JOURNAL OF BACTERIOLOGY, Sept. 2005, p. 5967–5976 Vol. 187, No. 17
0021-9193/05/$08.00+0 doi:10.1128/JB.187.17.5967–5976.2005
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The Heat Shock Genes *dnaK*, *dnaJ*, and *grpE* Are Involved in Regulation of Putisolvin Biosynthesis in *Pseudomonas putida* PCL1445

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Received 5 April 2005/Accepted 18 June 2005

Pseudomonas putida **PCL1445 produces two cyclic lipopeptides, putisolvins I and II, which possess surfactant activity and play an important role in biofilm formation and degradation. In order to identify genes and traits that are involved in the regulation of putisolvin production of PCL1445, a Tn*5luxAB* library was generated and mutants were selected for the lack of biosurfactant production using a drop-collapsing assay. Sequence analysis of the Tn*5luxAB* flanking region of one biosurfactant mutant, strain PCL1627, showed that the transposon had inserted in a *dnaK* homologue which is located downstream of *grpE* and upstream of *dnaJ*. Analysis of putisolvin production and expression studies indicate that *dnaK*, together with the *dnaJ* and *grpE* heat shock genes, takes part in the positive regulation (directly or indirectly) of putisolvin biosynthesis at the transcriptional level. Growth of PCL1445 at low temperature resulted in an increased level of putisolvins, and mutant analyses showed that this requires *dnaK* and *dnaJ* but not *grpE*. In addition, putisolvin biosynthesis of PCL1445 was found to be dependent on the GacA/GacS two-component signaling system. Expression analysis indicated that *dnaK* is positively regulated by GacA/GacS.**

Lipopeptides are produced by members of the genera *Bacillus*, *Serratia*, *Burkholderia*, and *Pseudomonas*. Lipopeptides are nonribosomally synthesized via multifunctional proteins, which are encoded by large gene clusters (3, 43, 46). Lipopeptides produced by *Pseudomonas* have been reported as agents for biocontrol of phytopathogenic fungi (35) or as phytotoxins (18). Lipopeptides produced by gram-positive *Bacillus* play a role in bacterial attachment to surfaces (34). Lipopeptides produced by *Serratia* (29) and *Burkholderia* (16) were shown to be essential for the stimulation of swarming motility and thus could contribute to the regulation of biofilm formation (16).

Lipopeptides function as biosurfactants (7) by stimulating swarming motility (26, 29), facilitating bacterial growth on water-insoluble carbon sources (40), or by altering the cell surface hydrophobicity and therefore influencing the interaction between the individual cells (40). However, the significance of lipopeptides for growth and survival of rhizobacteria remains unkown. The regulation of lipopeptides in soil *Pseudomonas* is poorly understood. The GacA/GacS two-component regulatory system was shown to control regulation of lipopeptides syringomycin (15), and lipopeptides of *Pseudomonas* sp. strain DSS73 (22). Whether the *gac* system controls directly the lipopeptide biosynthesis remains to be investigated as, to our knowledge, no intermediate involved in this regulation has been identified.

Pseudomonas putida PCL1445 was isolated from soil heavily polluted with polyaromatic hydrocarbons (24) and produces two surface-active compounds, putisolvin I and putisolvin II, which have been identified as cyclic lipopeptides (26). They represent a new class of lipodepsipeptides consisting of 12 amino acids linked to a hexanoic lipid chain. Strain PCL1445 produces putisolvin I and II via a putisolvin synthetase (26), later designated as *psoA*.

Putisolvins I and II have important functions for PCL1445 as they were shown (i) to reduce the surface tension of the medium, (ii) to increase the formation of an emulsion with toluene, (iii) to stimulate swarming motility, and (iv) to inhibit biofilm formation and to degrade existing biofilms (26).

Putisolvins are not constitutively produced. Surfactant activity appeared in the culture medium at the end of the exponential growth phase (26). The aim of the present work was to identify and characterize genes that are involved in the regulation of lipopeptide production and to investigate their function. To this end we generated a Tn*5luxAB* library of PCL1445 and screened for mutants defective in biosurfactant production using a drop-collapsing assay. We analyzed one biosurfactant mutant in detail. Its transposon appeared to be integrated in a *dnaK* homolog, encoding a heat shock protein. DnaK, DnaJ, and GrpE chaperones have been described to form the central regulatory system of the heat shock response in *Escherichia coli* (9, 17, 37). In this paper, we describe the analysis of the function of *dnaK*, *dnaJ*, and *grpE* in putisolvin biosynthesis, as well as their roles at different temperatures.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All bacterial strains used are listed in Table 1. *Pseudomonas* strains were grown in King's medium B (KB) (21) at 28°C under vigorous shaking (190 rpm). *E. coli* strains were grown in Luria-Bertani medium (41) at 37°C under vigorous shaking. Media were solidified with 1.8% agar (Select Agar; Invitrogen, Life Technologies, Paisley, United Kingdom). The antibiotics kanamycin, tetracycline, gentamicin, and carbenicillin were added, when necessary, to final concentrations of 50, 40, 2, and 100 μg ml^{-1}, respectively.

Generation, selection, and characterization of mutants defective in biosurfactant production. Transposon mutants were generated by triparental mating using pRL1063a, which harbors a Tn*5* transposon carrying the promoterless *luxAB*

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
Pseudomonas		
PCL1445	Wild-type *P. putida*; colonizes grass roots and produces biosurfactants	23
PCL1436	Tn*5luxAB* derivative of PCL1445; mutated in *psoA*, a lipopeptide synthetase homologue	26
PCL1622	Tn*5luxAB* derivative of PCL1445; mutated in a *gacA* homologue	This study
PCL1623	Tn*5luxAB* derivative of PCL1445; mutated in a *gacS* homologue	This study
PCL1627	Tn*5luxAB* derivative of PCL1445; mutated in a *dnaK* homologue	This study
PCL1628	PCL1445 derivative mutated in the *dnaJ* homologue; constructed by single homologous recombination	This study
PCL1629	PCL1445 derivative mutated in the *grpE* homologue; constructed by single homologous recombination	This study
Escherichia coli		
DH5α	*EndA1 gyrSA96 hrdR17* (r_K^- m_K^-) *supE44 recAI*; general purpose host strain used for transformation and propagation of plasmids	11
Plasmids		
pRL1063a	Plasmid harboring a promotorless Tn*5luxAB* transposon, Km^r	48
pRK2013	Helper plasmid for triparental mating, Km^r	42
pMP5505	pRL1063a-based plasmid recovered from chromosomal DNA of PCL1627 after digestion with EcoRI with Tn*5luxAB*, Km^r	This study
pME6010	Cloning vector which is maintained in *Pseudomonas* strains without selection pressure, Tc^r	12
pME3049	Cloning vector, used for homologous recombination, Tc^r Hg^r	8
pML103	pML10 derivative *lac*-fusion broad-host-range vector for gram-negative bacteria, Gm^r	27
pJBA89	pUC18 Not-*luxR*-P_{luxI}PBSII-*gfp* (ASV)- To -T1, Ap^r	1
pBBR1MCS-5	Broad-host-range cloning vector for gram-negative bacteria, Gm^r	23
pMP4669	pME6010 derivative harboring P_{tac} DsRed, Tc^r	4
pMP6562	pME6010 containing *gacS* gene of PCL1171, used for complementation, Tc^r	45
pMP5285	pME3049 derivative, missing the Hg^r gene, used for single homologous recombination, Km^r	25
pMP5512	pMP6010 containing a PCR fragment of 1.3 kb with *gacA* gene of PCL1445, Tc^r	This study
pMP5518	pME6010 containing a PCR fragment of 3.5 kb with the *dnaK* and *dnaJ* genes from pMP5505, used for complementation, Tc^r	This study
pMP5519	pMP5518 derivative containing *dnaK* gene and the 5′ 366 bp of *dnaJ* gene, Tc^r	This study
pMP5530	pMP5518 containing the 3′ 520 bp of *dnaK* and *dnaJ* gene Tc^r	This study
pMP5534	pME6010 containing a PCR fragment of 1.1 kb with the *grpE* gene from pMP5505, Tc^r	This study
pMP5524	pMP5285 containing a 0.6 kb EcoRI-*Eco*RI PCR fragment of the central part of *dnaJ* gene from pMP5505, Km^r	This study
pMP5532	pMP5285 containing a 0.5 kb *Eco*RI-*Eco*RI PCR fragment of the central part of *grpE* gene from pMP5505, Km^r	This study
pMP5535	pML103 containing the *dnaK*::*lacZ* promoter in transcriptionally active orientation, Gm^r	This study
pMP5536	pML103 containing the *dnaK*::*lacZ* promoter in transcriptionally inactive orientation, Gm^r	This study
pMP5537	pMP6516 derivative with *phzA* promoter replaced by *psoA* promoter in transcriptionally active orientation, Gm^r	This study
pMP5538	pMP6516 derivative with *phzA* promoter replaced by *psoA* promoter in transcriptionally inactive orientation, Gm^r	This study
pMP5539	pMP5537 derivative harboring *psoA*::*gfp* transcriptionally active fused to pMP4669 harboring P_{tac} DsRed, Gm^r Tc^r	This study
pMP5540	pMP5538 derivative harboring *psoA*::*gfp* transcriptionally inactive fused to pMP4669 harboring P_{tac} DsRed, Gm^r Tc^r	This study
pMP7551	pGemT cloning vector containing an amplified cDNA fragment of 0.75 kb with the beginning part of *dnaJ*, Cb^r	This study

