Inhibition of Quorum Sensing by a *Pseudomonas aeruginosa* *dksA* Homologue

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The *Pseudomonas aeruginosa* *las* (*lasR-lasI*) and *rhl* (*rhlR-rhlI*) quorum-sensing systems regulate the expression of several virulence factors, including elastase and rhamnolipid. *P. aeruginosa* strain PR1-E4 is a *lasR* deletion mutant that contains a second, undefined mutation which allows production of elastase and rhamnolipid despite a nonfunctional *las* system. We have previously shown that this strain accomplishes this by increasing the expression of the autoinducer synthase gene *rhlI*. In this report, we show that the elastolytic phenotype of mutant PR1-E4 can be complemented with a *P. aeruginosa* homologue of the *Escherichia coli dnaK* mutation suppressor gene *dksA*. When supplied in *trans* on a multicopy plasmid, this gene completely suppressed elastase production by mutant PR1-E4. Cloning and Northern blot analysis revealed that *dksA* was neither mutated nor less transcribed in mutant PR1-E4. When overexpressed, *dksA* also reduced rhamnolipid production by both mutant PR1-E4 and the wild type, PAO1. Using Northern blot analysis and *lacZ* reporter fusions, we show that *dksA* inhibits *rhlI*, *rhlAB*, and *lasB* transcription. Exogenous *N*-butyryl-ʟ-homoserine lactone overcame the reduced expression of *rhlI* and restored *rhlAB* and *lasB* expression, as well as elastase production. Our results suggest that the overproduction of the *P. aeruginosa* DksA homologue inhibits quorum-sensing-dependent virulence factor production by downregulating the transcription of the autoinducer synthase gene *rhlI*.

Pseudomonas aeruginosa is a major opportunistic human pathogen. In *P. aeruginosa* the quorum-sensing circuitry, composed of the *las* and the *rhl* quorum-sensing systems, regulates the expression of numerous genes, including *lasB* (elastase) and *rhlAB* (rhamnosyltransferase, required for rhamnolipid production) (35). The transcriptional activator LasR and the autoinducer molecule 3-oxo-C_{12}-HSL [*N*-(3-oxododecanoyl)-ʟ-homoserine lactone] constitute the *las* quorum-sensing system (21, 23). Similarly, the transcriptional activator RhlR and the autoinducer molecule C_4-HSL (*N*-butyryl-ʟ-homoserine lactone) constitute the *rhl* system (18, 24). The *lasI* and the *rhlI* genes encode the autoinducer synthases that synthesize the autoinducer molecules 3-oxo-C_{12}-HSL and C_4-HSL, respectively. In cell-to-cell signaling, or quorum sensing, the concentration of the autoinducer molecule increases with bacterial cell density until a threshold concentration is reached. At this point the autoinducer binds to its corresponding transcriptional activator. The autoinducer-protein complex then activates the transcription of specific target genes (7, 10). The *las* and *rhl* quorum-sensing systems interact with each other, as the complex 3-oxo-C_{12}-HSL–LasR activates *rhlR* transcription (14, 26), and the complex C_4-HSL–RhlR is necessary for optimal expression of *lasB* (3). *P. aeruginosa* strain PAO-R1 is a *lasR* deletion mutant (8) which is unable to produce elastase and rhamnolipid (36) and is significantly less virulent than the parent wild-type strain PAO1 (27, 33). We have previously described a strain PAO-R1-derived mutant, PR1-E4, which produces elastase and rhamnolipid despite the absence of a functional *las* quorum-sensing system (36). The precise site of the mutation in strain PR1-E4 is unknown; however, because of the extensive deletion in the *lasR* gene, a simple reversion is impossible. An increased expression of the autoinducer synthase encoding gene *rhlI* seems to compensate for the loss of the *las* quorum-sensing system in this strain.

To further characterize mutant PR1-E4, we complemented this strain with a wild-type *P. aeruginosa* gene bank and screened transformants for loss of elastase production. A complementing gene was identified and found to be a homologue of the *Escherichia coli* multicopy mutation suppressor gene *dksA* (13). In *trans*, *dksA* suppressed the elastase production of mutant PR1-E4 and reduced the rhamnolipid production of both PR1-E4 and PAO1. We demonstrate that overexpression of *dksA* inhibits the expression of the autoinducer synthase gene *rhlI*, leading to a secondary reduction in the production of quorum-sensing-dependent virulence factors.

MATERIALS AND METHODS

Media and culture conditions. Cultures were grown, with the appropriate antibiotics, at 37°C with shaking. PTSB medium (20) was used for *P. aeruginosa*

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TABLE 1. Strains and plasmids used

