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Regulation of Cyclic Lipopeptide Biosynthesis in *Pseudomonas fluorescens* by the ClpP Protease▽†

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Cyclic lipopeptides produced by *Pseudomonas* species exhibit potent surfactant and broad-spectrum antibiotic properties. Their biosynthesis is governed by large multimodular nonribosomal peptide synthetases, but little is known about the genetic regulatory network. This study provides, for the first time, evidence that the serine protease ClpP regulates the biosynthesis of massetolides, cyclic lipopeptides involved in swarming motility, biofilm formation, and antimicrobial activity of *Pseudomonas fluorescens* SS101. The results show that ClpP affects the expression of *luxR*(mA), the transcriptional regulator of the massetolide biosynthesis genes *massABC*, thereby regulating biofilm formation and swarming motility of *P. fluorescens* SS101. Transcription of *luxR*(mA) was significantly repressed in the *clpP* mutant, and introduction of *luxR*(mA) restored, in part, massetolide biosynthesis and swarming motility of the *clpP* mutant. Site-directed mutagenesis and expression analyses indicated that the chaperone subunit ClpX and the Lon protease are not involved in regulation of massetolide biosynthesis and are transcribed independently of *clpP*. Addition of Casamino Acids enhanced the transcription of *luxR*(mA) and *massABC* in the *clpP* mutant, leading to a partial rescue of massetolide production and swarming motility. The results further suggested that, at the transcriptional level, ClpP-mediated regulation of massetolide biosynthesis operates independently of regulation by the GacA/GacS two-component system. The role of amino acid metabolism and the putative mechanisms underlying ClpP-mediated regulation of cyclic lipopeptide biosynthesis, swarming motility, and growth in *P. fluorescens* are discussed.

Cyclic lipopeptides are versatile metabolites produced by a variety of bacterial genera, including *Pseudomonas* and *Bacillus* (54, 55, 60). They are composed of a short cyclic oligopeptide linked to a fatty acid tail and exhibit potent surfactant properties (60). Cyclic lipopeptides have received considerable attention for their antibiotic activities against a range of human- and plant-pathogenic organisms, including enveloped viruses, mycoplasmas, trypanosomes, bacteria, fungi, and oomycetes (60). For plant-associated *Pseudomonas* species, cyclic lipopeptides play important roles in swarming motility, biofilm formation, and virulence (2, 4, 14, 15, 18, 34, 45, 60, 61). Cyclic lipopeptide biosynthesis is governed by large, multimodular nonribosomal peptide synthetases via a thiotemplate process (23, 60). Compared to the understanding of cyclic lipopeptide biosynthesis in *Pseudomonas* and other bacterial genera, however, relatively little is known about the genetic network involved in the perception of external factors and the signal transduction pathways that drive transcription of the cyclic lipopeptide biosynthesis genes.

For pathogenic and saprophytic *Pseudomonas* species, only a few regulatory genes and mechanisms have been identified. The GacA/GacS two-component system functions as a master switch, as a mutation in either one of the two genes results in loss of cyclic lipopeptide production (14, 15, 20, 41, 42). For pathogenic *Pseudomonas syringae* pv. syringae, regulatory genes identified downstream of the Gac system include *salA* and *syrF*, two LuxR-type transcriptional regulators involved in syringomycin and syringopeptin biosynthesis (41, 47, 48, 66). For saprophytic *Pseudomonas putida* strain PCL1445, DnaK and DnaJ were also shown to regulate putisolvin biosynthesis (20). Although the exact roles of these heat shock proteins are not yet resolved, the authors speculated that they might be required for proper folding or activity of other regulators of the putisolvin biosynthesis gene *psoA* or that DnaK is required for proper assembly of the peptide synthetase complex (20). In addition, cell density plays a role in cyclic lipopeptide biosynthesis in some *Pseudomonas* strains. For plant-pathogenic *Pseudomonas fluorescens* strain 5064, Cui et al. (12) provided evidence that *N*-acyl homoserine lactone (*N*-AHL)-mediated quorum sensing is required for viscosin biosynthesis. Also, for *P. putida* strain PCL1445, it was shown that putisolvin production was regulated by the quorum-sensing system composed of *ppuI*, *rsaL*, and *ppuR* (22). In many other pathogenic and saprophytic *Pseudomonas* species and strains, however, cyclic lipopeptide production is not regulated via *N*-AHL-mediated quorum sensing (2, 14, 15, 40, 59). In this context, Nybroe and Sørensen (54) emphasized that although cyclic lipopeptide production is affected by the growth phase and nutritional conditions, the specific impacts of these factors and the underlying molecular mechanisms in relation to cyclic lipopeptide biosynthesis are still unknown and may differ considerably among species and strains.

This study focuses on the regulation of cyclic lipopeptide biosynthesis in the plant growth-promoting strain *P. fluorescens* SS101. Strain SS101 produces massetolide A, which consists of

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TABLE 1. Primers used in this study

Fragment	Orientation	Primer sequence[a]
Site-directed mutagenesis		
lon	Forward	5′-GAGCAGATGAAGGCCATTCAG-3′
	Reverse	5′-GCCACATCCGGCAAGGGCTC-3′
tig	Forward	5′-TCTGTGCAACGAGGAATATCC-3′
	Reverse	5′-CTTGTTCTTCCAGCACAACCG-3′
FRT	Forward	5′-CGAATTAGCTTCAAAAGCGCTCTGA-3′
	Reverse	5′-CGAATTGGGGATCTTGAAGTTCCT-3′
clpP-Up	Forward	5′-TCAAGCAAGCGGATCCTGACTACCAGAACCTGGAC-3′
	Reverse	5′-tcagagcgcttttgaagctaattcgGGAATTACGGAACATGCTCTG-3′
clpP-Dn	Forward	5′-aggaacttcaagatccccaattcgGTGATCTTTCTGGTTGGCC-3′
	Reverse	5′-TCAAGCAAGCGGATCCGATGTCATTGCACAGGTCGAC-3′
clpX-Up	Forward	5′-TCAAGCAAGCGGATCCGTGCGCAGTTGCTGTTCCTT-3′
	Reverse	5′-tcagagcgcttttgaagctaattcgCCTCACGGATGATGTCATTGC-3′
clpX-Dn	Forward	5′-aggaacttcaagatccccaattcgTCGATCACTCGGGACGTTTC-3′
	Reverse	5′-TCAAGCAAGCGGATCCTTGGACTTGCCTTCGATAACG-3′
pEX18Tc	Forward	5′-CCTCTTCGCTATTACGCCAG-3′
	Reverse	5′-GTTGTGTGGAATTGTGAGCG-3′
Complementation		
clpP	Forward	5′-ttttttgagctcCCGCACCGAAGTTCGCAAG-3′
	Reverse	5′-aaaaaaaggatccCCTGCTGCACGCCTTCAC-3′
luxR(mA)	Forward	5′-TGCTCCAGGGCGCTGTAGAG-3′
	Reverse	5′-CATGCCGAGGGTGCACAG-3′

[a] The 5′ end of the Up reverse and Dn forward primers for site-directed mutagenesis contain a 25-bp sequence (lowercase letters) complementary to the FRT-F and FRT-R primers for overlap extension in the second-round PCR. The 5′ end of the Up forward and Dn reverse primers contain a restriction site (underlined) for BamHI, which is required for cloning into pEX18Tc.

a 9-amino-acid cyclic peptide moiety linked to 3-hydroxydecanoic acid (14). Massetolide A was first identified in a marine *Pseudomonas* species isolated from Masset Inlet, BC, Canada (31), and showed surfactant and broad-spectrum antimicrobial activities. Massetolide A inhibits the growth of *Mycobacterium tuberculosis* and *Mycobacterium avium-M. intracellulare* (31) and has destructive effects on zoospores of multiple oomycete plant pathogens (15, 17). Furthermore, massetolide A induces a systemic resistance response in tomato plants and contributes to root colonization by strain SS101 (64). Massetolide A is produced in the early exponential growth phase and is essential for swarming motility and biofilm formation of strain SS101 (14). Its biosynthesis is governed by three nonribosomal peptide synthetases, designated MassA, MassB, and MassC, and is not regulated via *N*-AHL-based quorum sensing (14). Due to flexibility in amino acid selection by the nonribosomal peptide synthetases, strain SS101 produces several massetolide A derivatives that differ in the amino acid composition of the peptide moiety (14). To begin to identify the genetic networks and mechanisms underlying the regulation of cyclic lipopeptide biosynthesis, *P. fluorescens* strain SS101 was subjected to random mutagenesis. Among the massetolide-deficient mutants obtained, one mutant harbored a Tn*5* insertion in the caseinolytic protease gene *clpP*. The *clpP* gene of strain SS101 was cloned and sequenced, and its genomic context was assessed by primer walking. Site-directed mutagenesis, genetic complementation, and phenotypic and transcriptional analyses were performed to assess the functions of the ClpP protease in the regulation of massetolide biosynthesis and other bacterial traits, including swarming motility, growth, and biofilm formation. The effects of the *clpP* mutation on the expression of two LuxR-type transcriptional regulators, as well as the role of amino acids in ClpP-mediated regulation of massetolide biosynthesis, were investigated in detail.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *P. fluorescens* SS101 was grown on Pseudomonas agar F (Difco) plates or in liquid King's medium B (KB) at 25°C. The transposon mutants were obtained as described by De Souza et al. (17), and plasposon mutants were obtained with plasmid pTn*Mod*OKm (16). *Escherichia coli* strain DH5α was used as a host for the plasmids for site-directed mutagenesis and complementation. *E. coli* strains were grown on Luria-Bertani (LB) plates or in LB broth amended with the appropriate antibiotics.

Identification of the *clpP* cluster. *clpP* was identified by sequencing the regions flanking the transposon insertions as described by De Sousa et al. (17). The flanking regions of *clpP* were sequenced by primer walking, and open reading frames (ORFs) were identified with the Softberry FGENESB program. The ORFs were analyzed using Blastx in the NCBI database, PseudoDB (http://xbase.bham.ac.uk/pseudodb/), and Pseudomonas.com.

Site-directed mutagenesis. Site-directed mutagenesis of the *lon* and *tig* genes was performed with the pKnockout-G suicide vector (67) as described by De Bruijn et al. (14). The primers used for site-directed mutagenesis are listed in Table 1. Site-directed mutagenesis of the *clpP* and *clpX* genes was performed based on the method described by Choi and Schweizer (10). For each mutant construct, three fragments were amplified: a 5′ fragment, a Gm cassette flanked by FRT sites (FRT-Gm-FRT cassette), and a 3′ fragment. In the first-round PCR, the FRT-Gm-FRT cassette and the 5′ and 3′ fragments were amplified. In the second-round PCR, these three fragments were coupled by overlap extension PCR. The 5′ and 3′ fragments were chosen in such a way that, after homologous recombination in *Pseudomonas*, the FRT-Gm-FRT cassette was inserted around the 170-bp position of the *clpP* or *clpX* gene. For amplification of the FRT-Gm-FRT cassette, pPS854-GM, a derivative of pPS854 (37), was used as a template in the PCR with primers FRT-F and FRT-R. The first-round PCR was performed with KOD polymerase (Novagen) according to the manufacturer's protocol, but with the addition of 1 to 10% dimethyl sulfoxide for the *clpP* and *clpX* fragments. The program used for the PCR consisted of 2 min of denaturation at 95°C, followed by 5 cycles of 95°C, 55°C, and 68°C, each for 20 s. The PCR amplification was preceded by 25 cycles of 95°C, 60°C, and 68°C, each for 20 s. The last step of the PCR was 68°C for 7 min. All fragments were separated on a 1% (wt/vol) agarose gel and purified with a NucleoSpin kit (Macherey-Nagel).