reporter genes (48), and the helper plasmid pRK2013 (42). Transposants were initially screened for the decreased ability to flatten a droplet of water on Parafilm using cells of a single colony as described below. Culture supernatants of the selected mutants, obtained after growth overnight in King's B (KB) medium, were analyzed for the presence of surfactant production using the drop-collapsing assay.

To isolate the DNA region flanking the Tn*5*, total genomic DNA was isolated and digested with EcoRI, which does not cut pRL1063a. Digested genomic DNA fragments were recirculated and selected for kanamycin resistance, resulting in plasmids containing genomic DNA regions flanking the Tn*5luxAB*. All DNA techniques were performed as described in reference 41. Sequencing of the plasmids was performed by BaseClear (Leiden, The Netherlands). DNA sequences were analyzed with the software packages provided by the National Center for Biotechnology Information BLAST network server. Biolog SF-N microplates (Biolog, Hayward, CA) were used according to the protocol provided by the manufacturer. The plates were read after 24 h of incubation at 28°C using a microplate reader model 3550 (Bio-Rad Laboratories, Hercules, CA) at an optical density at 595 nm (OD_{595}).

Construction of *dnaJ* and *grpE* mutants. A *Pseudomonas putida* PCL1445 *dnaJ* mutant was constructed by homologous recombination. A 0.6-kb internal fragment of the *dnaJ*-homologous gene of strain PCL1445 was obtained by PCR using primers oMP862 (5′ CAGTTCAAGGAGGCCAACGAG 3′) and oMP863 (5′ CGGGCCACCATGGGTACC 3′), cloned into pGEM-T Easy Vector System I (Promega Corporation, Madison, WI), and ligated as an EcoRI-EcoRI insert with the pMP5285 (25) suicide plasmid derived from pME3049 (8), resulting in pMP5524. pMP5524 was transferred to *P. putida* PCL1445 by triparental mating using pRK2013 as a helper plasmid (42) and using selection on KB agar medium supplemented with kanamycin (50 μg ml^{-1}). Strain PCL1628 was obtained as a resistant colony resulting from single homologous recombination. The insertion of the suicide construct was confirmed by Southern hybridization.

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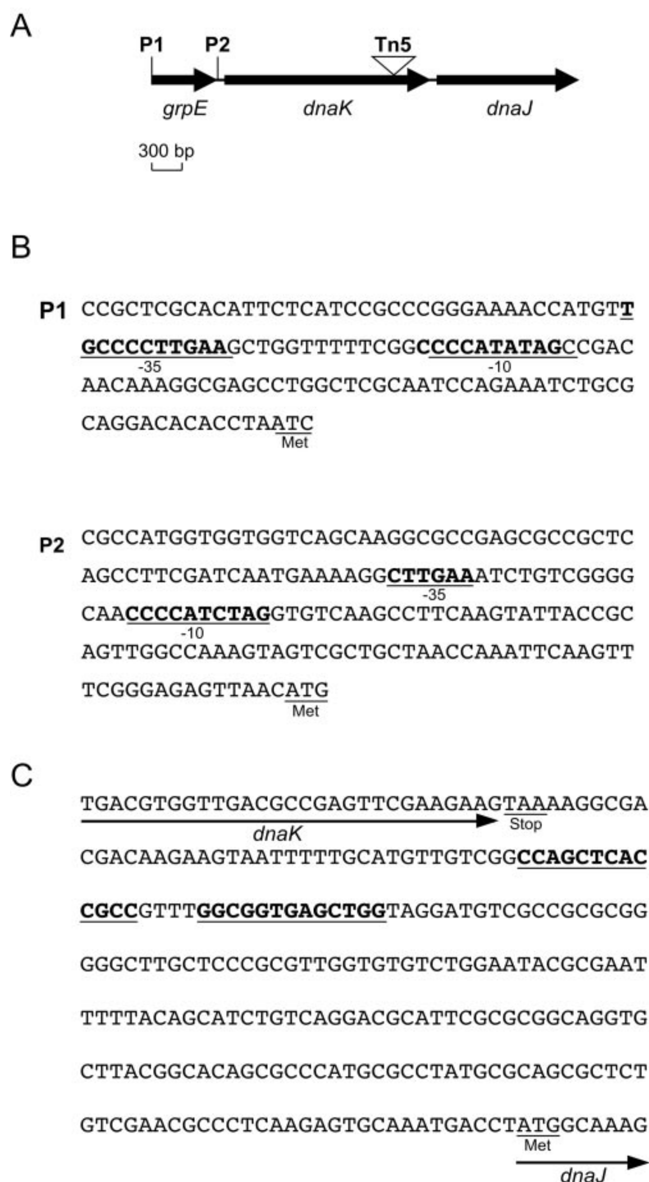

FIG. 1. (A) Schematic representation of the *grpE-dnaK-dnaJ* chromosomal region of *Pseudomonas putida* PCL1445 showing the location of the transposon insertion in *dnaK* of mutant strain PCL1627. (B) Sequence of the 5′ upstream region of *grpE* and the adjacent *dnaK* gene. Features of the putative promoters P1 and P2 are indicated. (C) Sequence of the *dnaK-dnaJ* intergenic region. Features of the putative terminator stem loop are indicated ($\Delta G = -21$ kcal mol^{-1}). Nucleotides forming the stem are indicated in bold and underlined.

A *P. putida* PCL1445 *grpE* mutant was constructed using a similar mutagenesis strategy. The *grpE* fragment for the construction of the suicide plasmid pMP5532 resulted from a PCR using primers oMP874 (5′ GAAGAGACTGGTGCAGCAGAT 3′) and oMP875 (5′ CATTGATCGAAGGCTGAGCGG 3′) and chromosomal DNA of strain PCL1445 as a template. Single homologous recombination in *grpE* resulted in strain PCL1629.