Strain or plasmid	Relevant genotype or phenotype	Reference or source
E. coli DH5α	F′/*endA1 hsdR17 supE44 thi-1 recA1 gyrA relA1* Δ(*lacZYA-argF*)*U169 deoR* [φ80 *dlacZ*ΔM15 *recA1*]	40
P. aeruginosa		
PAO1	Wild-type, elastolytic, prototroph	12
PAO-R1	Δ*lasR*::Tc^r, nonelastolytic derivative of strain PAO1	8
PR1-E4	Δ*lasR*::Tc^r, casein incubation-induced elastolytic mutant of strain PAO-R1	36
PDO100	Δ*rhlI*::Tn*501-2* derivative of PAO1	3
Plasmids		
pBluescript II SK$^+$	General-purpose cloning vector, *ori* (ColE1), Ap^r	Stratagene
pUC18, pUC19	General-purpose cloning vectors, *ori* (ColE1)	Gibco
pUCP18, pUCP19, pUCP24	*E. coli-P. aeruginosa* shuttle vectors	30
pRL1500	pUCP19 containing *rhlA* on a 1.5-kb *Bgl*I-*Nco*I fragment from strain PG201	18
pPAO-2	pUC19 containing a 280-bp *Sau*3A fragment of *pilA* from strain PAO1	29
pJPP41	pBluescript II SK$^+$ containing *rhlI* on a 950-bp *Bgl*II-*Sph*I fragment from strain PAO1	25
pRB1801	pUC18 containing a 1.75-kb *Eco*RI-*Sma*I fragment of *lasB* from strain PAO1	2
GB24	*P. aeruginosa* gene bank: partial *Sau*3A digest of strain PAO1 DNA inserted into the *Bam*HI site of pUCP24	U. Ochsner
pVD99.0	pUCP24 containing a 2.5-kb *Sau*3A genome fragment from strain PAO1	This study
pVD99.1	pUCP24 containing a 1.98-kb *Eco*RI fragment from pVD99.0	This study
pVD99.3	pUCP24 containing *dksA* on a 960-bp *Sma*I fragment from pVD99.0	This study
pVD99.4	pUCP24 containing a 523-bp *Eco*RI fragment from pVD99.0	This study
pVD99.5	pUCP24 containing a 520-bp *Sal*I fragment from pVD99.0	This study
pECP60	pSW205 containing a *rhlA′-lacZ* translational fusion	26
pDECP60	pECP60 containing *dksA* on a 960-bp *Sma*I fragment from pVD99.3	This study
pLPR1	pLP170 containing a *rhlI′-lacZ* transcriptional fusion	36
pVD	pLPRI containing *dksA* on a 960-*Sma*I fragment from pVD99.3	This study
pPBL25	pUCP18 containing *lasB′-lacZ* on a 3-kb *Eco*0109 fragment from pKDT37 (22)	This study
pPBLD26	pPBL25 containing *dksA* on a 960-bp *Sma*I fragment from pVD99.3	This study

cultures, and LB medium (28) was used for *E. coli* cultures. The defined, nitrogen-limited Guerra-Santos (GS) medium used for rhamnolipid determinations (11) was supplemented with 20% glycerol instead of 0.1 M glucose (17). Elastin-agar plates contained 0.5% elastin and 0.8% nutrient broth (20). For β-galactosidase (β-Gal) determinations, A medium (23) supplemented with 0.05% yeast extract, 0.4% glucose, and 1 mM MgSO$_4$ was used. Antibiotics were used at the following concentrations when required: for *P. aeruginosa*, carbenicillin (200 μg/ml), tetracycline (100 or 50 μg/ml in solid or liquid medium, respectively), and gentamicin (100 μg/ml); for *E. coli*, ampicillin (100 μg/ml), tetracycline (20 μg/ml), and gentamicin (15 μg/ml). 3-oxo-C$_{12}$-HSL and C$_4$-HSL autoinducers were synthesized previously (22, 24).

Bacterial strains and plasmids. Bacterial strains and plasmids are listed in Table 1. The *P. aeruginosa* gene bank, GB24, was kindly provided by U. Ochsner. This 95% complete gene bank contains a *Sau*3A partial digestion of the *P. aeruginosa* wild-type strain PAO1 genome, cloned into the *Bam*HI site of the multicopy vector plasmid pUCP24 (30). It is composed of approximately 5,000 independent DNA fragments with a median size of 3.3 kb (2 to 7 kb) (U. Ochsner, personal communication). Plasmid pVD was constructed by cloning a *dksA*-containing 960-bp *Sma*I fragment from pVD99.3 into the Klenow-repaired *Xho*I site of pLPRI (36). Plasmid pDECP60 was constructed by cloning the 960-bp *Sma*I fragment of pVD99.3 into the *Sma*I site of pECP60 (26). The orientation of *dksA* in pDECP60 is opposite that of the adjacent *rhlA′-lacZ* fusion. Plasmid pPBL25 was constructed by ligating a Klenow enzyme-treated 3-kb *Eco*0109 *lasB′-lacZ*-containing fragment obtained from pKDT37 (22) into the *Sma*I site of pUCP18. Plasmid pPBLD26 was obtained by ligating a 960-bp *Sma*I *dksA*-containing fragment of pVD99.3 into the Klenow enzyme-repaired *Xba*I site of pPBL25.

DNA techniques. We used standard techniques for DNA manipulations (28). Restriction endonucleases and DNA-modifying enzymes were purchased from Gibco/BRL or New England Biolabs. Plasmids were introduced into *E. coli* by transformation (28) and into *P. aeruginosa* by electroporation (31).

Cloning of the *dksA* gene from mutant PR1-E4. A *Bam*HI-*Not*I digest of mutant PR1-E4 DNA was separated by gel electrophoresis and screened for the presence of the *dksA* gene, using a ^{32}P-radiolabeled 350-bp *Sma*I-*Sph*I probe

obtained from pVD99.3. A 2,500-bp *Bam*HI-*Not*I chromosomal DNA fragment of mutant PR1-E4 containing the *dksA* gene was cloned into the *Bam*HI-*Not*I site of pBluescript SKII$^+$. Colony blot hybridization on nylon membranes was performed using a 350-bp *Sma*I-*Sph*I probe for *dksA* obtained from pVD99.3, using *E. coli* strain DH5α as a host. The *dksA* gene recovered from strain PR1-E4 was sequenced using a Li-Cor 4000L electrophoresis apparatus and Ladderman dideoxy sequencing kit.