The second-round PCR was performed by mixing equimolar amounts of the 5′, FRT-Gm-FRT, and 3′ fragments with milliQ, deoxynucleotide triphosphates, KOD buffer, and KOD polymerase to a total of 47 μl. The PCR was started by 2 min of denaturation at 95°C, followed by 3 cycles of 95°C, 55°C, and 68°C for 20, 30, and 60 s, respectively. In the third extension cycle, 1.5 μl each of the Up forward and Dn reverse primers (10 μM stock) was added. The PCR amplification was preceded by 25 cycles of 95°C, 58°C, and 68°C for 20, 20, and 120 s, respectively. The last step of the PCR was 68°C for 7 min. All fragments were separated on a 1% agarose gel, and bands of the right size were purified with a NucleoSpin kit. The fragments were digested with BamHI and cloned into pEX18Tc. *E. coli* DH5α was transformed with a pEX18Tc-*clpP* or pEX18Tc-*clpX* plasmid by heat shock transformation according to the method of Inoue et al. (39), and transformed colonies were selected on LB supplemented with 25 μg/ml gentamicin (Sigma). Integration of the inserts was verified by PCR analysis with pEX18Tc primers and by restriction analysis of the isolated plasmids. The plasmid inserts were verified by sequencing (BaseClear, Leiden, The Netherlands). The correct pEX18Tc-*clpP* and pEX18Tc-*clpX* constructs were subsequently electroporated into *P. fluorescens* strain SS101. Electrocompetent cells were obtained according to the method of Choi et al. (9), and electroporation occurred at 2.4 kV and 200 μF. After incubation in SOC medium (2% Bacto tryptone [Difco], 0.5% Bacto yeast extract [Difco], 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose [pH 7]) for 2 h at 25°C, the cells were plated on KB supplemented with gentamicin (25 μg/ml) and rifampin (50 μg/ml). The colonies obtained were grown in LB for 1 h at 25°C and plated on LB supplemented with gentamicin (25 μg/ml) and 5% sucrose to accomplish the double crossover. The plates were incubated at 25°C for at least 48 h, and colonies were restreaked on LB supplemented with gentamicin plus 5% sucrose and on LB supplemented with tetracycline (25 μg/ml). Colonies that grew on LB with gentamicin plus sucrose, but not on LB with tetracycline, were selected and subjected to colony PCR to confirm the presence of the gentamicin resistance cassette and the absence of the tetracycline resistance cassette. Positive colonies were confirmed by sequencing the PCR fragments obtained with the Up forward and Dn reverse primers. The *clpP* and *clpX* mutants obtained were tested for massetolide production in a drop collapse assay and by high-performance liquid chromatography (HPLC) analysis. HPLC analyses were performed as described previously (14) with the exception that in this study, samples of the crude surfactant extract (1 mg/ml) were analyzed isocratically (flow rate, 0.5 ml/min) using a solution of 45% acetonitrile and 15% milliQ, both containing 0.1% trifluoroacetic acid, and 40% methanol as eluents.

Construction of pME6031-based vectors for genetic complementation. A fragment of approximately 2 kb containing the *clpP* gene, including the promoter and terminator, was obtained by PCR with specific primers (Table 1) and the KOD polymerase. The pME6031-*luxR*(mA) construct was generated as follows: a 1,817-bp fragment was obtained by PCR with specific primers (Table 1) and Phusion DNA polymerase (Finnzymes). The PCR fragments were subcloned in pGEM-T Easy (Promega), and the plasmids obtained were digested with EcoRI. The *clpP* and *luxR*(mA) (see below) fragments were obtained from gels with the NucleoSpin kit and cloned into the shuttle vector pME6031 (36), which was digested, dephosphorylated (shrimp alkaline phosphatase; Promega), and purified with the NucleoSpin kit according to the manufacturer's instructions. *E. coli* DH5α was transformed with the plasmid obtained, pME6031-*clpP* or pME6031-*luxR*(mA), by heat shock transformation (39), and transformed colonies were selected on LB agar plates supplemented with tetracycline (25 μg/ml). Correct integration of the fragments was verified by PCR analysis and restriction analysis of isolated plasmids. The pME6031-*clpP* and pME6031-*luxR*(mA) constructs were subsequently electroporated into the *clpP* mutant and the wild-type strain SS101. Transformed cells were plated on KB supplemented with tetracycline (25 μg/ml), and the presence of pME6031-*clpP* or pME6031-*luxR*(mA) was verified by PCR analysis with primers specific for pME6031.

Surface tension measurements and transcriptional analysis. Cells were grown at 25°C (220 rpm) in a 24-well plate with 1.25 ml KB broth per well. At specific time points during growth, 100 μl culture was transferred to a 96-well plate, and cell density was measured at 600 nm with a microplate reader (Bio-Rad). Subsequently, 1 ml of cell culture was collected and spun down. The cells were frozen in liquid N_2 and stored at −80°C. For the RNA isolations and cDNA synthesis, four biological replicates were used for each time point. Massetolide production was measured qualitatively by the drop collapse assay and quantitatively by tensiometric analysis of the cell supernatant (K6 tensiometer; Krüss GmbH, Hamburg, Germany) at room temperature. To get sufficient volume for the tensiometric analysis, the supernatants of four biological replicates were collected and pooled for each time point. The surface tension of each sample was measured in triplicate.

For the transcriptional analyses, RNA was isolated from the frozen bacterial cells with Trizol reagent (Invitrogen), followed by DNase I (GE Healthcare) treatment. One μg RNA was used for cDNA synthesis with Superscript III (Invitrogen) according to the manufacturer's protocol. For the real-time quantitative PCR (Q-PCR), conducted with the 7300SDS system from Applied Biosystems, the SYBR Green Core kit (Eurogentec) with a final concentration of 3.5 mM $MgCl_2$ was used according to the manufacturer's protocol. The concentration of the primers was optimized (400 nM final concentration for the *mass* genes and *rpoD*; 500 nM for *clpP* and *clpX*), and a melting curve was performed to check the specificity of the primers. The primers used for the Q-PCR are listed in Table S1 in the supplemental material. To correct for small differences in the template concentration, *rpoD* was used as the housekeeping gene. The cycle in which the SYBR green fluorescence crossed a manually set cycle threshold (C_T) was used to determine transcript levels. For each gene, the threshold was fixed based on the exponential segment of the PCR curve. The C_T value of *clpP* was corrected for the housekeeping gene *rpoD* as follows: $\Delta C_T = C_T(clpP) - C_T(rpoD)$; the same formula was used for the other genes investigated. The relative quantification (RQ) values, were calculated by the following formula: $RQ = 2^{-[\Delta CT(\text{mutant}) - \Delta CT(\text{wild type})]}$. If there was no difference in transcript levels between the mutant and the wild type, then RQ was equal to 1 (2^0) and log RQ was equal to 0. Q-PCR analysis was performed in duplicate (technical replicates) on four independent RNA isolations (biological replicates). Statistically significant differences were determined for log-transformed RQ values by analysis of variance ($P < 0.05$), followed by Bonferroni post hoc multiple comparisons.

Swarming motility and biofilm formation. The swarming and swimming motility of the wild-type strain SS101, the massetolide-deficient mutants, and several transformants was assessed on soft (0.6% and 0.25% agar [wt/vol], respectively) standard succinate agar medium (SSM) consisting of 32.8 mM K_2HPO_4, 22 mM KH_2PO_4, 7.6 mM $(NH_4)_2SO_4$, 0.8 mM $MgSO_4$, and 34 mM succinic acid and adjusted to pH 7 with NaOH. After being autoclaved, the SSM was cooled down in a water bath to 55°C and kept at 55°C for 1 h. Twenty milliliters of SSM was pipetted into a 9-cm-diameter petri dish, and the plates were kept for 24 h at room temperature (~20°C) prior to inoculation with the bacterial suspensions. For all swarming assays, the same conditions (the agar temperature, the temperature at which the plates were stored, and the time between pouring the plates and inoculation) were kept constant to maximize reproducibility. Overnight cultures of the wild-type SS101, mutants, and transformants were washed three times with 0.9% NaCl, and 5 μl of the washed cell suspension (1×10^{10} cells/ml) was spot inoculated in the center of the soft SSM plate and incubated for 48 to 72 h at 25°C. For the assays with Casamino Acids (CAA), a filter-sterile stock solution of 20% CAA (Difco, Becton Dickinson and Co.) was prepared and diluted in SSM to obtain final concentrations of 0.1, 0.4, 1, and 4%. To test each amino acid present in the CAA separately, the amounts used were equivalent to those present in 1% CAA (see Table S2 in the supplemental material). Also, the effects of citric acid (citrate, 0.4%), $CaCl_2$ (14.7 μM), and $FeCl_3$ (0.24 μM) on the swarming motility of strain SS101 were tested. Biofilm formation was assessed according to the method described by De Bruijn et al. (14) and O'Toole et al. (57) using flat-bottom 96-well plates made of transparent polystyrene (Greiner) with 200 μl KB broth per well. Statistically significant differences were determined by Student's *t* test ($P < 0.05$).

Nucleotide sequence accession number. The sequences of *clpP* and its flanking genes have been deposited in GenBank under accession number FJ403110.

RESULTS

Role of *clpP* in regulation of massetolide biosynthesis. Screening of an initial 520 random transposon mutants of *P. fluorescens* SS101 for loss of massetolide production by a drop collapse assay (Fig. 1A) resulted in the selection of six putative mutants. All six mutants contained a single Tn*5* transposon insertion, as determined by Southern blot analysis of their genomic DNAs with the *km* gene as a probe (data not shown). The regions flanking the Tn*5* transposon insertion were cloned and sequenced for all six massetolide-deficient mutants. In five mutants, the Tn*5* insertion was located in the *massA*, *massB*, or *massC* gene (14). In the sixth mutant, designated mutant 13.3, the transposon was inserted in the caseinolytic protease gene, *clpP*. The complete *clpP* gene comprised 636 bp, and Blastx analysis showed 80 to 98% identity to *clpP* in other *Pseudomo-*