Complementation of *dnaK*, *dnaJ*, and *grpE* mutants of PCL1445. To complement *dnaK*, *dnaJ*, and *grpE* mutants, several plasmids were constructed. pMP5518 containing *dnaK* and *dnaJ*, pMP5519 containing *dnaK*, pMP5530 containing *dnaJ*, and pMP5532 containing *grpE*. Complementation of strain PCL1627 (*dnaK* mutant) and mutant PCL1628 (*dnaJ* mutant) was carried out using pMP5518, a shuttle vector derived from pME6010 (12) in which a 3.5-kb fragment containing *dnaK* and *dnaJ* of strain PCL1445 was inserted. This insert was obtained by PCR using primers oMP918 (5′ TGCTCAAGGTGTTCCAGAAGG 3′) and oMP919 (5′ GCGCCCATTACCGCAATA 3′). pMP5518 was transferred to strains PCL1627 and PCL1628 by triparental mating as described above, and transformants were selected on KB agar medium supplemented with tetracycline (40 μg ml^{-1}). To complement the *dnaK* insertion in PCL1627 with only *dnaK*, pMP5518 was digested with SphI to create a deletion removing the second part of the *dnaJ* gene, resulting in pMP5519. In order to be able to complement the mutation in the *dnaJ* gene of PCL1628 with only *dnaJ*, digestion of pMP5518 with *Sca*I was carried out to delete the first part of the *dnaK* gene, resulting in pMP5530. To complement the mutation in *grpE* of PCL1629, a 1.1-kb PCR fragment containing the *grpE* gene of strain PCL1445 was obtained using primers oMP876 (5′ GAGGGCGTCAAGCATGATCGA 3′) and oMP877 (5′ TGGTCCCCAAGTCGATACCGA 3′) and cloned into pME6010, resulting in pMP5534.

5′ RACE. A 5′ rapid amplification of cDNA ends (5′ RACE) system, second generation (Roche Diagnostics GmbH, Penzberg, Germany), was used to determine the length of the *dnaJ* mRNA. Briefly, total RNA (1 μg) isolated from log-phase PCL1445 cells by RNeasy silica gel membrane column (QIAGEN GmbH, Hilden, Germany) purification was reverse transcribed into cDNA with the 3′ *dnaJ* primer oMP899 (5′ GGATCTTCAGCTTCACCCGGCCAT 3′). The purified cDNA was subjected to PCR using the *dnaJ* gene-specific nested primer oMP900 (5′ TGTAGCTGATCGGCACTTCGCAGTA 3′) and the 5′ RACE anchor primer containing 3′sequence complementary to the homopolymeric poly(dC) tail. The resulting PCR product was reamplified using primer oMP901 (5′ AGATCTCGTGCTCACGCACGTTGAT 3′) and the 5′ RACE primer complementary to the homopolymeric poly(dC) tail. The length of the product was estimated by gel electrophoresis.

Biosurfactant production. The production of biosurfactant activity was detected using the drop-collapsing assay as described previously (19), in which the reduction of the water surface tension can be observed as the collapse of a round droplet placed on a hydrophobic surface (19).

To quantify the biosurfactant production in culture medium, the decrease of surface tension between culture medium and air was determined using a Du Nouy ring (K6 Krüss, GmbH, Hamburg, Germany) (26).

Extraction and HPLC analysis of putisolvins. To quantify the production of putisolvins in KB culture medium, 5 ml of a KB culture supernatant was extracted with 1 volume of ethyl acetate (Fluka Chemie, Zwijndrecht, The Netherlands) as described previously (26). Ethyl acetate extracts were evaporated under vacuum to dryness and dissolved in 55% acetonitrile (Labscan Ltd., Dublin, Ireland). Dry material obtained from 5 ml of culture was resuspended in 500 μl of 50/50 (vol/vol) acetonitrile-water and purified on a spinX centrifuge tube filter of 0.45-μm pore size (Corning Costar Corporation, Cambridge, MA). A volume of 500 μl of the samples was separated by high-performance liquid chromatography (HPLC) (Jasco International CO. Ltd., Japan), using a reverse-phase C_8 5-μm Econosphere column (Alltech, Deerfield, IL), a PU-980 pump system (Jasco, B&L systems, Boechout, Belgium), an LG-980-02 gradient unit (Jasco), and an MD 910 detector (Jasco). Separation was performed using a linear gradient, starting at 35/65 (vol/vol) acetonitrile-water and ending at 20/80 after 50 min at a flow rate of 1 ml min^{-1}. Chromatograms were analyzed in the wavelength range between 195 nm and 420 nm. Fractions that corresponded to the retention time of 34 min for putisolvin I and 36 min for putisolvin II were collected and tested for activity in the drop-collapsing assay. The amount of putisolvins produced was determined as the area of the peak detected in micro-absorbance units (μAU) at the wavelength of 206 nm.

Construction of *psoA*::*gfp* transcriptional fusions. A 1.2-kb HindIII fragment containing the *luxI* promoter and the gene encoding green fluorescent protein (*gfp*) from pJBA89 (1) was cloned into the broad-host-range vector pBBR1MCS-5 (23), resulting in pMP4670. Subsequently the SphI fragment containing *lac*, *luxR*, and *luxI* promoters was removed, resulting in pMP4683. Removal of one HindIII site at the end of the *gfp* gene in pMP4683 resulted in pMP4689. The N-terminal ASV tag from pMP4689 was removed using StuI and SmaI digestion followed by religation, which resulted in pMP6516. To construct a *psoA*::*gfp* transcriptional fusion, a 0.75-kb PCR fragment containing the *psoA* promoter of strain PCL1445 was obtained using primers oMP907 (5′ GCATGCAAGCGATGAAAGCAGATGACCCAG 3′) and oMP908 (5′ GCATGCGTCGGCAGGTCCTTCTGATTGATC 3′) in which SphI sites were incorporated (see underlined nucleotides). The *psoA* promoter was cloned into pMP6516 as an SphI fragment, resulting in pMP5537, containing *psoA*::*gfp* in the transcriptionally active orientation, and into pMP5538, containing *psoA*::*gfp* in the transcriptionally inactive orientation, by cloning the fragment in the reverse orientation. The constructs pMP5537 and pMP5538 were fused as BamHI fragments to BglII-digested pMP4669 harboring P_{tac} DsRed, resulting in rhizosphere-stable

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TABLE 2. Comparison of the putative *P. putida* PCL1445 *grpE* and *dnaK* heat-shock promoter sequences with promoters from *E. coli*, *P. syringae* pv. *glycinea*, and *C. crescentus*

Promoter (reference)	−35 region sequence	Spacing (bp)	−10 region sequence
E. coli σ^{32} consensus (16)	TCTC-CCCTTGAA	13–15	CCCCATTTA
P. syringae pv. *glycinea dnaK* (16)	GAGCAGGCTTGAA	13	CCCCATTTA
Caulobacter crescentus dnaK P1 (8)	TTATGGCCTTGCG	13	CCCCATATC
P. putida PCL1445			
grpE (P1)	TGCCCCTTGAA	14	CCCCATATA
dnaK (P2)	AAAGGCTTGAA	14	CCCCATCTA

plasmids pMP5539 and pMP5540, respectively. The constructs were transferred to PCL1445 and PCL1627 by triparental mating as described previously, and transformants were selected with gentamicin (2 μg ml^{-1}) and tetracycline (40 μg ml^{-1}). Expression of *gfp* was quantified using a HTS7000 Bio Assay reader (Perkin-Elmer Life Sciences, Oosterhout, The Netherlands). Bacterial strains were grown to an OD_{620} of 2.0 and diluted to an OD_{620} of 0.6. Fluorescence of the diluted cultures was quantified using a white 96-well microtiter plate containing 200-μl culture aliquots. Fluorescence of the cultures was determined at an excitation wavelength of 485 nm and an emission wavelength of 520 nm.