RNA preparation and Northern blot analysis. Total cellular RNA was prepared as previously described (5). In brief, *P. aeruginosa* cells were grown at 37°C in PTSB medium to stationary phase (optical density at 660 nm [OD$_{660}$] = 2.0 ± 0.2), when the *las* and *rhl* systems are active, collected, and lysed in 3.5% sodium dodecyl sulfate. RNA was recovered after centrifugation of cell lysates on a 5.7 M CsCl cushion and further purified by two phenol-chloroform extractions. Northern blot analyses were performed following standard protocols. After electrophoresis on a 2.2 M formaldehyde–1.2% agarose gel, RNA was transferred to Hybond nylon membranes and hybridized according to the Amersham protocol to ^{32}P-labeled double-stranded DNA probes. DNA probes were obtained as follows. A 560-bp *Bam*HI internal fragment of *rhlA* was obtained from pRL1500 (18), a 630-bp *Sal*I internal fragment of *lasB* was obtained from pRB1801 (2), and a 250-bp *Kpn*I-*Eco*RI internal fragment of *rhlI* was obtained from pJPP41 (25). A 350-bp *Sma*I-*Sph*I fragment from pVD99.3 was used as a *dksA* probe. For Northern analysis of *dksA* expression, total cellular RNA was obtained from PTSB cultures of strains PAO1, PAO-R1, and PR1-E4 in the early exponential phase of growth (OD$_{660}$ = 0.8) and late stationary phase of growth (OD$_{660}$ = 2.0). ^{32}P labeling was performed by nick translation, and probes were separated from unincorporated nucleotides using NucTrap push columns (Stratagene). RNA experiments were performed twice with independent RNA preparations. To ascertain that the RNA of each strain was intact and loaded equally, we used a probe for the *P. aeruginosa pilA* gene (a 280-bp *Eco*RI-*Bam*HI internal fragment of *pilA* from pPAO-2 [29]), which encodes the structural subunit of pilin. Previous studies have shown that *pilA* mRNA is not affected by the *lasR* deletion in strain PAO-R1 (9).

Elastase and rhamnolipid production assays. Elastase production was measured by elastin Congo red assays as previously described (25). Rhamnolipid
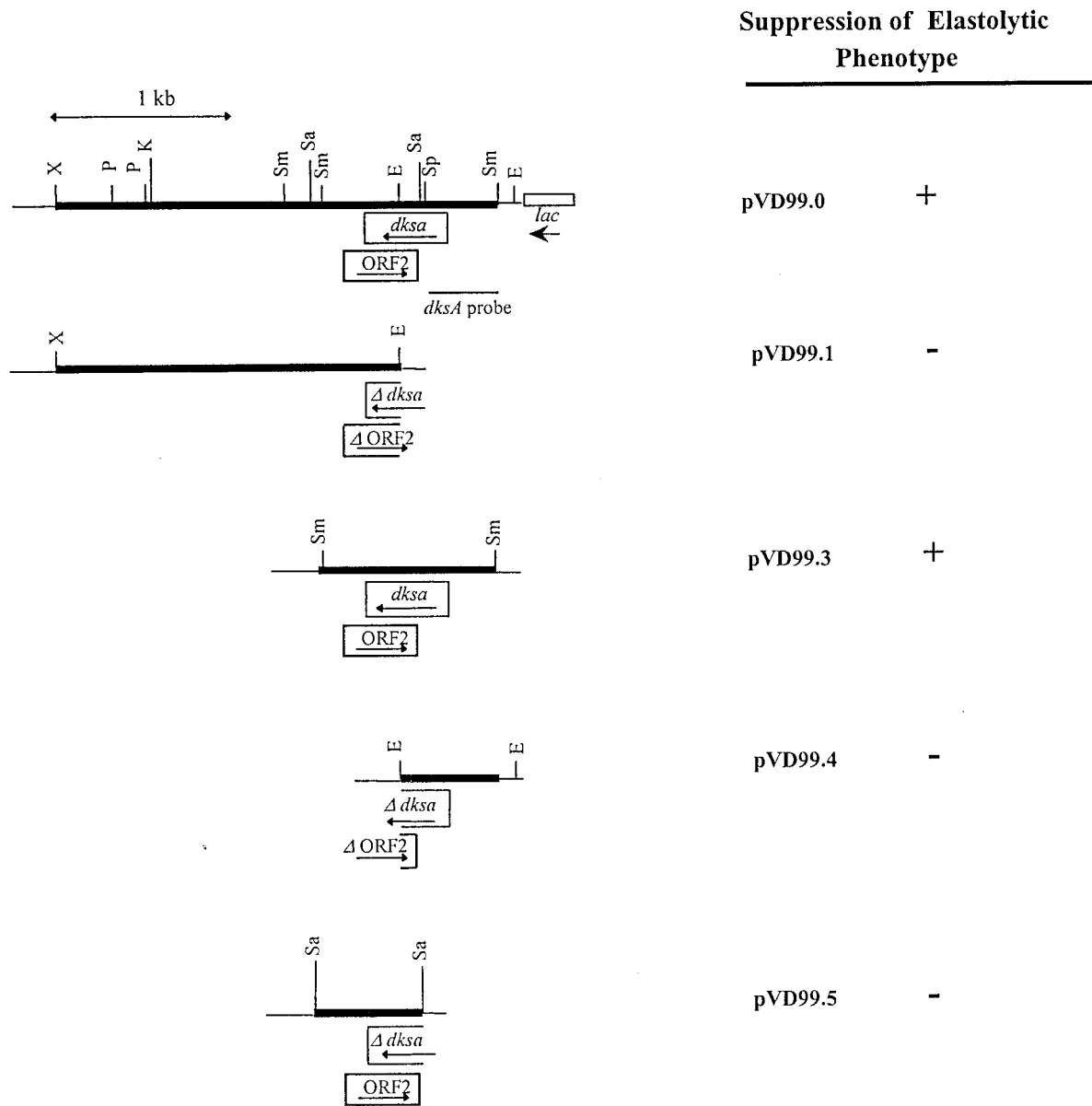
FIG. 1. Restriction endonuclease map and subcloning strategy for plasmid pVD99.0. Closed and open boxes represent complete and truncated genes, respectively. Important restriction endonuclease sites, the *dksA* probe, and the position and orientation of the *lac* promoter are indicated. The elastolytic phenotype suppressor activity of the different subclones was determined on elastin-agar plates. Abbreviations: E, *Eco*RI; K, *Kpn*I; P, *Pst*I; Sa, *Sal*I; Sm, *Sma*I; Sp, *Sph*I; X, *Xba*I.

concentration in *P. aeruginosa* culture fluids was determined as previously described by orcinol assays (25).