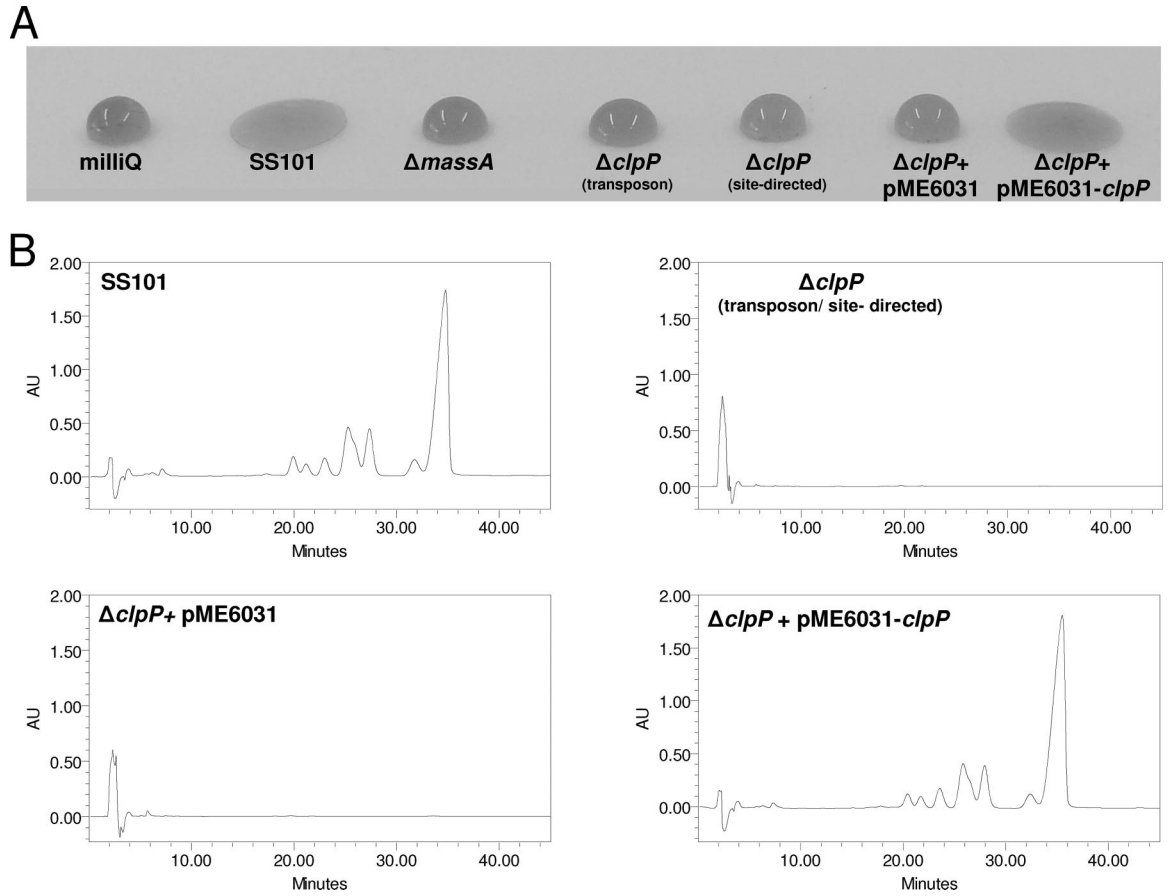


FIG. 1. Phenotypic and biochemical analyses of massetolide biosynthesis in *P. fluorescens* strain SS101 and several mutants obtained by random or site-directed mutagenesis. (A) Drop collapse assay with cultures of the wild-type strain SS101 and different mutants. Bacterial cells grown for 2 days at 25°C were resuspended in sterile water (1×10^{10} cells/ml), and 10-μl droplets were spotted on parafilm; crystal violet was added to the droplets to facilitate visual assessment. A flat droplet was indicative of massetolide production. (B) HPLC chromatograms of cell-free culture extracts of wild-type SS101, *clpP* mutants obtained by transposon or site-directed mutagenesis, *clpP*+pME6031 (empty-vector control), and *clpP*+pME6031-*clpP*. The wild-type strain SS101 produces massetolide A (retention time of approximately 35.5 min) and various other derivatives of massetolide A (peaks with retention times ranging from 21 to 33 min) differing in the amino acid composition of the peptide moiety (14).

nas genomes and 72% identity to *clpP* in *E. coli*. To confirm the role of *clpP* in the regulation of massetolide biosynthesis, site-directed mutagenesis of *clpP* was performed. Consistent with the phenotype of transposon mutant 13.3, the site-directed *clpP* mutant also lacked the ability to collapse a droplet of water (Fig. 1A). HPLC analysis confirmed that the *clpP* mutants obtained by random or site-directed mutagenesis did not produce detectable levels of massetolide A or its derivatives (Fig. 1B). Complementation of the *clpP* transposon mutant with the stable vector pME6031-*clpP* restored massetolide production to the wild-type level, whereas the empty-vector control had no effect (Fig. 1B). Taken together, these results indicate that *clpP* is required for massetolide biosynthesis in *P. fluorescens* SS101.

Genomic context of *clpP* in *P. fluorescens* SS101. By primer walking up- and downstream of the transposon insertion, a total 8,670-bp sequence was obtained from the regions flanking the *clpP* gene in strain SS101. Several ORFs were identified (Fig. 2), including the chaperone and protein-folding trigger factor (*tig*); the ATPase chaperone, *clpX*; the *lon* protease; the DNA binding and -bending *hupB*; and a partial sequence of *ppiD* isomerase, a gene involved in protein folding (3, 19, 24, 35). The organization of these genes in strain SS101 is identical to that found in various other fully sequenced *Pseudomonas* species and strains (Fig. 2). ClpX is known to act as a chaperone in the proteolytic complex with *clpP* (28) and is responsible for the recognition, unfolding, and translocation of substrates into the ClpP degradation chamber (51). Furthermore, Tig and Lon were shown to be substrates for Clp-dependent proteolysis (25, 32). To determine if the genes flanking *clpP* also play roles in the regulation of massetolide biosynthesis, site-directed mutagenesis was performed for *tig*, *clpX*, and *lon.* Drop collapse assays and HPLC analyses showed that disruptions of these three genes did not affect massetolide production (data not shown), suggesting that *clpP* acts independently of *clpX* in regulating massetolide biosynthesis.

Phenotypic characterization of the *clpP* mutant of *P. fluorescens*. Consistent with observations made previously for *E. coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* (13, 63, 65), a mutation in *clpP* adversely affected the growth of *P. fluorescens* SS101 (Fig. 3A). This reduced growth of the *clpP* mutant was not due to a lack of massetolide production, be-

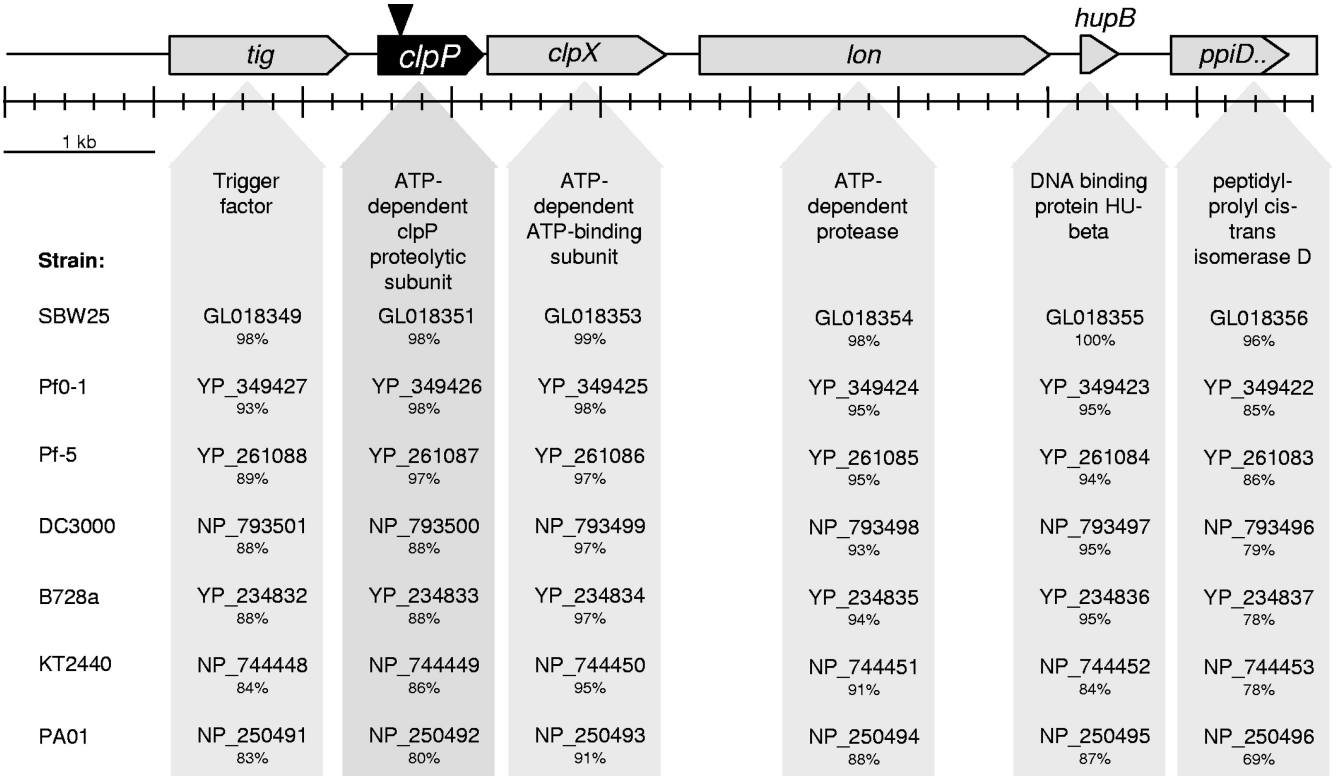


FIG. 2. Genomic organization of *clpP* and flanking genes in *P. fluorescens* SS101 and other fully sequenced *Pseudomonas* strains. For each of the genes of strain SS101, the percentages of amino acid identity with their corresponding genes in other *Pseudomonas* strains are given. The reference strains used were *P. fluorescens* strains SBW25 (15), Pf0-1 (15), Pf-5 (34), *P. syringae* pv. tomato strain DC3000 (4), *P. syringae* pv. syringae strain B728a (62, 70), *P. putida* strain KT2440, and *P. aeruginosa* strain PAO1. The codes for the genes of these reference strains correspond to those in the PseudoDB and NCBI databases.

cause the *massA* biosynthesis mutant showed growth comparable to that of the wild-type strain SS101 (Fig. 3A). Complementation of the *clpP* mutant with pME6031-*clpP* restored growth to the wild-type level, whereas the empty-vector control had no effect (Fig. 3A). Tensiometric analysis of cell-free culture supernatants of strain SS101 and mutants indicated that the wild-type strain SS101 started producing the massetolide surfactants at between 12 h and 16 h of incubation (Fig. 3B). A reduction in the surface tension of the growth medium was not observed for the *massA* mutant or for the *clpP* mutant, but surface tension was restored by complementation with pME6031-*clpP* (Fig. 3B).

Massetolide biosynthesis is essential for biofilm formation and swarming motility in strain SS101 (14). The capacity to form a biofilm was strongly reduced in the *clpP* mutant to a level similar to that observed for the *massA* biosynthesis mutant (Fig. 3C). Biofilm formation was fully restored to the wild-type level by complementation of the *clpP* mutant with pME6031-*clpP* (Fig. 3C). The *clpP* mutant also lost its ability to swarm on a soft agar surface (Fig. 3D). Swarming motility was restored in the *clpP* mutant by introduction of pME6031-*clpP*, although the extent of complementation, as well as the swarming pattern, was not identical to that of the wild-type strain (Fig. 3D). Introduction of pME6031-*clpP* into wild-type SS101 also resulted in reduced swarming (data not shown), suggesting that the altered swarming pattern of the complemented *clpP* mutant may have resulted from multiple copies of the *clpP* gene. In contrast to a complete loss of swarming motility, the *clpP* mutant was still able to swim on soft (0.25% [wt/vol]) agar plates (see Fig. S1 in the supplemental material). The observation that the swimming motility was similar to that of the *massA* biosynthesis mutant but reduced compared to the wild-type strain SS101 and the complemented *clpP* mutant (see Fig. S1 in the supplemental material) indicates that massetolide production also plays a (partial) role in swimming motility.

Transcriptional analysis of the *clpP* mutant of *P. fluorescens*. Q-PCR analyses were performed to study the effects of the *clpP* mutation on the expression of a range of genes, including the biosynthesis genes *massA*, *massB*, and *massC*. To prevent differences in growth rates between the wild-type strain SS101 and the *clpP* mutant from interfering with gene expression measurements, cells used for RNA isolation were collected when the wild type and mutant reached a specific density, i.e., early exponential (OD_{600}, ~0.2) and mid-exponential (OD_{600}, ~0.6) phases. Consistent with previous results (14), *massA*, *massB*, and *massC* were expressed in the wild-type strain SS101 during the early exponential and mid-exponential growth phases (data not shown). The transcript levels of all three *mass* genes were significantly decreased in the *clpP* mutant, especially in the mid-exponential growth phase (Fig. 4A). Mutations in *massA*, *massB*, or *massC* did not affect transcription of *clpP* (data not shown). Collectively, these results indicate that *clpP* regulates the transcription of the *mass* biosynthesis genes.