Construction of *dnaK*::*lacZ* transcriptional fusions. Plasmid pML103 (27), which contains a promoterless *lacZ* gene downstream of a multicloning site, was used to create a *dnaK*::*lacZ* transcriptional fusion. The region upstream of *dnaK* was amplified from PCL1445 by PCR using primer oMP870 (5′ TCAAGCGCT ACAACCTCGAGG 3′) and primer oMP871 (5′ GCATGCCATGTTAACTCT CCCGAAAC 3′) in which SphI sites were incorporated (see underlined nucleotides). The 0.35-kb PCR product was cloned as an SphI fragment into pML103, resulting in pMP5535 containing *dnaK*::*lacZ* in the transcriptionally active orientation and pMP5536 containing *dnaK*::*lacZ* in the transcriptionally inactive orientation (reverse orientation of the fragment). Plasmids pMP5535 and pMP5536 were transformed into PCL1445 and its derivatives PCL1622 and PCL1623 by triparental mating. Transformants were selected on KB agar medium supplemented with gentamicin (2 μg ml^{-1}) and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; 40 μg ml^{-1}) (Ophaero Q; Biosolve B.V., Valkenswaard, The Netherlands). The activity of *dnaK* transcriptional fusions was assayed by determining β-galactosidase activity (expressed in Miller units). Aliquots (200 μl) were removed from cultures diluted to an OD_{620} of 0.6 and analyzed for β-galactosidase activity by a standard method (30).

Nucleotide sequence accession number. The nucleotide sequences of the *P. putida* PCL1445 *grpE-dnaK-dnaJ* DNA region reported in this paper have been deposited in the GenBank database under accession number AY823737. The nucleotide sequences of the *P. putida* PCL1445 *gacS* and *gacA* DNA regions have been deposited in the GenBank database under accession numbers AY920315 and AY920316 respectively.

RESULTS

Isolation and characterization of the biosurfactant mutant PCL1627. In order to identify genes involved in putisolvin biosynthesis of *P. putida* PCL1445, 2,000 Tn*5luxAB* transposants were screened for loss of surfactant activity as judged by the drop-collapsing assay, using cells derived from a single colony. Strain PCL1627 was isolated together with two other mutants PCL1622 and PCL1623. After overnight growth in liquid KB, medium supernatant of strain PCL1627 was not able to decrease the surface tension between culture medium and air (54 mN m^{-1}) when compared to PCL1445 (32 mN m^{-1}).

Sequence analysis of the chromosomal regions flanking the Tn*5luxAB* showed that the transposon is inserted in an open reading frame (ORF) with 93% similarity at the amino acid level to the *dnaK* gene of *P. putida* KT2440 and 85% similarity to the *dnaK* gene of *P. aeruginosa* PAO1 (Fig. 1A). *dnaK* codes for a molecular chaperone belonging to the Hsp70 protein family, which is part of the heat shock response system (17, 20, 44). In *P. putida* PCL1445 a sequence similar to those of σ^{32}-dependent promoters was identified 78 bp upstream of the *dnaK* translational start (Fig. 1B). The *dnaK* promoter recognized by σ^{32} is located 121 bp upstream of the *dnaK* translational start in *E. coli* (6) and 86 bp upstream of the *dnaK* translational start in *Pseudomonas syringae* pv. *glycinea* PG4180 (20). The *E. coli* σ^{32} consensus sequences are TCTC-CCCTT GAA (−35) and CCCCAT-TA (−10). In *E. coli*, these two regions are separated by 13 to 17 bp. In *P. syringae* pv. *glycinea* and in *P. putida* PCL1445, the two putative −35 and −10 regions are separated by 14 bp (conserved nucleotides are underlined) (Table 2).

Downstream of *dnaK* a DNA sequence is present that contains complementary nucleotides, which can form a hairpin structure with 13 bp in the stem and 3 bp in the loop, resulting in a hairpin with ΔG of −21 kcal mol^{-1} (Fig. 1C). No consensus terminator sequence was found. Further downstream of *dnaK* the presence of an ORF was found (Fig. 1A) with amino acid homologies of 95% with the *dnaJ* gene product of *P. putida* KT2440 and 85% with *dnaJ* of *P. aeruginosa* PAO1, which encodes another molecular chaperone (17). The region upstream of *dnaK* revealed an ORF that showed 85% homology with *grpE* of *P. putida* and 73% homology with *grpE* of *Pseudomonas aeruginosa* PAO1 at the amino acid level (17). Upstream of this *grpE* homologue, a similar conserved nucleotide sequence as in the promoter region of the *dnaK* homologue, corresponding to the binding site for the σ^{32} subunit, was found. This suggests that *grpE* is also heat shock regulated in PCL1445 (Table 2). Comparison of the order of these genes in strain PCL1445 with those of *P. aeruginosa* (GenBank; www.pseudomonas.bit.uq.edu.au), *P. syringae* pv. *tomato* DC3000, and *P. putida* KT2440 showed the same gene arrangement. Two results suggest that *dnaK* and *dnaJ* are not cotranscribed in PCL1445. First, a putative terminator stem loop was identified in the region upstream of *dnaJ* (Fig. 1C). Second, the intergenic region between *dnaK* and *dnaJ* (213 bp) was found to be longer than in other *Pseudomonas* sp. (varying between 115 bp in *P. aeruginosa* PAO1 and 198 bp in *P. putida* KT2440). However, no typical heat shock promoter consensus was found in front of the *dnaJ* gene.

dnaJ-containing mRNA was amplified by PCR using a 3′-*dnaJ*-specific primer, which resulted in a 750-bp *dnaJ*-containing PCR product (data not shown). Thus, this indicates that *dnaJ* is transcribed as a single gene in PCL1445.

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Temperature tolerance of PCL1627 (*dnaK* mutant), PCL1628 (*dnaJ* mutant), and PCL1629 (*grpE* mutant). To test the tolerance to a temperature shift from low to high incubation temperature of cells from strains PCL1627 (*dnaK* mutant), PCL1628 (*dnaJ* mutant), PCL1629 (*grpE* mutant), and its wild type, PCL1445, the cells were precultured overnight in KB medium at 18°C under vigorous aeration. These cells were subsequently diluted to an OD_{620} of 0.1 in fresh KB medium and incubated at 28°C (the optimal growth temperature for *Pseudomonas*) (Fig. 2A) or at 35°C to follow cell growth in time (Fig. 2B). A temperature shift from 18°C to 28°C did not affect the growth rate of PCL1627 (*dnaK* mutant), PCL1628 (*dnaJ* mutant), and PCL1629 (*grpE* mutant) as compared with the wild type (Fig. 2A). However, when the incubation temperature was shifted to 35°C, mutants PCL1627 (*dnaK* mutant), PCL1628 (*dnaJ* mutant), and PCL1629 (*grpE* mutant) had a higher generation time (110.3 ± 1.8 min) than PCL1445 (74.6 ± 1.4 min) and the optical density of PCL1627 (*dnaK* mutant), PCL1628 (*dnaJ* mutant), and PCL1629 (*grpE* mutant) never reached the same value as that of the wild type (Fig. 2B). The determination of the number of CFU during growth at 35°C for PCL1627 (*dnaK* mutant) (Fig. 2C) strongly correlated with the cell density at 35°C (Fig. 2B). The growth phenotype of PCL1627 (*dnaK* mutant) could be restored only by introduction of a plasmid carrying functional *dnaK* and *dnaJ* but not with *dnaK* alone (Fig. 2C). This result suggests that at high incubation temperatures, *dnaK* and *dnaJ* regulation depends on a single heat shock promoter.