β-Gal activity assays. β-Gal activity was measured as previously described (16), with the following modifications. *P. aeruginosa* cultures were grown for 18 h at 37°C with vigorous shaking in PTSB medium supplemented with carbenicillin (200 μg/ml) and subcultured into the same medium to a starting OD_{660} of 0.15. Cultures were assayed for β-Gal activity at regular intervals during growth. Cells were washed twice and resuspended in A medium prior to β-Gal activity determinations. All experiments were done in triplicates and performed at least two times.

Determination of C_4-HSL concentrations. Culture supernatants were extracted with ethyl acetate, and C_4-HSL concentrations were determined in a previously described (25) bioassay using PAO-JP2(pECP61.5).

Nucleotide sequence accession number. The sequence of *dksA* is accessible in GenBank (accession number AF062653).

RESULTS

Isolation of a *P. aeruginosa dksA* homologue that suppresses elastase production of mutant PR1-E4. We wanted to determine whether the mutation restoring elastase production of the *lasR*-deficient strain PR1-E4 could be complemented by a wild-type gene or suppressed by overexpression of a different gene. We therefore electroporated the wild-type *P. aeruginosa* gene bank GB24 into the elastase-producing mutant PR1-E4; 6,700 isolated clones were screened on elastin-agar plates for the absence of elastase production. We found one non-elastase-producing clone, PR1-E4(pVD99.0). Isolation of pVD99.0 re-
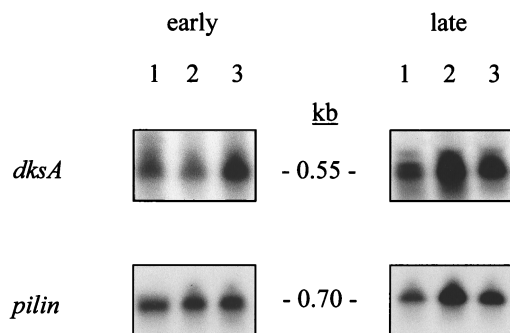
early late

1 2 3   1 2 3

dksA   kb

- 0.55 -

pilin   - 0.70 -

FIG. 2. *dksA* transcription in strains PAO1, PAOR-1, and PR1-E4. Total cellular RNA (12.5 μg) from early (exponential)- and late (stationary)-phase cultures were hybridized to a ^{32}P-labeled *dksA* probe. A *pilA* probe was used to ascertain equal loading and transfer of RNA. Lanes: 1, strain PAO1; 2, strain PAO-R1; 3, strain PR1-E4. Molecular sizes are indicated in the center.

vealed a 2.5-kb fragment from the wild-type PAO1 genome (Fig. 1). Neither plasmid pVD99.1, which contained a 1.98-kb fragment of pVD99.0, nor plasmid pVD99.4, which contained the other 523-bp fragment of pVD99.0, suppressed elastase production by mutant PR1-E4. Plasmid pVD99.3 was obtained during subcloning of the 2.5-kb fragment by ligating a 960-bp *Sma*I fragment of pVD99.0 into the *Sma*I site of pUCP24. This plasmid, when electroporated into PR1-E4, still completely suppressed its elastase production. Sequencing of the 960-bp insert of pVD99.3 revealed two complete putative open reading frames (ORFs), one from bases 292 to 735 (ORF1) and the second, in the opposite orientation, from bases 805 to 464 (ORF2). Subcloning a 520-bp *Sal*I fragment of pVD99.0 created pVD99.5, which contains an intact ORF2 but lacks the first 153 bp of ORF1. Plasmid pVD99.5 did not suppress elastase production by mutant PR1-E4. This result, together with the finding that ORF2 exhibits codon usage unusual (38) for *P. aeruginosa*, makes it unlikely that ORF2 is responsible for the suppression of elastase production by mutant PR1-E4. In contrast, the 443-bp ORF1 had a codon usage typical for *P. aeruginosa*, and accordingly the Genetics Computer Group program CODON PREFERENCE (38) indicated a high coding probability throughout its entire sequence. A search of the GenBank-European Molecular Biology Laboratory sequence database revealed amino acid homology between the gene product of this ORF and the *E. coli dnaK* mutation suppressor protein DksA (76% identity and 87% similarity) (13). We had therefore identified a new *P. aeruginosa* gene, which was homologous to the *E. coli* gene *dksA* and suppressed elastase production by mutant PR1-E4.

Cloning and transcription levels of the *dksA* gene from mutant PR1-E4. To examine whether strain PR1-E4 bears a mutated *dksA* gene that had been complemented by the wild-type *dksA* gene on plasmid pVD99.3, we cloned the *dksA* gene from mutant PR1-E4. The *dksA* gene from mutant PR1-E4 was recovered and sequenced, and its nucleotide sequence was compared with that of the wild-type *P. aeruginosa dksA* gene as described in Materials and Methods. We found no mutation in either the *dksA* ORF or the 350 bp of DNA upstream from *dksA* (data not shown). To determine whether a reduction of *dksA* transcription restored the elastase production of mutant