A mutation in *clpP* had only a minor effect on expression of

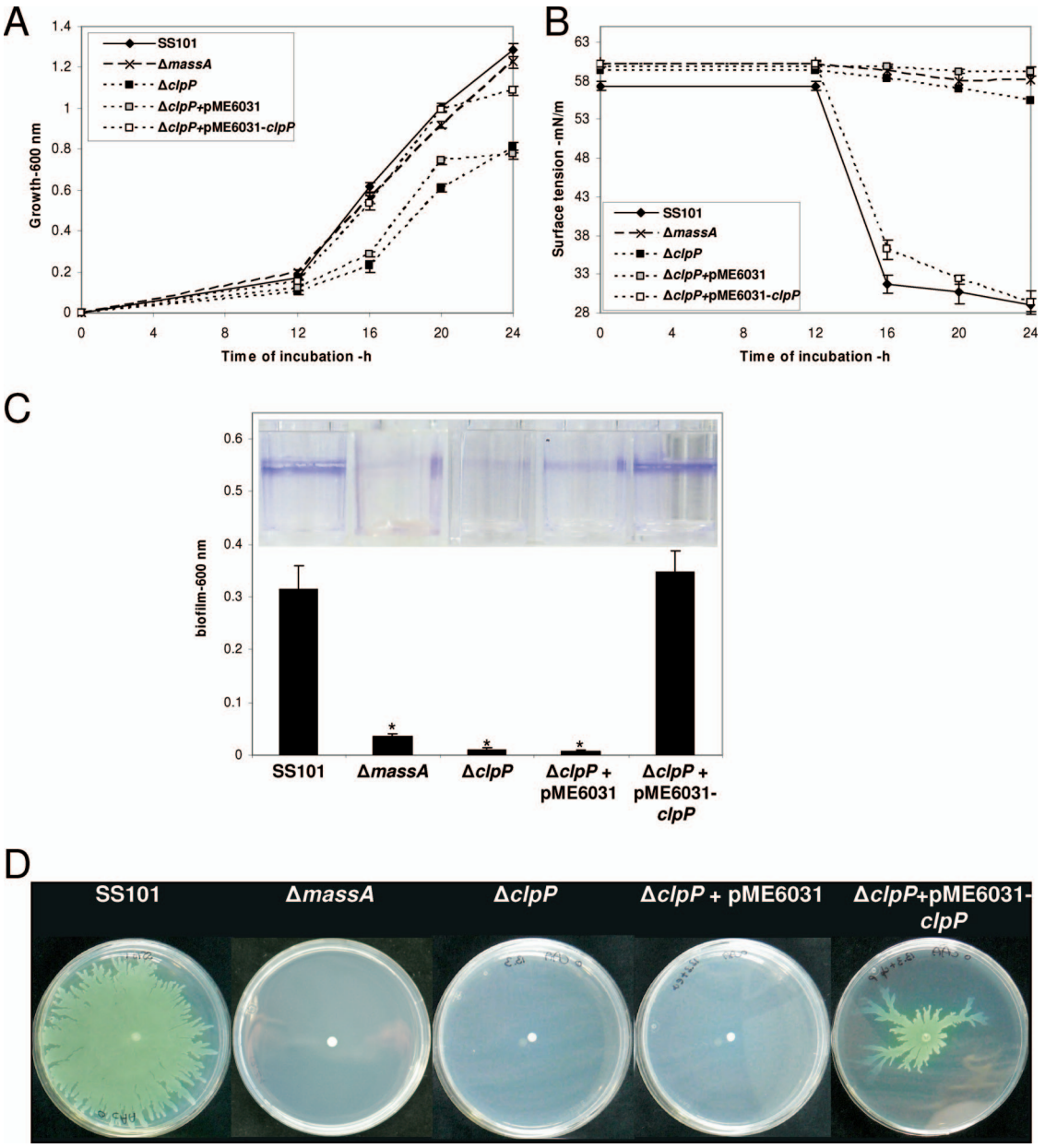


FIG. 3. Phenotypic characteristics of *P. fluorescens* strain SS101, the *massA* mutant, the *clpP* mutant, *clpP*+pME6031 (empty-vector control), and *clpP*+pME6031-*clpP*. (A) Growth at 25°C. At each time point, the cell density was measured spectrophotometrically (600 nm), and the mean values for four replicates are given; the error bars represent the standard errors of the mean. (B) Surface tension of cell-free culture supernatant of strain SS101 and the different mutants shown in panel A. (C) Biofilm formation by strain SS101 and the different mutants in a polystyrene 96-well plate containing 200 μl growth medium; cells firmly attached to the walls of the wells were stained with crystal violet, and their density was quantified spectrophotometrically at 600 nm. The mean values of four replicates are given. For each of the mutants, asterisks indicate statistically significant ($P < 0.05$) differences relative to wild-type SS101. (D) Swarming motility of wild-type strain SS101 and mutants on soft (0.6% [wt/vol]) agar plates. Five microliters of washed overnight cultures of wild-type SS101 and mutants was spot inoculated in the center of a soft agar plate and incubated for 48 to 72 h at 25°C.

clpX (Table 2), and also, a mutation in *clpX* only slightly reduced *clpP* transcript levels (data not shown), suggesting that under these conditions, *clpX* and *clpP* are transcribed independently. Moreover, the *clpP* mutation did not result in major or consistent changes in *tig*, *lon*, *hupB*, and *ppiD* transcript levels (Table 2). Since it was reported that *dnaK* regulates cyclic lipopeptide biosynthesis in *P. putida* (20) and that DnaK can influence proteolysis by ClpP (30), we also determined *dnaK*, *dnaJ*, and *gprE* transcript levels in the *clpP* mutant. No changes in transcript levels were observed (Table 2), indicating that *clpP* does not affect *dnaK* expression.

Effect of ClpP on expression of the transcriptional regulator *luxR*(mA). To further unravel the role of ClpP in transcriptional regulation of the *massABC* biosynthesis genes, we determined the transcript levels of two LuxR-type transcriptional-regulatory genes located upstream of *massA* (designated

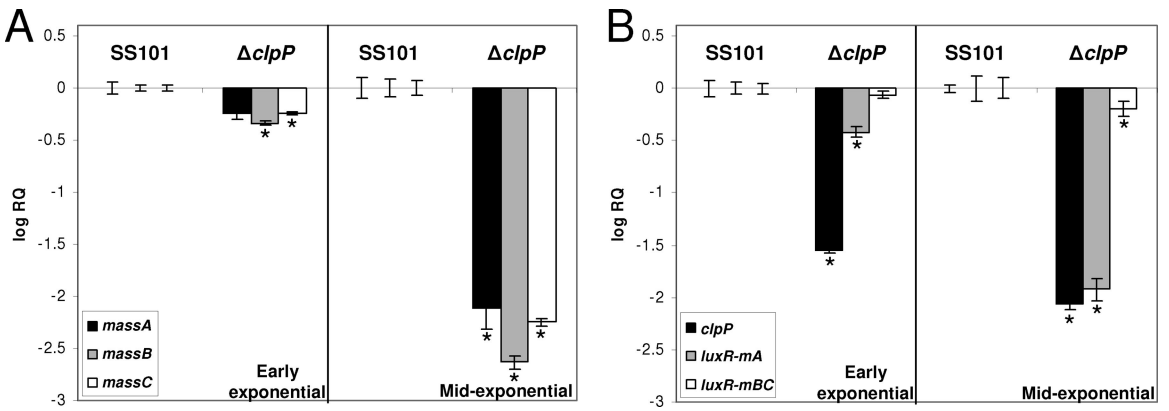


FIG. 4. (A) Transcript levels of *massA*, *massB*, and *massC* in cells of *P. fluorescens* SS101 and the *clpP* mutant obtained from the early and mid-exponential growth phases. (B) Transcript levels of *clpP*, *luxR*(mA), and *luxR*(mBC) in cells of *P. fluorescens* SS101 and the *clpP* mutant obtained from the early and mid-exponential growth phases. The transcript levels of each of the genes was corrected for the transcript level of the housekeeping gene *rpoD* [$\Delta C_T = C_T$ (gene *x*) $- C_T$(*rpoD*)] and is presented relative to the transcript levels in wild-type SS101 (log RQ), with RQ equal to $2^{-[\Delta CT(\text{mutant}) - \Delta CT(\text{wild type})]}$. For each time point, mean values of four biological replicates are given; the error bars represent the standard errors of the mean. The asterisks indicate statistically significant ($P < 0.05$) differences relative to wild-type SS101.

luxR(mA)) and downstream of *massBC* [*luxR*(mBC)] (14). The results showed that transcript levels of *luxR*(mA) were significantly decreased in the *clpP* mutant, whereas *luxR*(mBC) transcript levels were not or were only marginally reduced (Fig. 4B). Introduction of extra copies of *luxR*(mA) in the *clpP* mutant via pME6031-*luxR*(mA) restored massetolide production, based on the results of tensiometric analyses (Fig. 5B), drop collapse assays, and HPLC analysis (see Fig. S2 in the supplemental material). However, growth deficiency of the *clpP* mutant was not restored by pME6031-*luxR*(mA) (Fig. 5A), which in turn may explain why massetolide production was slightly delayed in the *clpP*+pME6031-*luxR*(mA) strain compared to the wild-type strain SS101 (Fig. 5B). Also, swarming motility was restored for the *clpP*+pME6031-*luxR*(mA) strain (Fig. 5C), but not to the same extent as in the wild type, most likely due to reduced growth. Gene expression analysis further showed that *massABC* transcript levels were partly restored in *clpP*+pME6031-*luxR*(mA), especially during early exponential growth (Fig. 5D). Collectively, these results strongly suggest that ClpP affects expression of the transcriptional-regulatory gene *luxR*(mA), thereby regulating massetolide biosynthesis and swarming motility in *P. fluorescens* SS101.

TABLE 2. Transcript levels of various genes in the *clpP* mutant of *P. fluorescens* strain SS101

Gene	Transcript level[a]			
	Early exponential		Mid-exponential	
	SS101	Δ*clpP*	SS101	Δ*clpP*
tig	0.00 ± 0.03	−0.29 ± 0.03[b]	0.00 ± 0.04	−0.36 ± 0.04[b]
clpX	0.00 ± 0.04	−0.30 ± 0.03[b]	0.00 ± 0.08	0.09 ± 0.11
lon	0.00 ± 0.02	0.06 ± 0.03	0.00 ± 0.02	−0.08 ± 0.02
hupB	0.00 ± 0.03	0.05 ± 0.03	0.00 ± 0.03	−0.46 ± 0.02[b]
ppiD	0.00 ± 0.08	−0.19 ± 0.03	0.00 ± 0.02	−0.61 ± 0.03[b]
dnaK	0.00 ± 0.03	0.12 ± 0.02[b]	0.00 ± 0.01	0.06 ± 0.02
dnaJ	0.00 ± 0.04	0.13 ± 0.02	0.00 ± 0.03	0.28 ± 0.05[b]
grpE	0.00 ± 0.04	−0.01 ± 0.03	0.00 ± 0.02	−0.02 ± 0.03

[a] The transcript level of each of the genes was corrected for the transcript levels of the housekeeping gene *rpoD* [$\Delta C_T = C_T$ (gene *x*) $- C_T$(*rpoD*)] and is presented relative to the transcript level in wild-type SS101 (log RQ ± standard errors of the mean), with RQ equal to $2^{-[\Delta Ct(\text{mutant}) - \Delta Ct(\text{wild type})]}$. For each time point, the mean values of four biological replicates are given.

[b] Significantly different from SS101 transcript levels ($P < 0.05$).

