymerase chain reaction (PCR) fragment of <i>dnaK</i> from PG4180	This study
pLH3	Tc <sup>r</sup> ; PG4180 library clone in pRK7813	This study
pDF5	Tc <sup>r</sup> ; PG4180 library clone in pRK7813	This study
pLK1	Km <sup>r</sup> ; 5-kb <i>EcoRI</i> fragment from pDF5 in pZErO2.1	This study
pLK2	Ap <sup>r</sup> ; 7-kb <i>HindIII</i> fragment from pLH3 in pBluescript SK <sup>+</sup>	This study
pLK3.Km	Tc <sup>r</sup> Km <sup>r</sup> ; contains <i>dnaK::Km<sup>r</sup></i> allele in pRK415	This study
pLK3.Cm	Ap <sup>r</sup> Cm <sup>r</sup> ; contains <i>dnaK::Cm<sup>r</sup></i> allele in pBluescript SK <sup>+</sup>	This study
pLK4	Ap <sup>r</sup> Km <sup>r</sup> ; 1-kb PCR fragment from PG4180; contains 730 bp of <i>dnaK</i> and 270 bp of upstream DNA in pCR2.1	This study
pLK5	Ap <sup>r</sup> Km <sup>r</sup> ; 0.4-kb PCR fragment from PG4180; contains 130 bp of <i>dnaK</i> and 270 bp of upstream DNA in pCR2.1	This study
pDNAKPR	Cm <sup>r</sup> 1.2-kb <i>EcoRI</i> -PstI fragment in pBBR.GUS; contains the <i>dnaK</i> promoter in transcriptionally inactive orientation	This study
pDNAKP1	Cm <sup>r</sup> ; 1-kb fragment from pLK4 in pBBR.GUS; contains <i>dnak::uidA</i> in transcriptionally active orientation	This study
pDNAKP2	Cm <sup>r</sup> ; 0.4-kb fragment from pLK5 in pBBR.GUS; contains <i>dnak::uidA</i> in transcriptionally active orientation	This study

native production (HSC) at 18°C (Palmer and Bender 1993). When appropriate, antibiotics were added at the following concentrations (µg/ml): ampicillin, 100; chloramphenicol, 25; kanamycin, 25; and tetracycline, 12.5. For analysis of DnaK by Western blotting, *P. syringae* pv. *glycinea* race 4 was maintained on KMB agar and stored at 4°C. Prior to use, a loopful of inoculum was transferred into fresh LB medium and incubated overnight at 26°C in a shaking water bath (250 rpm; Magni Whirl Incubator Shaker; Blue M Electric, Blue Island, IL). *P. syringae* pv. *glycinea* PG4180 was used in all cloning and complementation studies because it was easier to manipulate genetically than *P. syringae* pv. *glycinea* race 4.

#### DNA isolation procedures.

Plasmids were isolated from *E. coli* by the TENS mini-prep procedure (Sambrook et al. 1989). Plasmids submitted for sequencing were purified with the Promega Wizard Mini-Prep Kit (Madison, WI). PG4180 plasmid DNA was isolated by the procedure of Kado and Liu (1981). For library construction, genomic DNA of PG4180 was isolated as described by Staskawicz et al. (1984) and purified on CsCl-EtBr gradients (Sambrook et al. 1989).

#### PCR conditions.

Amplification of *dnaK* by PCR was accomplished with primers synthesized by the University of Nebraska-Lincoln DNA Synthesis Laboratory. Genomic DNA from PG4180 and plasmid pLK2 were used as templates and the following oligonucleotides were used as degenerate primers in the PCR—the variable bases S, B, N, D, and R were defined by the International Union of Biochemistry (Cornish-Bowden 1985): forward primer 5' GCGGAATTCATSACBGTNCCNGCA, the *EcoRI* recognition site is shown in bold and the underscored base pairs correspond to nucleotides 418 to 437 in Bardwell and Craig (1984); and reverse primer 5' ACGGG ATCCATDCCGTTDGCRT CRACRTCGAASGT, the *BamHI* recognition site is shown in bold and the underscored base pairs correspond to the complement of nucleotides 1423 to 1458 in Bardwell and Craig (1984). Conditions for PCR included 25 cycles at 94°C for 1 min, 60°C for 1 min, and 72°C for 3 min. Reaction mixtures (100 µl) contained reaction buffer, 1.5 mM MgCl<sub>2</sub>, 200 µM concentrations of each dNTP, 1 µg of chromosomal DNA or 0.1 µg of plasmid DNA, 50 pmol of each primer, and 10 U of *Taq* DNA polymerase (Promega, Madison, WI).