PR1-E4, we also examined the expression of *dksA* in both exponential (early) and stationary (late) growth phases. Using Northern blot analysis, we could not detect a difference in *dksA* mRNA levels between the wild-type strain PAO1 and mutants PAO-R1 and PR1-E4 (Fig. 2). (Note that the difference seen for *dksA* between lanes 1 and 2 for late RNA is identical to that seen with the pilin probe and is therefore presumed to be caused by loading differences.) Consequently, the *dskA* gene in strain PR1-E4 does not contain a mutation, nor is its expression downregulated. The suppression of elastase production of mutant PR1-E4 by *dksA* supplied in *trans* is therefore a phe-
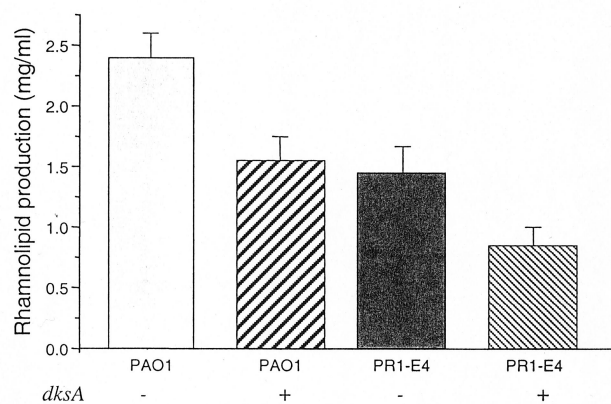
A
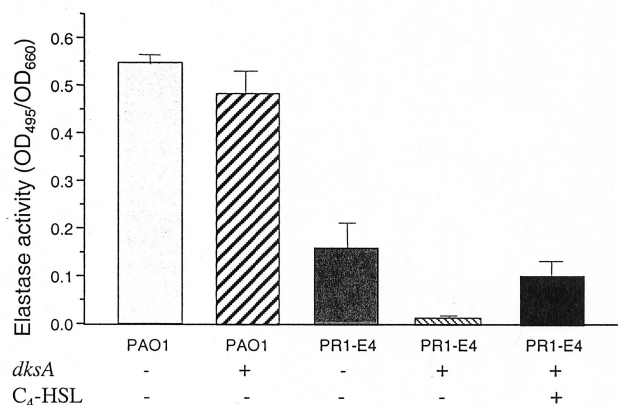
B

FIG. 3. Effect of *dksA* on elastase and rhamnolipid production in PAO1 and PR1-E4. Elastase (A) and rhamnolipid (B) production in wild-type strain PAO1 and mutant PR1-E4 were measured by the elastin Congo red and orcinol assays, respectively, in the presence of either the *dksA*-containing plasmid pVD99.3 (*dksA* +), or the vector control pUCP24 (*dksA* −). Supernatants were obtained from stationary-phase cultures from cells growing for 20 h in PTSB medium (OD_{660} = 2.0 ± 0.2) for determination of elastase production in the presence or absence of 10 μM C_4-HSL and from cells growing for 72 h in modified GS medium (OD_{660} = 3.2 ± 0.4) for determination of rhamnolipid production. All experiments were performed in triplicate and repeated at least two times.
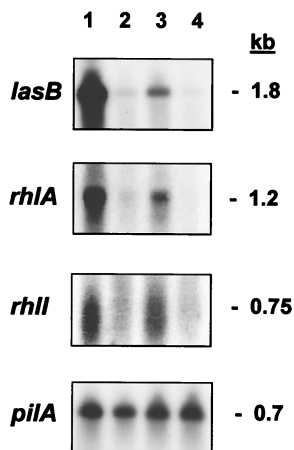
FIG. 4. Northern blot analysis of *lasB, rhlA,* and *rhlI* mRNA. Total cellular RNA (10 to 15 μg) was hybridized to ^{32}P-labeled *lasB, rhlA,* and *rhlI* probes. Equal loading and transfer of RNA were verified using a *pilA* probe. Lanes: 1, strain PAO1(pUCP24); 2, strain PAO-R1(pUCP24); 3, strain PR1-E4(pUCP24); 4, strain PR1-E4(pVD99.3). pVD99.3 contains the *P. aeruginosa dksA* gene cloned in the pUCP24 vector plasmid. Molecular sizes are indicated to the right.

notypic complementation, similar to the mutation suppressor effect of the *E. coli dksA* gene on chaperone mutations (13).

dksA affects the production of virulence factors in both PR1-E4 and PAO1. To quantify the inhibition of elastase and rhamnolipid production by *dksA*, we performed elastin Congo red and orcinol assays in the presence of the *dksA*-containing plasmid pVD99.3 or the vector control pUCP24 (see Materials and Methods). *dksA* completely abolished the elastase production of mutant PR1-E4 (Fig. 3A) and reduced its rhamnolipid production by 40% (Fig. 3B). We also wondered whether multiple copies of *dksA* could inhibit the elastase and/or rhamnolipid production of the wild-type strain PAO1. *dksA* only slightly reduced elastase production by strain PAO1 (Fig. 3A). However, the production of rhamnolipid was reduced by 35% when *dksA* was overexpressed in PAO1 (Fig. 3B). Therefore, *dksA* suppressed both elastase and rhamnolipid production in the absence of a functional *las* quorum-sensing system but mainly affected the production of rhamnolipid in a wild-type background.

dksA inhibits the expression of genes belonging to the quorum-sensing circuitry. Expression of the elastase gene (*lasB*), the rhamnosyltransferase genes (*rhlAB*), and the *rhl* autoinducer synthase gene (*rhlI*) is partially restored in mutant PR1-E4 compared to its parent strain, PAO-R1 (36). To determine whether overexpression of *dksA* affects the transcription of these genes, we performed Northern blot analysis of strains PAO1, PAO-R1, PR1-E4, and PR1-E4(pVD99.3), using *lasB, rhlA,* and *rhlI* probes, as described in Materials and Methods. *lasB, rhlA,* and *rhlI* mRNAs gave intense signals in the wild-type strain PAO1, contrasting with mutant PAO-R1, in which the mRNAs of these three genes were barely visible (Fig. 4, lanes 1 and 2, respectively). All three mRNAs were detected in mutant PR1-E4 (Fig. 4, lane 3). These results confirm our previous report that *lasB, rhlA,* and *rhlI* are barely transcribed in the absence of LasR in mutant PAO-R1 and that their expression is partially restored in mutant PR1-E4 (36).