Influence of amino acids on *clpP* expression and massetolide biosynthesis. Previous studies showed that various nutritional conditions, including specific sugars and amino acids, affect cyclic lipopeptide production in *P. fluorescens* and *P. putida* (21, 31, 54, 60). Furthermore, CAA, citrate, glutamate, and iron were shown to rescue biofilm and growth defects of a range of surface attachment (*sad*) mutants of *P. fluorescens* strain WCS365, including a *clpP* mutant (56). Based on these observations, swarming assays, Q-PCR, and tensiometric and HPLC analyses were performed to assess the effects of specific nutrients on growth, massetolide production, and gene expression in strain SS101 and the *clpP* mutant. The results showed that addition of CAA did not rescue the growth defect of the *clpP* mutant (Fig. 6A) but did restore, at concentrations of 1% and 4% (wt/vol), massetolide production, as evidenced by a reduction in surface tension (Fig. 6B) and by HPLC analysis (see Fig. S3 in the supplemental material). Consistent with this partial recovery of massetolide production, swarming motility of the *clpP* mutant was also partly restored when the mutant was grown on CAA-supplemented agar medium (Fig. 6C). In contrast, swarming motility of the *massA* mutant was not restored by addition of CAA to the growth medium (data not shown). For wild-type strain SS101, swarming motility increased with increasing CAA concentrations; however, growth was not affected by the addition of CAA to liquid KB (see Fig. S4 in the supplemental material). With increasing CAA concentrations, the motility patterns of wild-type SS101 changed from typical dendritic to more confluent (Fig. 6C). Moreover, compared to the other CAA concentrations, the drop in surface tension was delayed when 4% CAA was added to liquid KB (see Fig. S4 in the supplemental material). Gene expression analyses showed that addition of CAA led to an increase in *mass* transcript levels in wild-type SS101 (Fig. 6D). In the *clpP* mutant, addition of CAA restored transcription of *massA* to the wild-type level and led to an increase in *massBC* tran-

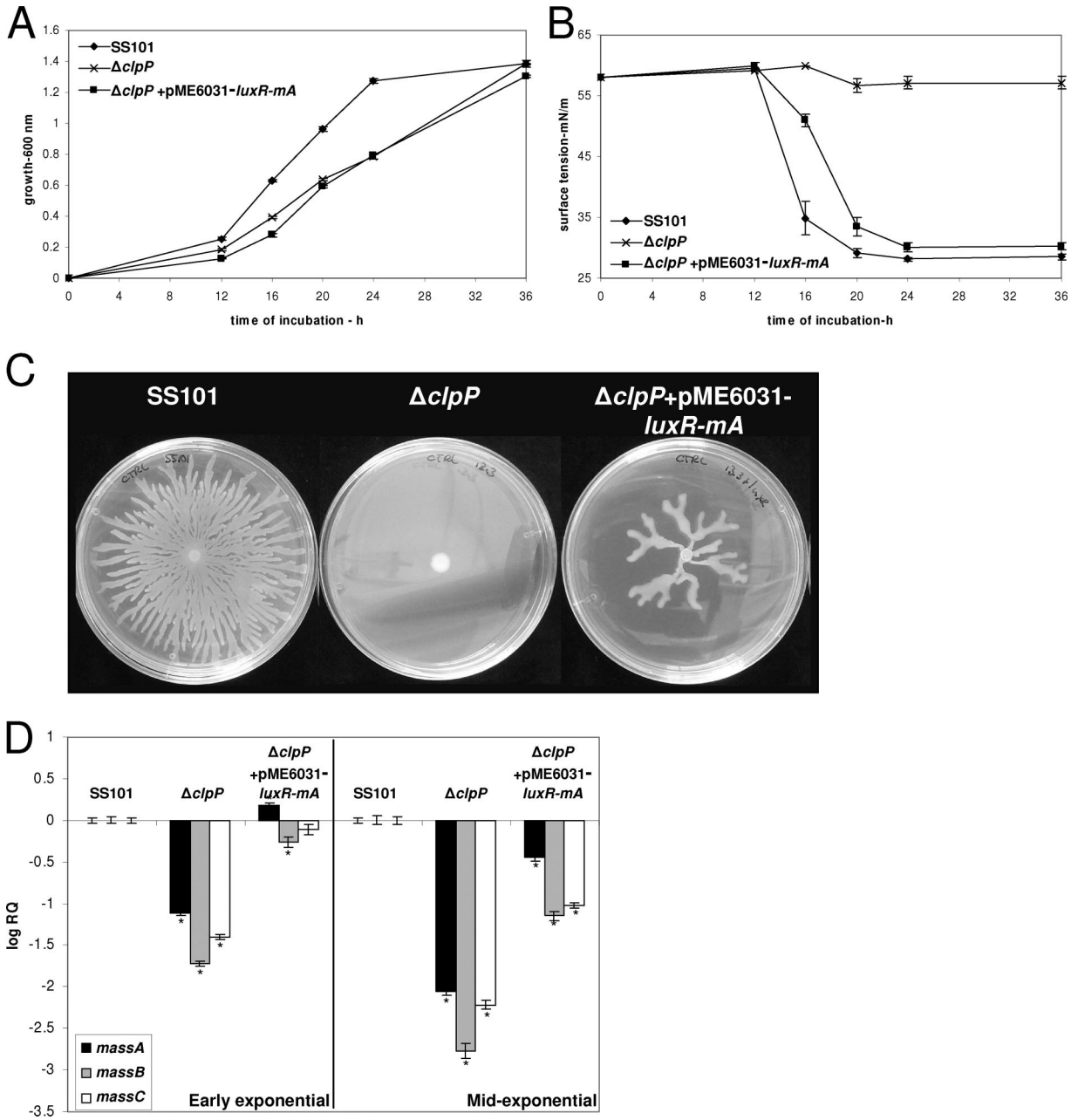
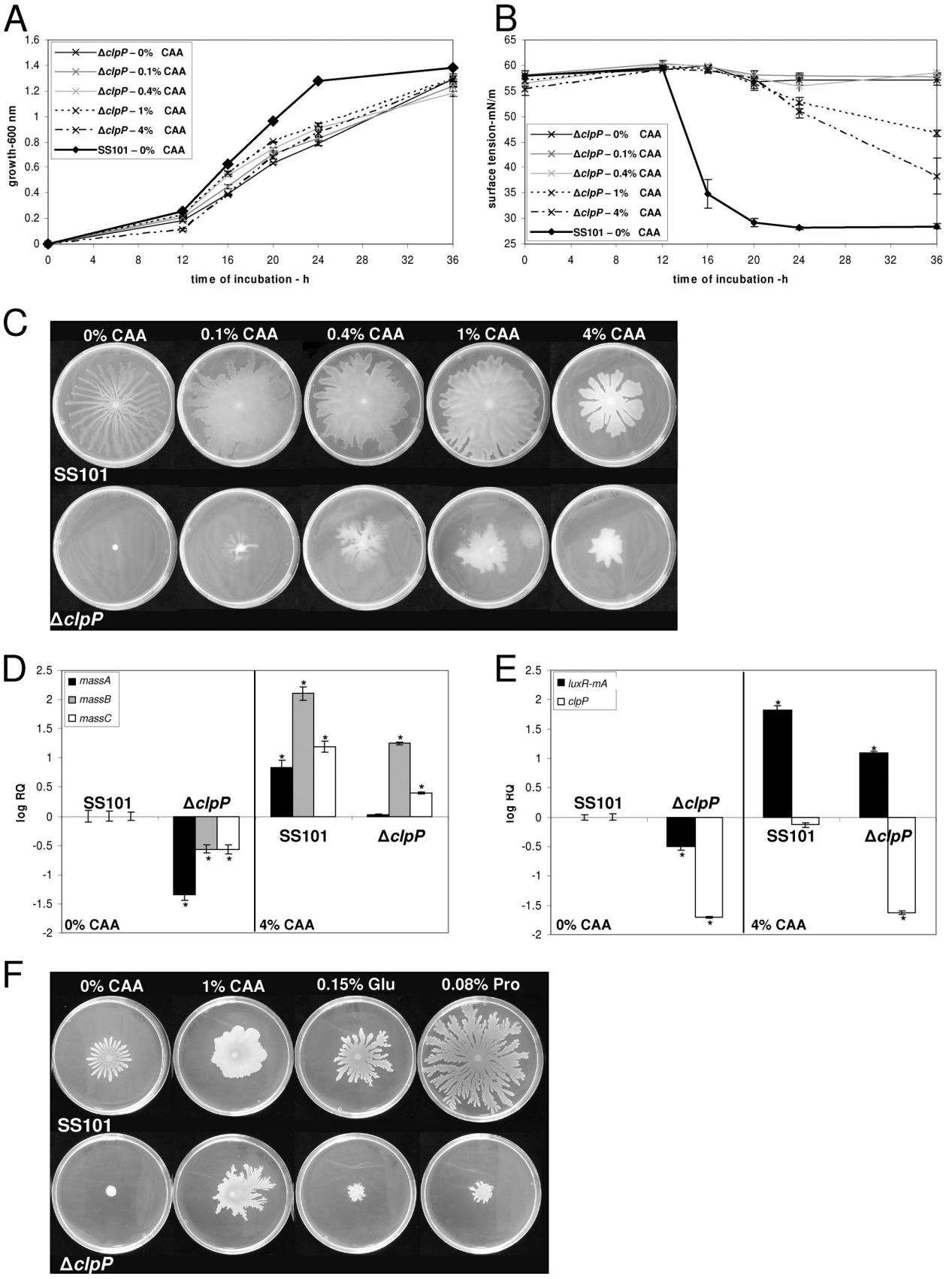


FIG. 5. (A) Growth of *P. fluorescens* strain SS101, the *clpP* mutant, and *clpP*+pME6031-*luxR*(mA) at 25°C. At each time point, the cell density was measured spectrophotometrically (600 nm), and the mean values of four replicates are given; the error bars represent the standard errors of the mean. (B) Surface tension of cell-free culture supernatant of strain SS101 and the different mutants shown in panel A. (C) Swarming motility of wild-type strain SS101 and mutants on soft (0.6% [wt/vol]) agar plates. Five microliters of washed overnight cultures of wild-type SS101 and mutants was spot inoculated in the center of a soft agar plate and incubated for 48 to 72 h at 25°C. (D) Transcript levels of the *massA*, *massB*, and *massC* genes in wild-type strain SS101, the *clpP* mutant, and *clpP*+pME6031-*luxR*(mA) in early and mid-exponential growth phases. The transcript level of each of the genes was corrected for the transcript level of *rpoD* [$\Delta C_T = C_T(\text{gene } x) - C_T(rpoD)$] and is presented relative to the transcript level in wild-type SS101 (log RQ), with RQ equal to $2^{-[\Delta CT(\text{mutant}) - \Delta CT(\text{wild type})]}$. For each time point, the mean values of four biological replicates are given; the error bars represent standard errors of the mean. The asterisks indicate statistically significant ($P < 0.05$) differences relative to wild-type SS101.

script levels (Fig. 6D), providing support at the transcriptional level to the idea that CAA restore, at least in part, massetolide biosynthesis in the *clpP* mutant. In the wild-type strain SS101 and the *clpP* mutant, addition of CAA increased *luxR*(mA) transcript levels but did not affect transcription of *clpP* (Fig. 6E). Addition of CAA to cultures of the *clpP* mutant modified with pME6031-*luxR*(mA) completely restored swarming motility (see Fig. S5 in the supplemental material). Taken together, these results show that CAA restore and enhance transcription of the *luxR*(mA) and *massABC* biosynthesis genes, leading to a


A
growth-600 nm
time of incubation - h
ΔclpP – 0% CAA
ΔclpP – 0.1% CAA
ΔclpP – 0.4% CAA
ΔclpP – 1% CAA
ΔclpP – 4% CAA
SS101 – 0% CAA
B
surface tension-mN/m
time of incubation -h
C
0% CAA
0.1% CAA
0.4% CAA
1% CAA
4% CAA
SS101
ΔclpP
D
log RQ
massA
massB
massC
SS101
ΔclpP
0% CAA
4% CAA
E
log RQ
luxR-mA
clpP
F
0% CAA
1% CAA
0.15% Glu
0.08% Pro

partial rescue of massetolide biosynthesis and swarming motility in the *clpP* mutant. Expression of the *clpP* gene, however, was not affected by CAA.

To identify which amino acid is responsible for the partial complementation of the swarming motility of the *clpP* mutant, each amino acid present in the CAA was tested separately at concentrations identical to their respective concentrations in 1% CAA (see Table S2 in the supplemental material). The results show that the amino acids proline and glutamic acid can partially complement the deficiency in swarming motility of the *clpP* mutant, but not to the same extent as provided by addition of 1% CAA (Fig. 6F). When proline and glutamic acid were combined, no significant additional effects were observed (data not shown). The other amino acids, as well as calcium, iron, and citrate, did not stimulate the swarming motility of the *clpP* mutant. In fact, addition of several amino acids (valine, isoleucine, and leucine) inhibited the swarming motility of the wild-type strain SS101 (data not shown).