Construction of a *dnaJ* mutant and a *grpE* mutant and complementation analyses of mutants for the production of lipopeptides. To investigate whether *dnaJ* and *grpE* (Fig. 3E and G) are also involved in putisolvin production, insertion mutants were constructed by single homologous recombination using suicide plasmids pMP5524 and pMP5532 (see Materials and Methods), resulting in PCL1628 and PCL1629, respectively. The integration of pMP5524 and pMP5532 was confirmed by Southern hybridization (data not shown).

Biosurfactant production of PCL1628 (*dnaJ* mutant) and PCL1629 (*grpE* mutant) grown in KB medium under standard conditions until the stationary phase was reached (28°C and vigorous aeration) was quantified by the Du Nouy ring method. Culture supernatant of PCL1628 (*dnaJ* mutant) was not able to decrease the surface tension between culture medium and air (54 mN m^{-1}), indicating a lack of biosurfactant production. Culture supernatant of PCL1629 (*grpE* mutant) caused a slight decrease of the surface tension (48 mN m^{-1}). In comparison the surface tension of PCL1445 was decreased to the value of 32 mN m^{-1}.

Complementation analyses were conducted using the constructs pMP5519 (*dnaK*), pMP5530 (*dnaJ*), and pMP5534 (*grpE*). The production of putisolvins by PCL1445, PCL1627 (*dnaK* mutant), PCL1628 (*dnaJ* mutant), and PCL1629 (*grpE* mutant) was tested by HPLC analysis (Fig. 3). Putisolvins were extracted from overnight KB culture supernatant, and production was quantified by determination of the area of the peaks with surfactant activity as tested by the drop-collapsing assay. Putisolvins I and II were eluted at 34 and 36 min, respectively (Fig. 3). Mutant PCL1627 (*dnaK* mutant) showed a significant reduction (90%) in putisolvin production (Fig. 3). Introduction of pMP5519, harboring a functional *dnaK* gene, restored putisolvin production in PCL1627 (*dnaK* mutant) (Fig. 3D). This result shows that the insertion of the transposon in *dnaK* is responsible for the decrease of lipopeptide production and that this decrease is not due to a downstream effect on *dnaJ* (Fig. 3). Production of putisolvins by mutant PCL1628 (*dnaJ* mutant) was almost completely abolished (Fig. 3E), while mu-

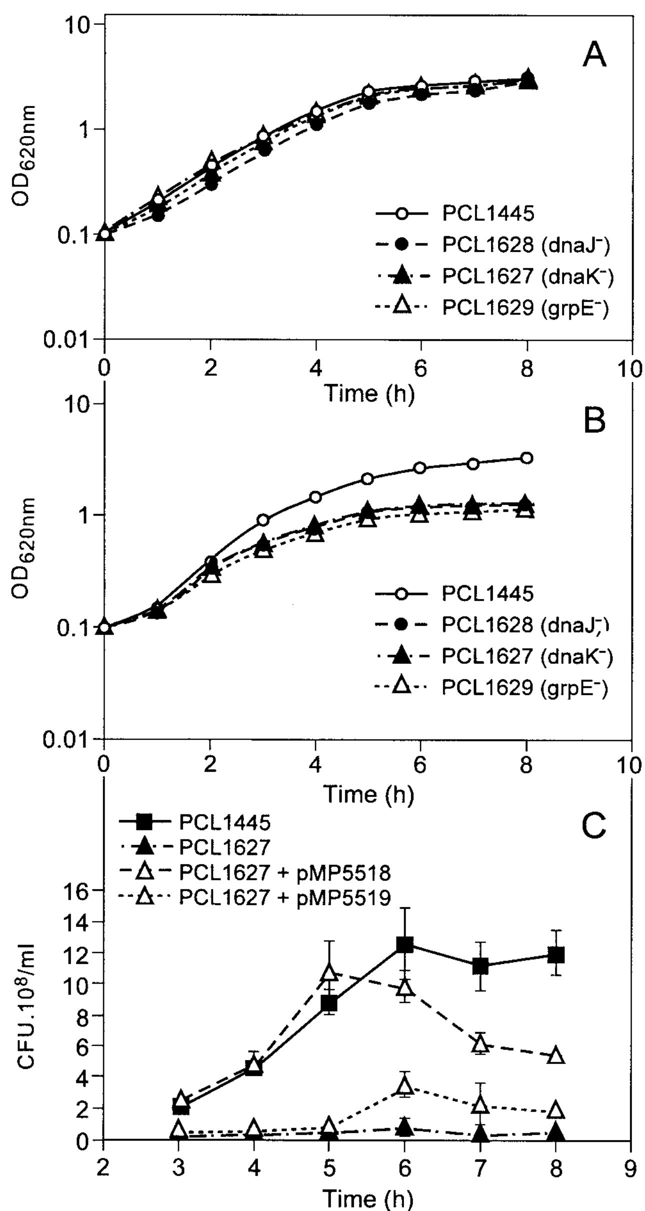

FIG. 2. Growth of *P. putida* PCL1445 and its mutants PCL1436 (*psoA* mutant), PCL1627 (*dnaK* mutant), PCL1628 (*dnaJ* mutant), and PCL1629 (*grpE* mutant). Cells were precultured overnight at 18°C in KB medium, adjusted to OD_{620} of 0.1, and then grown at 28°C (A) or 35°C (B) with vigorous aeration (190 rpm). Panel C represents the number of CFU ml^{-1} during growth at 35°C of PCL1627 (*dnaK* mutant), PCL1627 (*dnaK* mutant) harboring pMP5518 containing *dnaK* followed by *dnaJ*, and PCL1627 (*dnaK* mutant) harboring pMP5519 containing *dnaK* followed by part of *dnaJ*. Samples were taken at regular time points to determine the optical density or CFU ml^{-1}. Standard deviations are based on the mean values of two parallel cultures.