However, when *dksA* was supplied in *trans* on pVD99.3 to mutant PR1-E4, neither *lasB, rhlA,* nor *rhlI* message was detected (Fig. 4, lane 4). In contrast, the *pilA* message, used as a control, was not affected by the presence of *dksA*.

To confirm the suppressive effect of *dksA* on *rhlI* mRNA levels, we measured the expression of *rhlI'-lacZ* in the presence and absence of multiple copies of *dksA*, using plasmids pLPRI (*rhlI'-lacZ*) and pVD (*rhlI'-lacZ dksA*). The expression of *rhlI'-lacZ* in mutant PR1-E4 was reduced to 17% in the presence of *dksA* [PR1-E4(pLPRI), 18,201 ± 909 Miller units; PR1-E4(pVD), 3,135 ± 586 Miller units; measured at an OD_{660} of 1.8]. As PR1-E4 is an undefined mutant, we wanted to confirm the inhibition of *rhlI* expression in a defined genetic background. We therefore compared the expression of *rhlI'-lacZ* from plasmid pLPRI and pVD in the defined Δ*lasR* mutant PAO-R1 and in the wild type, PAO1. *rhlI* is barely transcribed in mutant PAO-R1 because of a nonfunctional *las* quorum-sensing system (36). To compensate for this defect, we measured the expression of *rhlI* in mutant PAO-R1 in the presence of 10 μM C_4-HSL. In both strains, the presence of *dksA* reduced the expression of *rhlI'-lacZ* (Fig. 5A). Therefore, *dksA* interferes with the transcription of *rhlI* not only in the undefined mutant PR1-E4 but also in the Δ*lasR* mutant PAO-R1, despite constant C_4-HSL levels, and in a wild-type background.

The effect of dksA on rhlAB and lasB transcription is indirect. *dksA* could primarily affect the expression of *rhlI*, leading to an indirect reduction of *rhlAB* and *lasB* expression. However, it could also directly inhibit the expression of these three genes. To address this question, we cloned *dksA* under its own promoter on plasmid pECP60, which contains a *rhlA'-lacZ* reporter fusion, to obtain plasmid pDECP60 (see Materials and Methods). In wild-type strain PAO1, the presence of *dksA* in *trans* reduced the expression of *rhlA'-lacZ* (Fig. 5B). These results confirm those of the orcinol assays that showed reduced rhamnolipid production in the presence of *dksA* (Fig. 3B). To determine whether the inhibition of *rhlI* transcription by *dksA* is responsible for this effect, we determined the effect of *dksA* on *rhlAB* expression in the defined *rhlI* mutant PDO100 (3). As *rhlAB* is normally not transcribed in this mutant, these experiments had to be performed in the presence of 10 μM exogenous C_4-HSL (26). These experimental conditions allow distinction of a direct effect of *dksA* on *rhlAB* expression from an indirect effect, due primarily to an inhibition of *rhlI* expression. Indeed, if *rhlAB* transcription is not directly affected by *dksA*, then its expression should not be diminished in this strain, as the source of the C_4-HSL autoinducer is exogenous. As shown in Fig. 5B, *rhlAB* was expressed similarly from plasmids pECP60 (*rhlA'-lacZ*) and pDECP60 (*rhlA'-lacZ dksA*), suggesting that *dksA* does not directly affect *rhlAB* expression. To demonstrate that the inhibitory effect of *dksA* can be overcome by exogenous C_4-HSL, we also determined the expression of *rhlAB* in the presence of exogenous C_4-HSL in PAO1 and PR1-E4 (Table 2). The addition of 10 μM exogenous C_4-HSL restored the expression of *rhlAB* in both strains PAO1(pDECP60) and PR1-E4(pDECP60). These results show that exogenous C_4-HSL can compensate for the inhibition of *rhlI* expression by *dksA* and confirm the hypothesis that *dksA* does not affect *rhlAB* expression directly. The expression of *rhlR* is reduced to 18% of the wild-type level in both strains PAO1 and PR1-E4 (36) and is not further reduced in PR1-E4 by the addition of *dksA* in *trans* (assayed by
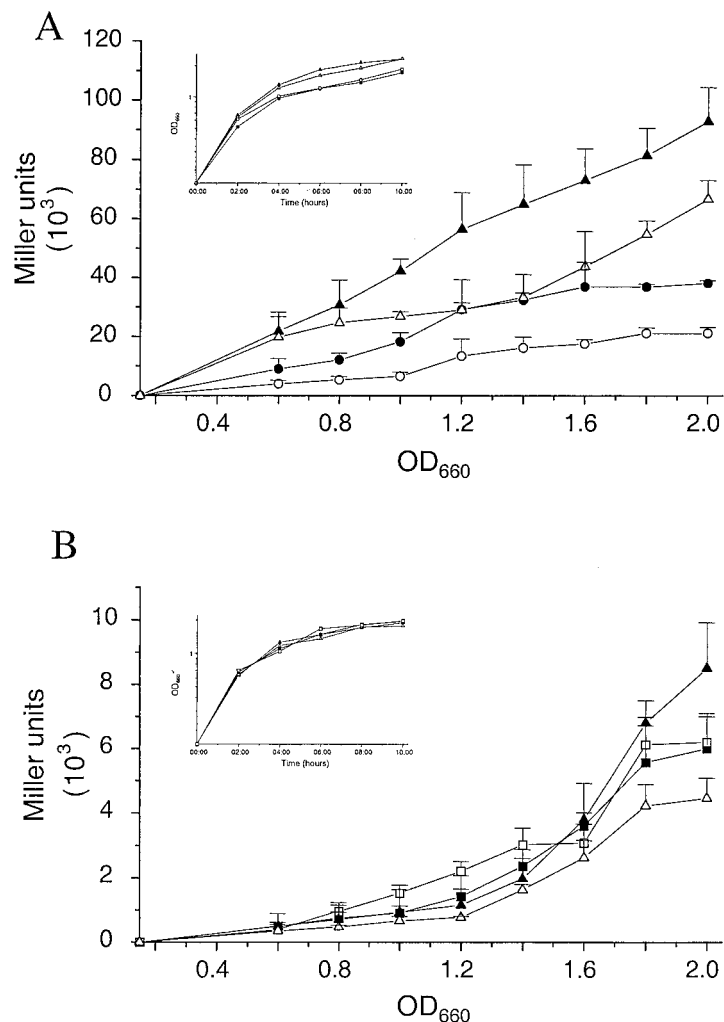
FIG. 5. Effect of *dksA* on the expression of *rhlI* and *rhlAB*. (A) Expression of *rhlI* was monitored by β-Gal determinations during growth of the Δ*lasR* mutant PAO-R1 in the presence of 10 μM C$_4$-HSL (circles) and the wild-type strain PAO1 (triangles), using either plasmid pLPRI (*rhlI'-lacZ*; solid symbols) or plasmid pVD (*rhlI'-lacZ dksA*; open symbols). (B) Expression of *rhlAB* was determined during the growth of PAO1 (triangles) and the Δ*rhlI* mutant PDO100 in the presence of 10 μM C$_4$-HSL (squares) by β-Gal assays, using either plasmid pECP60 (*rhlA'-lacZ*; solid symbols) or plasmid pDECP60 (*rhlA'-lacZ dksA*; open symbols). Growth of the strains was not influenced by the presence of either pVD or pDECP60, as shown in the insets.