Interplay between GacA/GacS and ClpP. For *P. fluorescens* SS101, a mutation in the sensor kinase gene *gacS* significantly reduced the expression of the *massABC* genes (Fig. 7) and shut down massetolide production. Also, *luxR*(mA) transcript levels were reduced but *clpP* transcription was not affected in the *gacS* mutant of strain SS101 (Fig. 7). Furthermore, transcript levels of *gacA*/*gacS* were not affected in the *clpP* mutant (Fig. 7), suggesting that, at the transcriptional level, ClpP-mediated regulation of massetolide biosynthesis is independent of regulation by GacA/GacS.

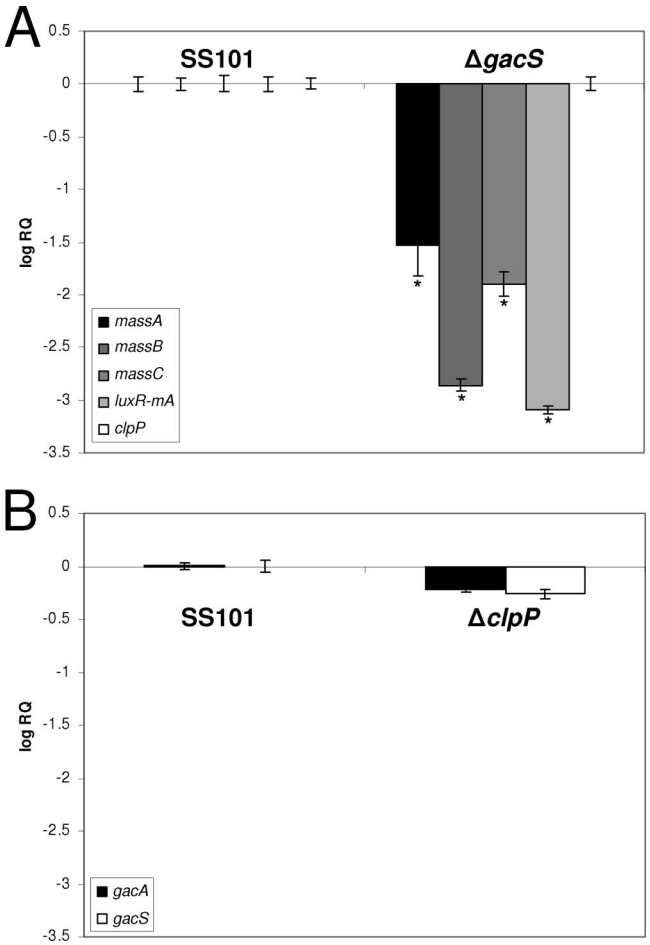


FIG. 7. (A) Transcript levels of *massA*, *massB*, *massC*, *luxR*(mA), and *clpP* in a *gacS* mutant of *P. fluorescens* SS101 at mid-exponential growth phase. (B) Transcript levels of *gacA* and *gacS* in the *clpP* mutant of *P. fluorescens* SS101 at mid-exponential growth phase. The transcript levels were corrected for the transcript levels of *rpoD* [$\Delta C_T = C_T(\text{gene } x) - C_T(rpoD)$] and are presented relative to the transcript level in wild-type SS101 (log RQ), with RQ equal to $2^{-[\Delta CT(\text{mutant}) - \Delta CT\ (\text{wild type})]}$. The mean values of four biological replicates are given. The error bars represent the standard errors of the mean. The asterisks indicate statistically significant ($P < 0.05$) differences relative to wild-type SS101.

DISCUSSION

ClpP is a serine protease that is highly conserved in bacteria and eukaryotes (68, 69). Together with other proteases, ClpP plays a crucial role in intracellular refolding and degradation of proteins, which is an essential process for the viability and growth of cells. In this study, we cloned and sequenced *clpP* from plant growth-promoting *P. fluorescens* strain SS101 and showed that *clpP* plays an important role in the regulation of cyclic lipopeptide biosynthesis, swarming motility, biofilm formation, and growth. These results confirm and extend observations made for other *Pseudomonas* species and bacterial genera. For example, biofilm formation was reduced in *clpP* mutants of *P. fluorescens* WCS365 and *S. aureus* but enhanced in a *clpP* mutant of *P. aeruginosa* (26, 56, 63, 65). ClpP is also important for virulence in several bacterial pathogens, like *Streptococcus pneumoniae*, *S. aureus*, *Salmonella enterica* serovar Typhimurium, *Yersinia enterocolitica*, *Listeria monocytogenes*, and *Porphyromonas gingivalis* (7, 29, 38, 58). In *Listeria*, the hemolytic activity, but not the production, of the virulence factor listeriolysin O was strongly reduced in a *clpP* mutant (29). In *Bacillus subtilis*, ClpP plays a role in competence de-

FIG. 6. (A) Growth of the *clpP* mutant of *P. fluorescens* strain SS101 at 25°C in growth medium supplemented with 0 to 4% (wt/vol) CAA. At each time point, the cell density was measured spectrophotometrically (600 nm); the mean values of four replicates are given, and the error bars represent the standard errors of the mean. (B) Surface tension of cell-free culture supernatant of strain SS101 and the *clpP* mutant grown in media supplemented with different concentrations of CAA. (C) Swarming motility of strain SS101 and the *clpP* mutant on soft (0.6% [wt/vol]) agar plates supplemented with different concentrations of CAA. Five microliters of washed overnight cultures of wild-type SS101 and the mutants was spot inoculated in the center of a soft agar plate and incubated for 48 to 72 h at 25°C. (D and E) Transcript levels of the *massA*, *massB*, and *massC* genes (D) and *luxR*(mA) and *clpP* (E) in wild-type SS101 and the *clpP* mutant when grown on soft-agar plates supplemented with 0 or 4% CAA. Cells were collected from the periphery of the swarming colony. The transcript levels were corrected for the transcript levels of *rpoD* [$\Delta C_T = C_T(\text{gene } x) - C_T(rpoD)$] and are presented relative to the transcript level in wild-type SS101 grown at 0% CAA (log RQ), with RQ equal to $2^{-[\Delta CT(\text{sample X}) - \Delta CT\ (\text{wild type 0\% CAA})]}$. For each sample, the mean values of four biological replicates are given, and the error bars represent the standard errors of the mean. The asterisks indicate statistically significant ($P < 0.05$) differences relative to wild-type SS101. (F) Swarming motility of strain SS101 and the *clpP* mutant on soft agar medium supplemented with glutamic acid and proline.

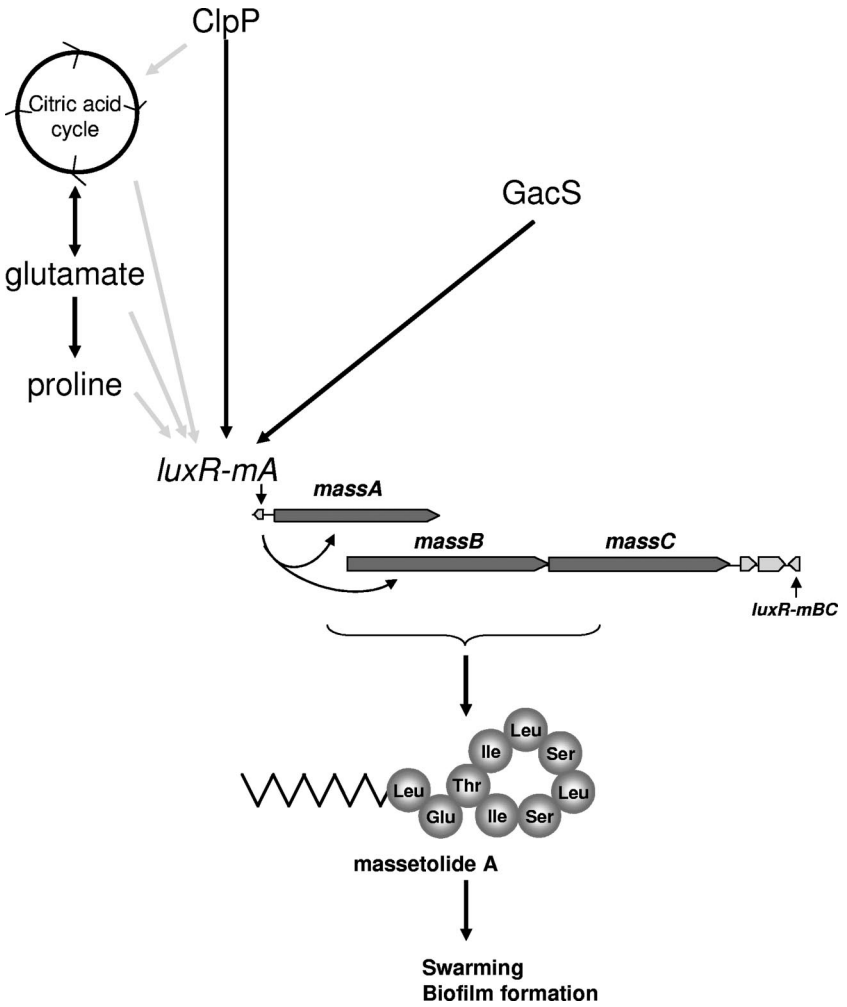


FIG. 8. Proposed model for ClpP-mediated regulation of massetolide biosynthesis and swarming motility in *P. fluorescens* strain SS101. The darkly shaded arrows indicate interactions based on observations in this study; the lightly shaded arrows indicate putative interactions based on previous findings in other studies.

velopment, motility, and sporulation (52). Although specific extracellular metabolites of *Pseudomonas* strains are known to play roles in swarming motility and biofilm formation, the involvement of ClpP in regulation of the biosynthesis genes encoding these metabolites has, to our knowledge, not been demonstrated conclusively. This study provides for the first time evidence that the ClpP protease regulates the biosynthesis of cyclic lipopeptide surfactants that play an important role in swarming the motility, biofilm formation, and antimicrobial activity of *P. fluorescens*. More specifically, ClpP was shown to affect expression of the transcriptional-regulatory gene *luxR*(mA), thereby regulating massetolide biosynthesis and concomitantly biofilm formation and swarming motility in *P. fluorescens* SS101. Whether this is typical for the *Pseudomonas* strain under study remains to be addressed, but the observation by Nakano et al. (53) that expression of the surfactin gene *srfA* in *B. subtilis* is affected in a *clpP* mutant suggests that a similar role of ClpP may apply to other bacterial genera and species producing lipopeptide antibiotics.

Based on the results of this and previous studies, several hypotheses can be proposed for the mechanisms underlying ClpP-mediated regulation of *luxR*(mA) expression, massetolide biosynthesis, and swarming motility in *P. fluorescens* (Fig. 8). In *E. coli*, ClpP consists of two heptameric rings that form a barrel-shaped core with active sites in an interior chamber (69). ClpP forms a proteolytic complex with Clp-ATPases, i.e., ClpX and ClpA, that carry one or two nucleotide binding domains (28). These ATPases belong to the Hsp100 protein family and unfold the substrates so they can be translocated to the active sites of the ClpP protease, which then leads to protein degradation and release of protein fragments (51, 69). Besides ClpXP and ClpAP, other ATP-dependent proteolytic complexes, like HslUV, Lon, and FtsH, have been identified in bacteria, particularly in *E. coli* (33, 68). However, based on site-directed mutagenesis and transcriptional analyses performed in this study, the chaperone subunit ClpX and also the Lon protease do not appear to be involved in regulation of massetolide biosynthesis in *P. fluorescens* SS101. Whether other Clp-ATPases are required as chaperones in ClpP-mediated regulation of these processes was not determined and will be investigated in more detail as soon as the whole genome of strain SS101 is sequenced. Alternatively, ClpP may also act as

a peptidase in the absence of the Clp-ATPases, thereby hydrolyzing short peptides of up to 6 amino acids (5). Studies of *B. subtilis* further showed that in addition to its function in the degradation of misfolded and defective proteins, ClpP is also involved in targeted proteolysis of specific protein substrates, including key regulators and transcriptional factors involved in competence and developmental programs (5, 26, 32, 43, 52). Based on these observations in *B. subtilis*, we postulate that in *P. fluorescens* strain SS101 ClpP may degrade, alone or in concert with a Clp-ATPase, proteins that repress or interfere with transcription of the massetolide-regulatory gene *luxR*(mA). To identify the cellular substrates and target proteins of the ClpP protease in *P. fluorescens*, an extensive proteomic analysis, as was performed previously for *E. coli* (25), will be required to support this hypothesis.