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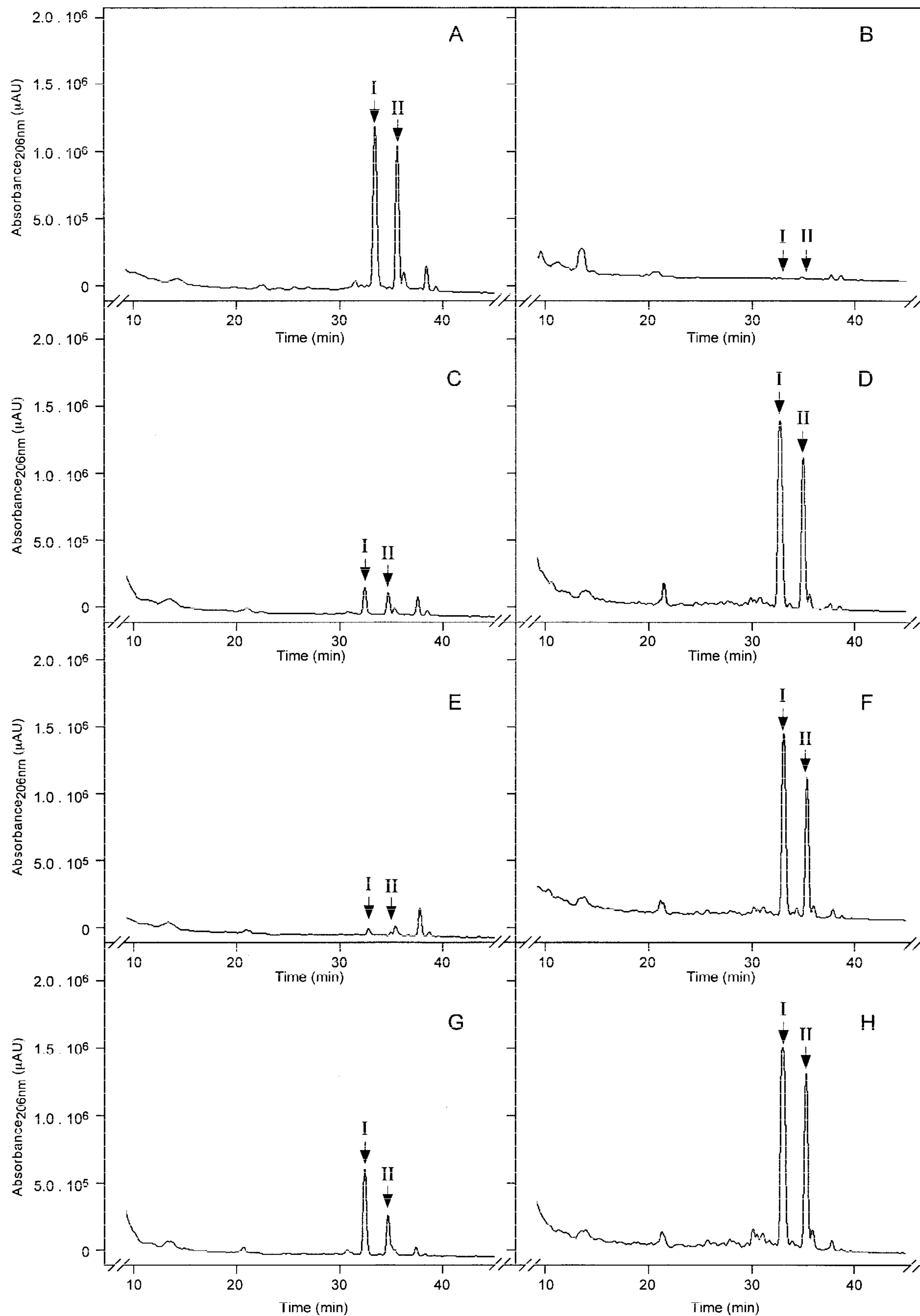

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tant PCL1629 (*grpE* mutant) showed a 50% reduction in putisolvin production (Fig. 3G). Introduction of pMP5530 containing the 3′ *dnaK* end and *dnaJ* into PCL1628 (*dnaJ* mutant) strain restored biosurfactant activity and putisolvin production (Fig. 3F). Finally, introduction of pMP5534 carrying *grpE* into PCL1629 (*grpE* mutant) complemented for the reduced putisolvin production (Fig. 3H). These results show that *dnaK*, *dnaJ*, and *grpE* take part in the regulation of putisolvin I and II biosynthesis at 28°C. Complementation analysis of *dnaK* and *dnaJ* mutations supports the result of the 5′ RACE indicating that *dnaK* and *dnaJ* are independently transcribed at normal growth temperature (28°C).

Effect of temperature on production of putisolvins I and II. Low incubation temperature had hardly any effect on the growth of the three mutants (data not shown). The effect of temperature (32°C, 28°C, 21°C, 16°C, and 11°C) on the production of putisolvins was analyzed in stationary-phase liquid cultures of PCL1445, PCL1627 (*dnaK* mutant), PCL1628 (*dnaJ* mutant), and PCL1629 (*grpE* mutant) (Fig. 4). HPLC analysis showed that the level of putisolvin production decreases with increasing growth temperature. Moreover, a mutation in *dnaK* (PCL1627) decreased putisolvin production at 21°C and 16°C and practically abolished putisolvin production at higher and lower temperatures (Fig. 4A). Production of putisolvins by mutant PCL1627 (*dnaK* mutant) at low and high temperatures could be restored by introduction of pMP5519 carrying the functional *dnaK* gene. Analysis of the *dnaJ* mutant for the production of putisolvins at the same range of temperatures showed that DnaJ has a similar effect as DnaK (Fig. 4B). The mutation in *dnaJ* was complemented for the production of putisolvins using pMP5530 carrying 3′ 520 bp of the *dnaK* and *dnaJ* genes. Although a mutation in *grpE* had a significant effect on putisolvin production at temperatures higher than 21°C, the level of putisolvins was comparable to that of PCL1445 at lower temperatures (Fig. 4C). Thus, these results show that (i) putisolvin production is up-regulated at low temperatures and (ii) DnaK and DnaJ are required for the production of putisolvins at low temperatures.

Effect of a *dnaK*, *dnaJ*, or *grpE* mutation on *psoA* expression. To analyze a possible effect of DnaK on the expression of *psoA*, a *psoA*::*gfp* transcriptional fusion was constructed. Transcriptional activity of the putisolvin promoter was analyzed in strains PCL1445, PCL1627 (*dnaK* mutant), PCL1628 (*dnaJ* mutant), and PCL1629 (*grpE* mutant) in liquid culture at different temperatures (28°C, 21°C, 16°C, and 11°C) (Fig. 5). Our data showed a strong correlation between *psoA*::*gfp* transcriptional activity and the production of putisolvins in culture medium. The absence of transcriptional activity in PCL1627 (*dnaK* mutant) and PCL1628 (*dnaJ* mutant) at 28°C and 11°C and its reduction at 16°C indicate that DnaK and DnaJ regulate (directly or indirectly) putisolvin synthesis at the transcriptional level. Furthermore, a mutation in *grpE* had hardly any effect on the expression of *psoA*::*gfp* at 11°C (Fig. 5). This result, which supports HPLC analysis (Fig. 4), shows that *grpE* does not take part in the regulation of putisolvins at low temperature.

Effect of a *gacA* or *gacS* mutation on *dnaK* expression. Transposon mutants PCL1622 and PCL1623 were isolated together with PCL1627 after an initial screening for surface tension-decreasing ability using the drop-collapsing assay. Strains PCL1622 and PCL1623 did not produce detectable amounts of putisolvin (data not shown). Sequence analysis of the DNA region flanking Tn*5luxAB* showed that the transposons of PCL1622 and PCL1623 were inserted in a *gacA* homolog encoding a global response regulator and a *gacS* homolog encoding a sensor kinase, respectively (13). Mutations in *gacA* and *gacS* genes could be complemented for the production of putisolvins using plasmids pMP5512 (*gacA*) and pMP6562 (*gacS*), respectively (data not shown). Since putisolvin production is induced during the late exponential phase and since DnaK appears to regulate transcriptional activity of putisolvin synthetase gene promoter (Fig. 5), we tested whether *dnaK* is regulated by the GacA/GacS two-component system. To test this hypothesis, a *dnaK*::*lacZ* transcriptional fusion was constructed and its expression was analyzed in both PCL1622 (*gacA* mutant) and PCL1623 (*gacS* mutant) (Table 3). Strains PCL1445, PCL1622 (*gacA* mutant), and PCL1623 (*gacS* mutant) harboring *dnaK*::*lacZ* were cultured at 28°C in liquid KB medium to the stationary phase. The expression of β-galactosidase activity was reduced in the *gac* mutants PCL1622 (*gacA* mutant) and PCL1623 (*gacS* mutant) and was restored to wild-type levels in the complemented derivatives (Table 3). This result shows that the GacA/GacS two-component regulatory system has a positive effect on DnaK in PCL1445.