Northern blotting [data not shown]. Apparently the concentration of RhlR is high enough to support, in the presence of an adequate C$_4$-HSL concentration, the level of expression of *rhlAB* observed in mutant PR1-E4.

To determine whether *dksA* inhibits elastase production directly by inhibition of *lasB* expression, we used a similar strategy and cloned *dksA* under its own promoter on plasmid pPBL25, which contains a *lasB'-lacZ* reporter fusion to obtain plasmid pPBLD26 (see Materials and Methods). To avoid the interference of a reduced expression of *rhlI*, these experiments were also first performed in the *rhlI* mutant PDO100. Similar to the case for *rhlAB, lasB* expression is reduced in mutant PDO100 due to the lack of C$_4$-HSL production (3). The transcription of *lasB* in mutant PDO100 was therefore measured in the presence of 10 μM exogenous C$_4$-HSL. In these experimental conditions, the expression of *lasB* was not suppressed by *dksA* [PDO100(pPBL25), 16,640 ± 885 Miller units;

PDO100(pPBLD26), 15,347 ± 697 Miller units; mean of five independent experiments ± standard errors, measured at an OD$_{660}$ of 2.0). To confirm these results, we determined the expression of *lasB* in mutant PR1-E4, using the reporter fusions pPBL25 and pPBLD26, in the absence and the presence of exogenous C$_4$-HSL (Table 2). As expected, the expression of *lasB* was reduced in the presence of *dksA*. This inhibition was overcome by the addition of 10 μM exogenous C$_4$-HSL. These results suggest that *dksA* does not inhibit *lasB* expression directly but reduces the production of the C$_4$-HSL autoinducer, which leads indirectly to a reduction of *las B* and *rhlAB* expression. To support this hypothesis, we measured the C$_4$-HSL concentrations in the culture supernatants obtained from the same experiments (Table 3). Not surprisingly, mutant PR1-E4 produced less C$_4$-HSL autoinducer than PAO1, confirming the partial restoration of *rhlI* expression observed previously in this strain (36). The concentration of C$_4$-HSL in supernatants of

TABLE 2. Restoration of *rhlA* and *lasB* expression by exogenous C_4-HSL[a]

Fusion	Enzyme activity (Miller units)					
	PAO1			PR1-E4		
	−*dksA*	+*dksA*	+*dksA* + 10 μM C_4-HSL	−*dksA*	+*dksA*	+*dksA* + 10 μM C_4-HSL
rhlA'-lacZ	6,830 ± 625	4,200 ± 600	7,350 ± 650	390 ± 45	54 ± 8	372 ± 32
lasB'-lacZ	ND	ND	ND	379 ± 40	110 ± 10	594 ± 42

[a] Cultures of PAO1 and mutant PR1-E4 carrying the indicated reporter fusions were grown for 8 h (OD_{660} = 1.8 ± 0.1), and β-Gal activity was assayed. Data are the mean ± 1 standard deviation of at least two separate experiments performed in triplicate. −*dksA*, pECP60 (*rhlA'-lacZ*) or pPBL25 (*lasB'-lacZ*); +*dksA*, pDECP60 (*rhlA'-lacZ dksA*) or pPBLD26 (*lasB'-lacZ dksA*). ND, not determined.

mutant PAO-R1 was at the limit of detection of our bioassay (the sensitivity of our bioassay was 0.04 μM). In the presence of *dksA*, no C_4-HSL was detected by our bioassay in supernatants of strain PR1-E4. Therefore, *dksA* in *trans* severely reduced the production of the C_4-HSL autoinducer, as expected from the inhibition of *rhlI* expression by *dksA*. To finally confirm that exogenous C_4-HSL can overcome the inhibitory effect of *dksA*, we also measured the production of elastase by strain PR1-E4 in the presence of *dksA* and exogenous C_4-HSL. As shown in Fig. 3A, the addition of 10 μM C_4-HSL in elastin Congo red assays restored the production of elastase by strain PR1-E4(pVD99.3). It therefore appears that *dksA* does not inhibit the expression of *lasB* and *rhlAB* directly but affects both elastase and rhamnolipid production indirectly by the inhibition of *rhlI* transcription, leading to reduced C_4-HSL levels.