#### Library construction and screening.

A PG4180 genomic library was constructed in pRK7813 as described previously (Barta et al. 1992). Tc<sup>r</sup> *E. coli* transfectants were screened for *dnaK* by colony hybridization with the 721-bp probe containing a portion of *dnaK* amplified from PG4180 (Fig. 1E). The PCR product was approximately 1 kb but, when digested with *EcoRI* and *BamHI*, the fragment was reduced to 0.7 kb because of an internal *EcoRI* site. For probing the cosmid library, the *dnaK* probe was labeled with [ $\alpha$ -<sup>32</sup>P]dCTP with the Rad Prime DNA Labeling System (Gibco BRL, Gaithersburg, MD). For all other experiments, the probe was labeled nonradioactively by incorporation of digoxigenin-11-dUTP (Genius Labelling and Detection Kit, Boehringer Mannheim, Indianapolis, IN). Selected restriction fragments containing *dnaK* were

subcloned into pBluescript SK<sup>+</sup> or pZErO2.1 according to standard protocols (Sambrook et al. 1989).

#### DNA sequencing and analysis.

Automated nucleotide sequencing was performed with Ampli *Taq* DNA polymerase, an ABI 373A apparatus, and the ABI PRISM Dye Primer Cycle Sequencing Kit (Perkin Elmer, Foster City, CA). Automated sequencing was provided by the Oklahoma State University Recombinant DNA/Protein Resource Facility. The DNA region sequenced and the deduced proteins were analyzed with the software packages provided by UWGCG or the NCBI (National Center for Biotechnology Information) BLAST network server.

#### Complementation of an *E. coli dnaK* mutant.

Competent cells of *E. coli* K-12 strains GW4813 (*dnaK* mutant) and PBL500 (wild type) were prepared by standard procedures (Sambrook et al. 1989) and transformed with pLK2 (Fig. 1B), pLK.721 (Fig. 1E), or pBluescript SK<sup>+</sup>. Growth was compared at 37 and 42°C for the *E. coli* wild-type and mutant strains with and without the plasmid constructs.

#### Construction of a PG4180 *dnaK* mutant.

To facilitate the construction of mutant *dnaK* alleles, the 1.4-kb *DdeI* fragment containing an internal portion of *dnaK* (Fig. 1D) was subcloned in pBluescript SK<sup>+</sup>, creating pLK3. The Km<sup>r</sup> cassette in pBSL15 was then inserted into the *EcoRI* site in the central region of *dnaK* (Fig. 1D), and the 2.6-kb fragment containing the *dnaK::Km<sup>r</sup>* allele was excised and cloned into pRK415. This clone, which was designated pLK3.Km, was introduced into PG4180 by triparental mating, and selection for the vector antibiotic marker (Tc<sup>r</sup>) was removed to facilitate homologous recombination. A similar approach was used to introduce the Cm<sup>r</sup> cassette from pMCM into the *EcoRI* site of the truncated *dnaK* gene in pLK3. This recombinant was designated pLK3.Cm and introduced into PG4180 by electroporation (Sambrook et al. 1989), and Cm<sup>r</sup> transformants were selected.