DISCUSSION

The aim of this work was to identify and characterize genes involved in regulating the production of the cyclic lipopeptides putisolvin I and II by *Pseudomonas putida* PCL1445. Putisolvins are biosurfactants that are required for swarming motility and are able to inhibit biofilm formation and degrade existing biofilms (26). We have screened a Tn*5* library for mutants defective in lipopeptide production. One of the mutants, PCL1627, carried the transposon in a *dnaK* homolog. DnaK is a member of the Hsp70 heat shock protein family. In *E. coli*, the *rpoH* gene product, σ^{32}, positively regulates heat shock genes by directing the core RNA polymerase to the *dnaK* promoter (6, 17). A sequence similar to the *E. coli* consensus σ^{32}-dependent promoters was identified in the *dnaK* promoter region of *P. putida* PCL1445 indicating that *dnaK* is also regulated by σ^{32} in PCL1445.

FIG. 3. C_8 reverse-phase HPLC analysis of putisolvin production by *P. putida* PCL1445 and its mutants, PCL1436 (*psoA* mutant), PCL1627 (*dnaK* mutant), PCL1628 (*dnaJ* mutant), and PCL1629 (*grpE* mutant). The panels depict: PCL1445 (A), PCL1436 (B), PCL1627 (C), PCL1628 (E), PCL1629 (G), PCL1627 harboring pMP5519 containing *dnaK* of PCL1445 (D), PCL1628 harboring pMP5530 containing the last part of *dnaK* followed by *dnaJ* of PCL1445 (F), and PCL1629 harboring pMP5534 containing *grpE* of PCL1445 (H). Cells were grown to the stationary phase in 5 ml KB medium at 28°C under vigorous aeration. Compounds from the ethyl acetate-extracted culture supernatant were separated and analyzed at a wavelength of 206 nm. HPLC fractions of 1 ml were collected and tested for surfactant activity using the drop-collapsing assay.

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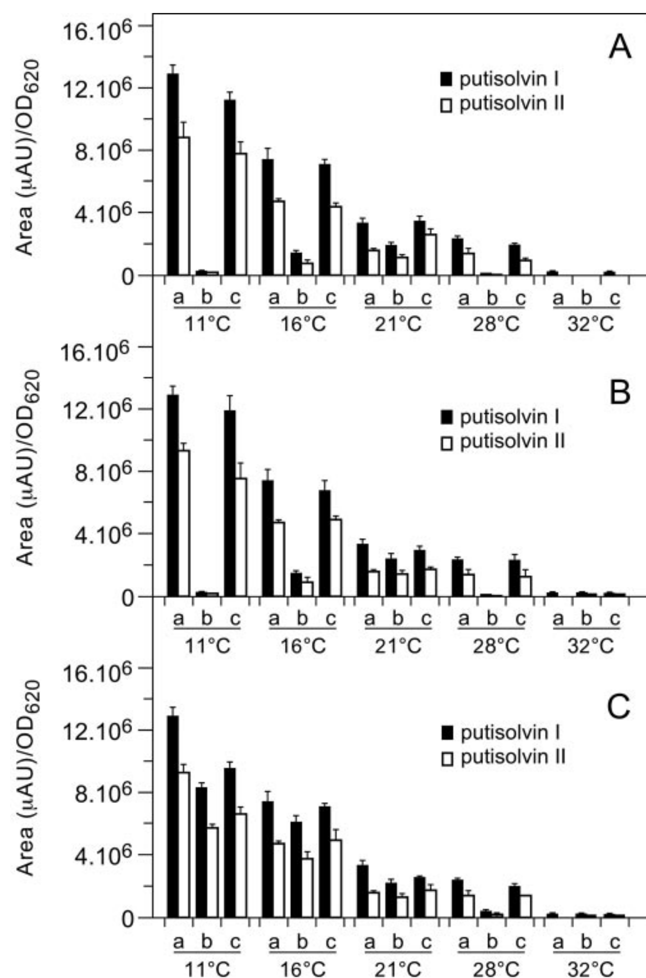

FIG. 4. Effect of growth temperature on the production of putisolvins I and II by *Pseudomonas putida* PCL1445 and its mutant derivatives. PCL1445, PCL1627 (*dnaK* mutant), PCL1627(*dnaK* mutant) with pMP5530 (*dnaK*), PCL1628 (*dnaJ* mutant), PCL1628 (*dnaJ* mutant) with pMP5530 (*dnaJ*), PCL1629, and PCL1629 (*grpE* mutant) with pMP5532 (*grpE*) were cultured at 11, 16, 21, 28, and 32°C to stationary phase. The production of putisolvins was quantified by C_8 reverse-phase HPLC analysis. The values depicted represent the area of the peaks over cell density (μAU/OD_{620}). (A) PCL1445 (column a), PCL1627 (*dnaK* mutant) (column b), and PCL1627 (*dnaK* mutant) complemented using pMP5519 (*dnaK*) (column c). (B) PCL1445 (column a), PCL1628 (*dnaJ* mutant) (column b), and PCL1628 (*dnaJ* mutant) complemented using pMP5530 (*dnaJ*) (column c). (C) PCL1445 (column a), PCL1629 (*grpE* mutant) (column b), and PCL1629 (*grpE* mutant) complemented using pMP5532 (*grpE*) (column c).

Sequencing of the region downstream of *dnaK* revealed *dnaJ*, an organization that is conserved in *Staphylococcus aureus* (36), *Xanthomonas campestris* (47), *Neisseria gonorrhoeae* (28), or *Clostridium acetobutylicum* (32). In many organisms, *dnaK* and *dnaJ* are organized as an operon and the gene products are part of an equimolar protein complex which is formed with the cochaperone GrpE. Sequencing of the region upstream of *dnaK* localized a *grpE* homolog (Fig. 1A).

Deletion of *grpE* in *E. coli* (2) and *dnaK* or *grpE* in *P. syringae* pv. *glycinea* (20) results in a loss of viability due to a severely compromised physiological function. In contrast, our results

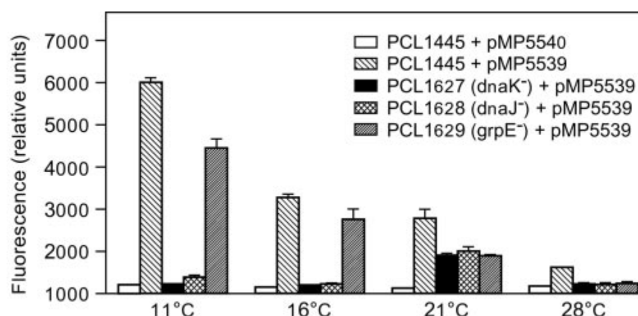

FIG. 5. Expression of *psoA* in *Pseudomonas putida* PCL1445, PCL1627 (*dnaK* mutant), PCL1628 (*dnaJ* mutant), and PCL1629 (*grpE* mutant). Expression was determined by measuring fluorescence from cells containing the putisolvin synthetase promoter fused to *egfp* (pMP5539). pMP5540 in which the *psoA* promoter was cloned in the reverse orientation was used as a control vector. Strains were grown at 11, 16, 21, and 28°C in KB medium. Mean values of duplicate cultures are given.