Another scenario for how ClpP may regulate massetolide biosynthesis is by influencing the citric acid cycle and amino acid metabolism (Fig. 8). In *E. coli*, ClpAP plays a role in the degradation of L-glutamate dehydrogenase (49), and ClpXP associates with the two principal enzymes (AceA and GlcB) of the glyoxylate shunt, which replenishes the pool of citric acid cycle intermediates (25). The results of other studies showed that the degradation rate of enzymes involved in amino acid metabolism was significantly reduced in a *clpP* mutant of *B. subtilis* (32). More specifically, one of the ClpP substrates in *B. subtilis* was PycA, a pyruvate carboxylase that catalyzes the conversion of pyruvate into oxaloacetate, which replenishes the citric acid cycle (32). For *P. fluorescens* SS101, preliminary results of Q-PCR analyses showed that the transcript levels of a *pycA* homologue are indeed significantly reduced (log RQ = −1.76) in the *clpP* mutant (data not shown). However, the role of this gene and other enzymes involved in the amino acid metabolism of *P. fluorescens* SS101, as well as their effects on massetolide biosynthesis and swarming motility, remain to be investigated. Assuming that ClpP adversely affects the citric acid cycle and amino acid metabolism in *P. fluorescens* SS101, it also may provide an explanation for the reduced growth observed for the *clpP* mutant. At higher temperatures, a condition known to increase the levels of misfolded proteins (33), growth was reduced in *clpP* mutants of *Campylobacter jejuni*, *L. monocytogenes*, and *B. subtilis* (11, 29, 52), but at regular temperatures, growth deficiencies were also observed for *clpP* mutants of *E. coli*, *S. aureus*, and *P. aeruginosa* (13, 63, 65). In this context, Chandu and Nandi (8) suggested that the ClpP protease degrades proteins, resulting in the release of amino acids that are subsequently recycled in the cellular pool and used for growth. For example, in *E. coli*, the growth deficiency of *clpP* mutant colonies was restored by the addition of CAA (13). For *P. fluorescens* SS101, however, growth of the *clpP* mutant was not restored by addition of CAA, suggesting that this effect may be strain specific. When the effects of individual amino acids were analyzed, the results of our study showed that glutamic acid and proline restored, in part, the swarming deficiency of the *clpP* mutant of strain SS101. The possibility that these amino acids may have served as building blocks for the nonribosomal peptide synthetases MassABC to synthesize the peptide moieties of the massetolide compounds seems unlikely. Although glutamic acid is a constituent of the massetolide compounds, proline is not (14). Furthermore, valine, leucine, and isoleucine, three amino acids in the peptide moieties of massetolides (14), did not complement the swarming deficiency in the *clpP* mutant and even adversely affected swarming in the wild-type strain SS101. Alternatively, glutamic acid and proline may have served as chemical signals that triggered, directly or indirectly, the expression of *luxR*(mA) and the *mass* biosynthesis genes, leading to a partial rescue of massetolide biosynthesis and swarming motility in the *clpP* mutant (Fig. 8). It is well known that specific amino acids, including glutamate and proline, can promote swarming in *P. aeruginosa* (44) and *Proteus mirabilis* (1) and act as a chemoattractant (1). Moreover, glutamine can serve as a signal for the cellular nitrogen state; in *E. coli*, glutamine is sensed by enzymes that trigger a signal transduction cascade that activates the glutamine synthase gene *glnA* (46). Also, exogenously provided proline can release the transcriptional repressor PutA from the proline utilization (*put*) genes (6, 71). These studies demonstrate that these amino acids can induce gene transcription.

Finally, we looked into the possible interplay between ClpP and the two-component regulatory system GacA/GacS (Fig. 8). In other systems, ClpP affects global regulation. For example, in *S. aureus*, the global regulator *agr* was repressed in the *clpP* mutant, which resulted in reduced alpha-toxin and extracellular protease activities (27, 50). Also in *Bacillus*, ClpP-dependent proteolysis is regulated in response to environmental signals (nutrients) and transmitted via the two-component signal transduction system ComK/ComS (28). For *P. fluorescens* SS101, *gacS* regulates transcription of the *massABC* and *luxR*(mA) genes and thereby massetolide production, but *clpP* transcription is not affected. Furthermore, expression of *gacA*/*gacS* was not affected in the *clpP* mutant, suggesting that, at the transcriptional level, ClpP-mediated regulation of massetolide biosynthesis is independent of regulation by GacA/GacS.

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REFERENCES

1. **Allison, C., H. C. Lai, D. Gygi, and C. Hughes.** 1993. Cell differentiation of *Proteus mirabilis* is initiated by glutamine, a specific chemoattractant for swarming cells. Mol. Microbiol. **8:**53–60.
2. **Andersen, J. B., B. Koch, T. H. Nielsen, D. Sorensen, M. Hansen, O. Nybroe, C. Christophersen, J. Sorensen, S. Molin, and M. Givskov.** 2003. Surface motility in *Pseudomonas* sp. DSS73 is required for efficient biological containment of the root-pathogenic microfungi *Rhizoctonia solani* and *Pythium ultimum.* Microbiology **149:**37–46.
3. **Bartels, F., S. Fernandez, A. Holtel, K. N. Timmis, and V. de Lorenzo.** 2001. The essential HupB and HupN proteins of *Pseudomonas putida* provide redundant and nonspecific DNA-bending functions. J. Biol. Chem. **276:** 16641–16648.
4. **Berti, A. D., N. J. Greve, Q. H. Christensen, and M. G. Thomas.** 2007. Identification of a biosynthetic gene cluster and the six associated lipopeptides involved in swarming motility of *Pseudomonas syringae* pv. tomato DC3000. J. Bacteriol. **189:**6312–6323.
5. **Brotz-Oesterhelt, H., D. Beyer, H. P. Kroll, R. Endermann, C. Ladel, W. Schroeder, B. Hinzen, S. Raddatz, H. Paulsen, K. Henninger, J. E. Bandow, H. G. Sahl, and H. Labischinski.** 2005. Dysregulation of bacterial proteolytic machinery by a new class of antibiotics. Nat. Med. **11:**1082–1087.
6. **Brown, E. D., and J. M. Wood.** 1993. Conformational change and membrane association of the PutA protein are coincident with reduction of its FAD cofactor by proline. J. Biol. Chem. **268:**8972–8979.