#### Construction of *dnaK* transcriptional fusions.

Plasmid pBBR.GUS, which contains a promoterless *uidA* gene downstream of a multi-cloning site, was used to create *dnaK::uidA* transcriptional fusions. To obtain *dnaK::uidA* fusions in the transcriptionally active orientation, the *dnaK* upstream region was amplified from pLK2 by PCR with restriction sites incorporated into the primers to facilitate cloning. The forward primer, 5' GCGAAAGCTTTGCAGGAAAGTG CCGACG, was used for amplification of both 1.0- and 0.4-kb PCR products and contained a *HindIII* site (see underscored nucleotides). Reverse primers had *PstI* sites and were 5' GGCAGCTGCAGTCAGACGCTGCAT and 5' CCAGCTG CAGACCGTCATTGGCATAAGC for the 1.0- and 0.4-kb PCR products, respectively. PCR products were cloned into pCR2.1, resulting in pLK4 (1-kb fragment) and pLK5 (0.4-kb fragment). pLK4 and pLK5 were then digested with *HindIII* and *PstI* and cloned into pBBR.GUS, resulting in pDNAKP1 and pDNAKP2, respectively (Fig. 1C). The 1.2-kb *PstI/EcoRI* fragment of pLK2 was cloned in pBBR.GUS in the transcriptionally inactive orientation resulting in pDNAKPR (Fig. 1C). pCFLP, which contains a *cfl::uidA* transcriptional fusion, and pBBR.GUS were also used in GUS assays.

## GUS assays.

Transcriptional activity was initially assayed by spotting bacterial suspensions ( $OD_{600} = 0.1$ ) on LB or MG plates containing 20  $\mu$ g of X-Gluc per ml and incubating at 18 or 28°C for 1 to 2 days, followed by further incubation at 4°C for 2 days. The activity of *dnaK* and *cfl* transcriptional fusions was monitored at various time points by measuring GUS activity. A 50-ml culture of PG4180 containing the constructs described above was grown in KMB or HSC broth (starting concentration  $OD_{600} = 0.1$ ) and incubated at 18 or 28°C, 250 rpm (Queue Radial Shaker; Queue Systems, Parkersburg, WV). Aliquots (1 ml) were removed at various time points and analyzed for GUS activity as described previously (Palmer et al. 1997). GUS activity was expressed in U per mg of protein with 1 U equivalent to 1 nmol of methylumbelliferone formed per min. In temperature shift experiments, *P. syringae* pv. *glycinea* PG4180 containing *dnaK::uidA* or *cfl::uidA* fusions was incubated for 24 h in HSC broth at 18°C, and then shifted to 24 or 35°C.

## Detection of DnaK.

Overnight cultures of *P. syringae* pv. *glycinea* race 4 grown at 26°C for 16 to 20 h were diluted in LB medium (50 ml) to obtain an  $OD_{600} = 0.1$ . These cultures were shifted to 26, 32, or 38.5°C; after a 2-min equilibration period at the new temperature, the  $OD_{600}$  was measured, and a sample representing  $1 \times 10^9$  CFU was removed. The cells were harvested by centrifugation at  $10,000 \times g$  for 5 min and then suspended in 1 $\times$  sample buffer (3 $\times$ : 125 mM Tris [pH 6.8], 6% sodium dodecyl sulfate (SDS), 30% glycerol, 5 mg of bromophenol blue per ml). This procedure was repeated every hour for 6 h. Samples were temporarily stored at -20°C and then boiled for 10 min prior to SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

## Gel electrophoresis and Western blotting.

SDS-PAGE was performed in 12.5% (wt/vol) acrylamide overlaid with a 10% (wt/vol) stacking gel (Sambrook et al. 1989). Electrophoresis was at room temperature with constant voltage (125 V) until the tracking dye reached the end of the separating gel. Gels were stained with Coomassie blue (2 mg/ml) in 50% methanol-10% acetic acid, destained in 50% methanol-10% acetic acid, and dried under vacuum. Electrophoretic transfer of polypeptides to nitrocellulose and immunoblotting were performed as described by Sambrook et al. (1989). Blots were incubated with an anti-DnaK monoclonal antibody provided by Paul Blum (University of Nebraska-Lincoln) and then with a goat anti-mouse IgG alkaline phosphatase conjugate (Gibco BRL, Gaithersburg, MD). Bound alkaline phosphatase was detected with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate. DnaK levels were compared by image analysis with a Visage 110 (BioImage, Ann Arbor, MI) image analyzer (Dougherty et al. 1989). Images were acquired by a solid-state CCD array (512  $\times$  512) 8-bit video camera, providing a resolution of approximately 0.17 mM per pixel. Images were analyzed with BioImage E.Q. software in a Sun 110-3 computer. The software captured the Western blot image and constructed a database, in which the *x/y* location, area, and shape of each band were reported. The integrated intensity (absorbance  $\times$  mm<sup>2</sup>) was calculated for each band, and the relative densities of DnaK were in the linear range of detection.

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