DISCUSSION

An upregulation of *rhlI* expression has been suggested to be responsible for the partial restoration of elastase and rhamnolipid production by the *P. aeruginosa* starvation mutant PR1-E4 (36). In the present study, we have complemented mutant PR1-E4 with a wild-type *P. aeruginosa* gene bank carried on a multicopy plasmid. By screening for the loss of elastase production, we isolated a *P. aeruginosa* homologue to the *E. coli dnaK* multicopy suppressor gene *dksA*. We have shown that *dksA* is neither mutated nor expressed at a reduced level in mutant PR1-E4. *dksA* in *trans* not only abolishes elastase production in mutant PR1-E4 but also downregulates rhamnolipid production in mutant PR1-E4 and in the wild type, PAO1. Using Northern blot analysis and plasmids carrying both *dksA* and *rhlI'-lacZ, rhlA'-lacZ*, and *lasB'-lacZ* reporter fusions, we have shown that *dksA* reduces the expression of these three genes, as well as the production of the C_4-HSL

TABLE 3. C_4-HSL concentrations in cultures of wild-type PAO1 and mutant PR1-E4 carrying or not carrying *dksA*

Culture density (OD_{660})	C_4-HSL concn (μM)[a]		
	PAO1/pPBL25	PRI-E4 carrying:	
		pPBL25	pPBLD26
1.4	5.1 ± 0.2	0.4 ± 0.1	<0.04
1.8	6.3 ± 0.4	1.2 ± 0.2	<0.04

[a] Mean ± 1 standard deviation of at least two separate experiments performed in triplicate.

autoinducer. The addition of exogenous C_4-HSL autoinducer compensates for the inhibitory effect of *dksA* and restores elastase production, as well as *rhlAB* and *lasB* transcription. These results suggest that overexpression of *dksA* inhibits *rhlI* expression, leading to a secondary reduction of rhamnolipid and elastase production.

dksA homologues with strikingly conserved sequences have been isolated from *E. coli* (13), *Haemophilus influenzae* (4), *Salmonella enterica* serovar Typhimurium (34), and now *P. aeruginosa*. The cellular localization and function of DksA are still unknown. DksA shares amino acid similarity with the *E. coli* TraR protein, a putative transcriptional regulator (6). Both proteins contain a zinc finger domain ($Cx_2Cx_{17}Cx_2C$) that facilitates binding to DNA. Interestingly, the putative zinc finger motif is the region most conserved between the known DksA homologues, suggesting that these proteins might also function as gene expression regulators. In *E. coli, dksA* expressed from multicopy plasmids suppresses the temperature-sensitive phenotype associated with deletion mutations in the heat shock genes *dnaK, dnaJ*, and *grpE280n* (13) and in the *mukB* gene (41). It is also a multicopy suppressor of the conditional lethal phenotype associated with a *prc* null mutation in *E. coli* (1) and has been suggested to be involved in plasmid replication (19). The *S. enterica* serovar Typhimurium DksA homologue has been linked to virulence of this strain, as a Δ*dksA S. enterica* serovar Typhimurium mutant was impaired in the ability to colonize chickens (34). Both *E. coli* and *S. enterica* serovar Typhimurium *dksA* mutants show poor growth in minimal media and defects in glutamine-glutamate biosynthesis (13, 34). The *S. enterica* serovar Typhimurium *dksA* mutant also yielded a higher RNA amount in stationary-phase cultures than the wild type (34). For these reasons, DksA has been suggested to be involved in stress responses such as the stringent response (34). Recently DksA has also been shown to be required for optimal translation of the stationary-phase sigma factor *rpoS*, as well as for the expression of several other genes in *S. enterica* serovar Typhimurium (37). DksA might therefore regulate the expression of genes which products are required for the stabilization of proteins during the stringent response and entrance into stationary phase (34)

The relationship between *rpoS* and the *rhl* quorum-sensing system in *P. aeruginosa* is a matter of debate. Whereas previous data suggested that the transcription of *rpoS* might be regulated by the *rhl* system (14), recent experiments have suggested that RpoS might repress *rhlI*, rather than the *rhl* system influencing the expression of *rpoS* (39). Could an upregulation of RpoS, secondary to the overexpression of *dksA*, explain the

effects observed in this study? The repression of *rhlI* by RpoS manifests essentially during early exponential growth, whereas the inhibition of *rhlI* by *dksA* occurs mainly during stationary growth. Moreover, an *rpoS* mutation results in increased production of pyocanin, a secondary metabolite dependent on the *rhl* quorum-sensing system (whether this mutation also affects rhamnolipid production is unknown), but more importantly also in a 20% reduction in the production of elastase (32). This effect of RpoS on elastase production is opposite what would be needed to explain the inhibition of elastase production by *dksA*. It is therefore unlikely that the results presented in this study are due to an upregulation of RpoS alone. The overexpression of *dksA* could also affect another regulator of *rhlI* expression that has been previously suspected (14). This regulator could be the recently described *Pseudomonas* quinolone signal, which increases the expression of *rhlI* (15). This third *P. aeruginosa* cell-to-cell signal seems to acts as a link between the *las* and *rhl* quorum-sensing systems.

Our data show for the first time that DksA can interfere with quorum sensing. Further work is required to determine the physiologic function of DksA and whether it plays a significant role in the regulation of virulence factor production by *P. aeruginosa*.

ACKNOWLEDGMENTS

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