7. **Capestany, C. A., G. D. Tribble, K. Maeda, D. R. Demuth, and R. J. Lamont.** 2008. Role of the Clp system in stress tolerance, biofilm formation, and intracellular invasion in *Porphyromonas gingivalis*. J. Bacteriol. **190:**1436–1446.
8. **Chandu, D., and D. Nandi.** 2004. Comparative genomics and functional roles of the ATP-dependent proteases Lon and Clp during cytosolic protein degradation. Res. Microbiol. **155:**710–719.
9. **Choi, K. H., A. Kumar, and H. P. Schweizer.** 2006. A 10-min method for preparation of highly electrocompetent *Pseudomonas aeruginosa* cells: application for DNA fragment transfer between chromosomes and plasmid transformation. J. Microbiol. Methods **64:**391–397.
10. **Choi, K. H., and H. P. Schweizer.** 2005. An improved method for rapid generation of unmarked *Pseudomonas aeruginosa* deletion mutants. BMC Microbiol. **5:**30.
11. **Cohn, M. T., H. Ingmer, F. Mulholland, K. Jorgensen, J. M. Wells, and L. Brondsted.** 2007. Contribution of conserved ATP-dependent proteases of *Campylobacter jejuni* to stress tolerance and virulence. Appl. Environ. Microbiol. **73:**7803–7813.
12. **Cui, X., R. Harling, P. Mutch, and D. Darling.** 2005. Identification of *N*-3-hydroxyoctanoyl-homoserine lactone production in *Pseudomonas fluorescens* 5064, pathogenic to broccoli, and controlling biosurfactant production by quorum sensing. Eur. J. Plant Pathol. **111:**297–308.
13. **Damerau, K., and A. C. St John.** 1993. Role of Clp protease subunits in degradation of carbon starvation proteins in *Escherichia coli*. J. Bacteriol. **175:**53–63.
14. **De Bruijn, I., M. J. de Kock, P. de Waard, T. A. van Beek, and J. M. Raaijmakers.** 2008. Massetolide A biosynthesis in *Pseudomonas fluorescens*. J. Bacteriol. **190:**2777–2789.
15. **De Bruijn, I., M. J. D. de Kock, M. Yang, P. de Waard, T. A. van Beek, and J. M. Raaijmakers.** 2007. Genome-based discovery, structure prediction and functional analysis of cyclic lipopeptide antibiotics in *Pseudomonas* species. Mol. Microbiol. **63:**417–428.
16. **Dennis, J. J., and G. J. Zylstra.** 1998. Plasposons: modular self-cloning minitransposon derivatives for rapid genetic analysis of gram-negative bacterial genomes. Appl. Environ. Microbiol. **64:**2710–2715.
17. **De Souza, J. T., M. De Boer, P. De Waard, T. A. Van Beek, and J. M. Raaijmakers.** 2003. Biochemical, genetic, and zoosporicidal properties of cyclic lipopeptide surfactants produced by *Pseudomonas fluorescens*. Appl. Environ. Microbiol. **69:**7161–7172.
18. **Deziel, E., F. Lepine, S. Milot, and R. Villemur.** 2003. *rhlA* is required for the production of a novel biosurfactant promoting swarming motility in *Pseudomonas aeruginosa*: 3-(3-hydroxyalkanoyloxy)alkanoic acids (HAAs), the precursors of rhamnolipids. Microbiology **149:**2005–2013.
19. **Dougan, D. A., A. Mogk, and B. Bukau.** 2002. Protein folding and degradation in bacteria: to degrade or not to degrade? That is the question. Cell Mol. Life Sci. **59:**1607–1616.
20. **Dubern, J.-F., E. L. Lagendijk, B. J. J. Lugtenberg, and G. V. Bloemberg.** 2005. The heat shock genes *dnaK*, *dnaJ*, and *grpE* are involved in regulation of putisolvin biosynthesis in *Pseudomonas putida* PCL1445. J. Bacteriol. **187:**5967–5976.
21. **Dubern, J. F., and G. V. Bloemberg.** 2006. Influence of environmental conditions on putisolvins I and II production in *Pseudomonas putida* strain PCL1445. FEMS Microbiol. Lett. **263:**169–175.
22. **Dubern, J. F., B. J. J. Lugtenberg, and G. V. Bloemberg.** 2006. The *ppuI-rsaL-ppuR* quorum-sensing system regulates biofilm formation of *Pseudomonas putida* PCL1445 by controlling biosynthesis of the cyclic lipopeptides putisolvins I and II. J. Bacteriol. **188:**2898–2906.
23. **Finking, R., and M. A. Marahiel.** 2004. Biosynthesis of nonribosomal peptides. Annu. Rev. Microbiol. **58:**453–488.
24. **Fischer, G., T. Tradler, and T. Zarnt.** 1998. The mode of action of peptidyl prolyl *cis*/*trans* isomerases in vivo: binding vs. catalysis. FEBS Lett. **426:**17–20.
25. **Flynn, J. M., S. B. Neher, Y. I. Kim, R. T. Sauer, and T. A. Baker.** 2003. Proteomic discovery of cellular substrates of the ClpXP protease reveals five classes of ClpX-recognition signals. Mol. Cell **11:**671–683.
26. **Frees, D., A. Chastanet, S. Qazi, K. Sorensen, P. Hill, T. Msadek, and H. Ingmer.** 2004. Clp ATPases are required for stress tolerance, intracellular replication and biofilm formation in *Staphylococcus aureus*. Mol. Microbiol. **54:**1445–1462.
27. **Frees, D., S. N. Qazi, P. J. Hill, and H. Ingmer.** 2003. Alternative roles of ClpX and ClpP in *Staphylococcus aureus* stress tolerance and virulence. Mol. Microbiol. **48:**1565–1578.
28. **Frees, D., K. Savijoki, P. Varmanen, and H. Ingmer.** 2007. Clp ATPases and ClpP proteolytic complexes regulate vital biological processes in low GC, Gram-positive bacteria. Mol. Microbiol. **63:**1285–1295.
29. **Gaillot, O., E. Pellegrini, S. Bregenholt, S. Nair, and P. Berche.** 2000. The ClpP serine protease is essential for the intracellular parasitism and virulence of *Listeria monocytogenes*. Mol. Microbiol. **35:**1286–1294.
30. **Garcia-Fruitos, E., M. Martinez-Alonso, N. Gonzalez-Montalban, M. Valli, D. Mattanovich, and A. Villaverde.** 2007. Divergent genetic control of protein solubility and conformational quality in *Escherichia coli*. J. Mol. Biol. **374:**195–205.
31. **Gerard, J., R. Lloyd, T. Barsby, P. Haden, M. T. Kelly, and R. J. Andersen.** 1997. Massetolides A-H, antimycobacterial cyclic depsipeptides produced by two pseudomonads isolated from marine habitats. J. Nat. Prod. **60:**223–229.
32. **Gerth, U., H. Kock, I. Kusters, S. Michalik, R. L. Switzer, and M. Hecker.** 2008. Clp-dependent proteolysis down-regulates central metabolic pathways in glucose-starved *Bacillus subtilis*. J. Bacteriol. **190:**321–331.
33. **Gottesman, S.** 1996. Proteases and their targets in *Escherichia coli*. Annu. Rev. Genet. **30:**465–506.
34. **Gross, H., V. O. Stockwell, M. D. Henkels, B. Nowak-Thompson, J. E. Loper, and W. H. Gerwick.** 2007. The genomisotopic approach: a systematic method to isolate products of orphan biosynthetic gene clusters. Chem. Biol. **14:**53–63.
35. **Hartl, F. U., and M. Hayer-Hartl.** 2002. Molecular chaperones in the cytosol: from nascent chain to folded protein. Science **295:**1852–1858.
36. **Heeb, S., Y. Itoh, T. Nishijyo, U. Schnider, C. Keel, J. Wade, U. Walsh, F. O'Gara, and D. Haas.** 2000. Small, stable shuttle vectors based on the minimal pVS1 replicon for use in gram-negative, plant-associated bacteria. Mol. Plant-Microbe Interact. **13:**232–237.
37. **Hoang, T. T., R. R. Karkhoff-Schweizer, A. J. Kutchma, and H. P. Schweizer.** 1998. A broad-host-range Flp-FRT recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. Gene **212:**77–86.
38. **Ibrahim, Y. M., A. R. Kerr, N. A. Silva, and T. J. Mitchell.** 2005. Contribution of the ATP-dependent protease ClpCP to the autolysis and virulence of *Streptococcus pneumoniae*. Infect. Immun. **73:**730–740.
39. **Inoue, H., H. Nojima, and H. Okayama.** 1990. High efficiency transformation of *Escherichia coli* with plasmids. Gene **96:**23–28.
40. **Kinscherf, T. G., and D. K. Willis.** 2002. Global regulation by *gidA* in *Pseudomonas syringae*. J. Bacteriol. **184:**2281–2286.
41. **Kitten, T., T. G. Kinscherf, J. L. McEvoy, and D. K. Willis.** 1998. A newly identified regulator is required for virulence and toxin production in *Pseudomonas syringae*. Mol. Microbiol. **28:**917–929.
42. **Koch, B., T. H. Nielsen, D. Sorensen, J. B. Andersen, C. Christophersen, S. Molin, M. Givskov, J. Sorensen, and O. Nybroe.** 2002. Lipopeptide production in *Pseudomonas* sp. strain DSS73 is regulated by components of sugar beet seed exudate via the Gac two-component regulatory system. Appl. Environ. Microbiol. **68:**4509–4516.
43. **Kock, H., U. Gerth, and M. Hecker.** 2004. MurAA, catalysing the first committed step in peptidoglycan biosynthesis, is a target of Clp-dependent proteolysis in *Bacillus subtilis*. Mol. Microbiol. **51:**1087–1102.
44. **Kohler, T., L. K. Curty, F. Barja, C. van Delden, and J. C. Pechere.** 2000. Swarming of *Pseudomonas aeruginosa* is dependent on cell-to-cell signaling and requires flagella and pili. J. Bacteriol. **182:**5990–5996.
45. **Kuiper, I., E. L. Lagendijk, R. Pickford, J. P. Derrick, G. E. M. Lamers, J. E. Thomas-Oates, B. J. J. Lugtenberg, and G. V. Bloemberg.** 2004. Characterization of two *Pseudomonas putida* lipopeptide biosurfactants, putisolvin I and II, which inhibit biofilm formation and break down existing biofilms. Mol. Microbiol. **51:**97–113.
46. **Leigh, J. A., and J. A. Dodsworth.** 2007. Nitrogen regulation in bacteria and archaea. Annu. Rev. Microbiol. **61:**349–377.
47. **Lu, S. E., B. K. Scholz-Schroeder, and D. C. Gross.** 2002. Characterization of the *salA*, *syrF*, and *syrG* regulatory genes located at the right border of the syringomycin gene cluster of *Pseudomonas syringae* pv. *syringae.* Mol. Plant-Microbe Interact. **15:**43–53.
48. **Lu, S. E., N. Wang, J. Wang, Z. J. Chen, and D. C. Gross.** 2005. Oligonucleotide microarray analysis of the *salA* regulon controlling phytotoxin production by *Pseudomonas syringae* pv. *syringae.* Mol. Plant-Microbe Interact. **18:**324–333.
49. **Maurizi, M. R., and F. Rasulova.** 2002. Degradation of L-glutamate dehydrogenase from *Escherichia coli*: allosteric regulation of enzyme stability. Arch. Biochem. Biophys. **397:**206–216.
50. **Michel, A., F. Agerer, C. R. Hauck, M. Herrmann, J. Ullrich, J. Hacker, and K. Ohlsen.** 2006. Global regulatory impact of ClpP protease of *Staphylococcus aureus* on regulons involved in virulence, oxidative stress response, autolysis, and DNA repair. J. Bacteriol. **188:**5783–5796.
51. **Mogk, A., R. Schmidt, and B. Bukau.** 2007. The N-end rule pathway for regulated proteolysis: prokaryotic and eukaryotic strategies. Trends Cell Biol. **17:**165–172.
52. **Msadek, T., V. Dartois, F. Kunst, M. L. Herbaud, F. Denizot, and G. Rapoport.** 1998. ClpP of *Bacillus subtilis* is required for competence development, motility, degradative enzyme synthesis, growth at high temperature and sporulation. Mol. Microbiol. **27:**899–914.
53. **Nakano, M. M., Y. Zhu, J. Liu, D. Y. Reyes, H. Yoshikawa, and P. Zuber.** 2000. Mutations conferring amino acid residue substitutions in the carboxy-terminal domain of RNA polymerase alpha can suppress *clpX* and *clpP* with respect to developmentally regulated transcription in *Bacillus subtilis*. Mol. Microbiol. **37:**869–884.
54. **Nybroe, O., and J. Sorensen.** 2004. Production of cyclic lipopeptides by fluorescent pseudomonads, p. 147–172. *In* J.-L. Ramos (ed.), *Pseudomonas*, vol. 3. Biosynthesis of macromolecules and molecular metabolism. Kluwer Academic Publishers, New York, NY.

55. **Ongena, M., and P. Jacques.** 2008. *Bacillus* lipopeptides: versatile weapons for plant disease biocontrol. Trends Microbiol. **16:**115–125.
56. **O'Toole, G. A., and R. Kolter.** 1998. Initiation of biofilm formation in *Pseudomonas fluorescens* WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis. Mol. Microbiol. **28:**449–461.
57. **O'Toole, G. A., L. A. Pratt, P. I. Watnick, D. K. Newman, V. B. Weaver, and R. Kolter.** 1999. Genetic approaches to study of biofilms. Methods Enzymol. **310:**91–109.
58. **Porankiewicz, J., J. Wang, and A. K. Clarke.** 1999. New insights into the ATP-dependent Clp protease: *Escherichia coli* and beyond. Mol. Microbiol. **32:**449–458.
59. **Quinones, B., G. Dulla, and S. E. Lindow.** 2005. Quorum sensing regulates exopolysaccharide production, motility, and virulence in *Pseudomonas syringae*. Mol. Plant-Microbe Interact. **18:**682–693.
60. **Raaijmakers, J. M., I. De Bruijn, and M. J. D. De Kock.** 2006. Cyclic lipopeptide production by plant-associated *Pseudomonas* spp.: diversity, activity, biosynthesis, and regulation. Mol. Plant-Microbe Interact. **19:**699–710.
61. **Roongsawang, N., K. Hase, M. Haruki, T. Imanaka, M. Morikawa, and S. Kanaya.** 2003. Cloning and characterization of the gene cluster encoding arthrofactin synthetase from *Pseudomonas* sp. MIS38. Chem. Biol. **10:**869–880.
62. **Scholz-Schroeder, B. K., J. D. Soule, and D. C. Gross.** 2003. The *sypA*, *sypB*, and *sypC* synthetase genes encode twenty-two modules involved in the nonribosomal peptide synthesis of syringopeptin by *Pseudomonas syringae* pv. *syringae* B301D. Mol. Plant-Microbe Interact. **16:**271–280.
63. **Shanks, R. M., N. C. Caiazza, S. M. Hinsa, C. M. Toutain, and G. A. O'Toole.** 2006. *Saccharomyces cerevisiae*-based molecular tool kit for manipulation of genes from gram-negative bacteria. Appl. Environ. Microbiol. **72:**5027–5036.
64. **Tran, H., A. Ficke, T. Asiimwe, M. Hofte, and J. M. Raaijmakers.** 2007. Role of the cyclic lipopeptide massetolide A in biological control of *Phytophthora infestans* and in colonization of tomato plants by *Pseudomonas fluorescens*. New Phytol. **175:**731–742.
65. **Wang, C., M. Li, D. Dong, J. Wang, J. Ren, M. Otto, and Q. Gao.** 2007. Role of ClpP in biofilm formation and virulence of *Staphylococcus epidermidis*. Microbes Infect. **9:**1376–1383.
66. **Wang, N., S. E. Lu, A. R. Records, and D. C. Gross.** 2006. Characterization of the transcriptional activators SalA and SyrF, which are required for syringomycin and syringopeptin production by *Pseudomonas syringae* pv. syringae. J. Bacteriol. **188:**3290–3298.
67. **Windgassen, M., A. Urban, and K. E. Jaeger.** 2000. Rapid gene inactivation in *Pseudomonas aeruginosa*. FEMS Microbiol. Lett. **193:**201–205.
68. **Wong, P., and W. A. Houry.** 2004. Chaperone networks in bacteria: analysis of protein homeostasis in minimal cells. J. Struct. Biol. **146:**79–89.
69. **Yu, A. Y., and W. A. Houry.** 2007. ClpP: a distinctive family of cylindrical energy-dependent serine proteases. FEBS Lett. **581:**3749–3757.
70. **Zhang, J. H., N. B. Quigley, and D. C. Gross.** 1995. Analysis of the *syrB* and *syrC* genes of *Pseudomonas syringae* pv. syringae indicates that syringomycin is synthesized by a thiotemplate mechanism. J. Bacteriol. **177:**4009–4020.
71. **Zhou, Y., W. Zhu, P. S. Bellur, D. Rewinkel, and D. F. Becker.** 2008. Direct linking of metabolism and gene expression in the proline utilization A protein from *Escherichia coli*. Amino Acids **35:**711–718.