show that mutation in any of these three genes does not affect growth of PCL1445 at 28°C (Fig. 2A). Southern blot analysis of the PCL1445 wild type and its *dnaK* mutant, using a *dnaK* probe, did not indicate the presence of a second *dnaK* homolog in the genome (data not shown). This suggests that under the growth conditions used, at 28°C, *dnaK* is not important or the loss of *dnaK* can be compensated for by the production of other heat shock proteins such as GroEL-GroES (31). Growth of *dnaK*, *dnaJ*, and *grpE* mutants is reduced at 35°C (Fig. 2B), indicating that functioning of *dnaK*, *dnaJ*, and *grpE* becomes important for PCL1445 at high temperature and is (at least) not completely compensated for by the production of other chaperones. Furthermore, the growth deficiency of a *dnaK* mutant can be restored only by introduction of both *dnaK* and *dnaJ* and not with *dnaK* alone, indicating that *dnaK* and *dnaJ* are coregulated at high temperature (Fig. 2C). The results on the growth of *dnaK*, *dnaJ*, and *grpE* mutants demonstrate that DnaK, DnaJ, and GrpE are not essential for growth of PCL1445.

dnaK, *dnaJ*, or *grpE* mutants were analyzed for putisolvin

TABLE 3. Expression of the *dnaK* promoter fused to *lacZ* in *P. putida* PCL1445, PCL1622 (*gacA* mutant) and PCL1623 (*gacS* mutant)

Strain[a]	Relative β-galactosidase activity (Miller units)
Wild type	
PCL1445 (empty vector)	9.68 ± 0.93
PCL1445-pMP5536 (P_{dnaK} *lacZ* mutant)[b]	24.71 ± 2.83
PCL1445-pMP5535 (P_{dnaK} *lacZ*$^+$)[c]	75.43 ± 1.36
PCL1622 (*gacA* mutant)	
PCL1622-pMP5535 (P_{dnaK} *lacZ*$^+$)	32.18 ± 1.79
PCL1622-*gacA*$^+$-pMP5535 (P_{dnaK} *lacZ*$^+$)	70.80 ± 3.11
PCL1623 (*gacS* mutant)	
PCL1623-pMP5535 (P_{dnaK} *lacZ*$^+$)	30.94 ± 2.44
PCL1623-*gacS*$^+$-pMP5535 (P_{dnaK} *lacZ*$^+$)	69.55 ± 3.46

[a] pML103 (empty reporter vector) and pMP5536 containing the *dnaK* promoter cloned in the reverse orientation were used as control vectors. Cells were grown in KB medium to stationary phase under the normal growth condition (28°C).
[b] *lacZ* mutant, *lacZ* reporter gene in transcriptionally inactive orientation.
[c] *lacZ*$^+$, *lacZ* reporter gene in transcriptionally active orientation.

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production to assess the significance of the three heat shock genes for putisolvin production. Putisolvin production was almost eliminated in *dnaK* and *dnaJ* mutant strains, while production was decreased in a *grpE* mutant (Fig. 3). This implicates that DnaK, DnaJ, and GrpE act as a complex in the regulation of putisolvin production. In addition, expression analysis of the putisolvin synthetase gene *psoA*, tested by a *psoA*::*gfp* transcriptional fusion in the wild type and in *dnaK*, *dnaJ*, and *grpE* mutants, showed that transcriptional activity (Fig. 4) correlated with putisolvin production, as determined by HPLC (Fig. 5). Finally, we also showed that the GacA/GacS two-component regulatory system is important for putisolvin production and interestingly that expression of *dnaK* was also regulated by the *gac* system (Table 3). This provides genetic evidence that DnaK could play a role in temperature sensing via the GacA/GacS two-component regulatory system in PCL1445.

We do not know yet how the DnaK, DnaJ, and GrpE complex is involved in transcription of *psoA* and if GacA/GacS directly or indirectly regulates *dnaK*. However, the heat shock response does not seem to take part in this regulation. Although complementation of phenotypic growth at high temperature (35°C) shows that *dnaK*-*dnaJ* may function as an operon (Fig. 2C), two results, (i) complementation of *dnaK* and *dnaJ* mutations for the production of putisolvins at 28°C (Fig. 3) (ii) and transcriptional analysis using 5′ RACE (data not shown), strongly suggest that *dnaK* and *dnaJ* are transcribed separately at lower temperatures. This is in accordance with two previous studies, which showed that in *Pseudomonas syringae* pv. *glycinea dnaK* and *dnaJ* are not organized as an operon (20) and in *Neisseria gonorrhoeae* a promoter is present in front of *dnaJ* (28).

A number of possible mechanisms involving the DnaK complex in the regulation of putisolvins can be predicted. DnaK, DnaJ, and GrpE may be required for the proper folding or activity of an unknown positive regulator of *psoA*. One particularly appealing possibility is that the GacA/GacS two-component system positively regulates *psoA*. In that case, DnaK, DnaJ, and or GrpE may regulate proper folding of some known small RNA (sRNA) mediators regulated by a *gac* system, such as RsmZ and RsmY, and which have been shown to control biosynthesis of antibiotics of *P. fluorescens* (10). Another possible target for the DnaK complex is σ^S, which is encoded by *rpoS* and which plays a crucial role in gene regulation during entry into stationary phase and was suggested to be regulated by DnaK in previous study (14, 31, 39). Alternatively, DnaK-DnaJ-GrpE may be required for the proper assembly of the large lipopeptide synthase complex. Finally, the effect on lipopeptide synthesis may be an indirect consequence of other cellular changes in *dnaK*, *dnaJ*, and *grpE* mutant strains.

In this report, we have demonstrated that the synthesis of the surfactants putisolvins at low temperatures requires the DnaK chaperone complex in *P. putida* (Fig. 4) and that consequently the putisolvin synthetase gene *psoA* is up-regulated (Fig. 5).

It is still unknown how the DnaK chaperone complex controls transcription of the *psoA* gene at low temperatures. However, GrpE does not take part in the regulation, indicating that the functioning of the DnaK complex differs at 11°C and at 28°C. Performance of *dnaK*::*lacZ* expression analysis in PCL1445 indicated in accordance with a study in *E. coli* (49) that *dnaK* expression decreases gradually at lower temperatures, with respective values of 67.38 ± 1.4 Miller units at 28°C, 28.89 ± 0.48 Miller units at 21°C, 7.421 ± 0.50 Miller units at 16°C, and 2.88 ± 0.08 Miller units at 11°C. Although the expression of *dnaK* decreases at lower temperatures, the presence of a functional DnaK is required since mutation results in loss of putisolvin production. This hypothesis is supported by the results in *E. coli* indicating that DnaK is not only involved in the regulation of heat shock response but could also take part in the regulation of environmental stress response, such as temperature and stationary phase (14, 38, 39).

Temperature and heat shock proteins have been reported to play an important role in the modulation of virulence in phytopathogenic bacteria—for example, for tumor induction by *Agrobacterium tumefaciens* (5, 33) and for phytotoxin production by *P. syringae* pv. *glycinea* (20). Low temperature restricts growth of *P. putida* PCL1445 and positively regulates putisolvin production during late exponential phase via the DnaK stress response system. Low temperature could constitute a challenge for the dissemination of *Pseudomonas putida* due to, for instance, a reduction of metabolic functions or a reduction of nutrient availability such as root exudates or intermediates of the polyaromatic hydrocarbon degradation process (24). Production of biosurfactants could confer an ecological advantage for bacteria at low temperature. Their specific activity could be involved in important functions such as (i) creating a protective microenvironment by reducing surface tension, (ii) taking part in the solubilization of nutrient (hydrophobic carbon sources), (iii) forming an emulsion as a result of reduction of the interfacial tension between water and oil at low temperatures, which in turn could increase the available surface for growth; or (iv) taking part in swarming motility in order to colonize a more favorable environment.

ACKNOWLEDGMENTS

This research was supported by the Council for Chemical Sciences of The Netherlands Foundation for Scientific Research (NWO/CW).

We thank I. Kuiper for valuable discussions and P. Hock for assistance in processing the graphs.

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