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Analysis of the *Pseudomonas aeruginosa* Elastase (*lasB*) Regulatory Region

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The enzyme elastase is an important virulence factor of the opportunistic human pathogen *Pseudomonas aeruginosa*. Previous studies have shown that expression of the *P. aeruginosa* elastase gene (*lasB*) requires both an activator protein, LasR, and an *N*-acylhomoserine lactone compound termed *Pseudomonas* autoinducer (PAI). In this study, we analyzed the *lasB* promoter region to learn more about *lasB* activation by LasR and PAI. We report that the *lasB* transcriptional start is located 141 nucleotides upstream from the *lasB* translational start. It was also discovered that the *lasB* promoter region contains two putative operator sequences (OP1 and OP2) that are similar to each other and the *Vibrio fischeri lux* operator. OP1 is located directly upstream from, and may overlap with, the *lasB* promoter region, and OP2 is centered 102 nucleotides upstream from the *lasB* transcriptional start site. To study the effects of these putative operators and other sequences upstream from the *lasB* transcriptional start site on *lasB* activation, a series of transcriptional *lasBp-lacZ* gene fusions was constructed. Data from these fusions indicate that both putative operators are involved in LasR- and PAI-mediated *lasB* activation, with OP1 being more important than OP2.

Pseudomonas aeruginosa produces a variety of extracellular enzymes contributing to its pathogenesis (22, 32, 33, 36). One of these enzymes, elastase, is well established as a *P. aeruginosa* virulence factor. *P. aeruginosa* elastase is produced during the course of clinical infections, and it contributes to the development of infections in animal models (5, 8, 14, 18, 19, 22, 23, 25, 26). This enzyme is capable of degrading or inactivating important biologic tissues and immune system components, including immunoglobulin (9, 15), serum complement factors (21, 43), α_1-proteinase inhibitor (34, 35), collagen (16), fibrin (32), and elastin (32, 52). Elastase cleaves host proteases MMP9 and MMP2 in corneal culture (50) and acts synergistically with alkaline protease to inactivate the human cytokines gamma interferon and tumor necrosis factor alpha (38).

The elastase structural gene, *lasB*, has been cloned and sequenced (3), and its expression was shown to require an intact *lasR* gene (12) and *P. aeruginosa* autoinducer (PAI) (39). PAI has been identified as *N*-(3-oxododecanoyl) homoserine lactone and is one of a family of *N*-acylhomoserine lactone compounds that, together with a regulatory protein (e.g., LasR), are involved in cell density signalling and gene activation (41). The best-characterized member of this family of quorum-sensing systems is the LuxR and *Vibrio* autoinducer (VAI) autoinduction system of *Vibrio fischeri* (11). The properties of this family of autoinducers have been recently reviewed by Fuqua et al. (11) and Swift et al. (48).

Two recent reports indicate a complexity of *lasB* gene regulation beyond the requirement of LasR and PAI (37, 42). A second activator with homology to LasR (RhlR) is required for rhamnolipid synthesis in *P. aeruginosa* (37). An *rhlR* mutant strain was shown to be deficient not only for rhamnolipid synthesis but also for elastase activity, indicating that regulation of elastase synthesis is multifactorial (37). In addition, a second *N*-acylhomoserine lactone (factor 2) is required for *lasB* gene activation when *lasR* is under the control of its own promoter (42). Factor 2 has been identified as *N*-butyrylhomoserine lactone (42). The authors suggest the possibility of *lasB* being subject to a complex circuit of regulatory events involving multiple activators and autoinducers (42). However, existing evidence indicates that LasR and PAI are sufficient for high-level expression of *lasB* (39, 41).

Herein, we identify critical elements in the *lasB* upstream region that are involved in LasR- and PAI-mediated transcriptional regulation. We report the location of the *lasB* transcriptional start site and identify a second putative operator within the *lasB* regulatory region. We also identify base pairs within the two putative *lasB* operators that are involved in LasR- and PAI-mediated transcriptional activation of *lasB*. Finally, we show that a region upstream from the *lasB* promoter and operator 1 (OP1) is also necessary for *lasB* expression.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are shown in Table 1.

Media and culture conditions. For experiments in which β-galactosidase activity was assayed as a measure of *lasB* promoter activity, *P. aeruginosa* PAO1 and PAO-R1 containing plasmids with *lasBp-lacZ* transcriptional fusions were cultured in chelated trypticase soy broth (29) containing carbenicillin (200 μg/ml) (TSDB cb200). *P. aeruginosa* cultures were grown overnight at 32°C and subcultured into fresh, prewarmed TSDB cb200 (starting inoculation at an A_{540} of 0.04) and grown for 19 h at 32°C. Growth of the cultures was monitored by measuring the A_{540}. Cultures of *Escherichia coli* MG4 containing plasmids with *lasBp-lacZ* transcriptional fusions were grown overnight at 37°C in modified A medium (A medium [31] supplemented with 0.4% glucose, 1 mM $MgSO_4$, and 0.05% yeast extract). The selective antibiotics ampicillin (100 μg/ml) and chloramphenicol (30 μg/ml) were included in media when appropriate. Secondary cultures were inoculated to an A_{600} of 0.04 into fresh, prewarmed modified A medium, and 68 nM synthetic PAI was added where indicated. Synthetic PAI (41) was kindly supplied by A. S. Kende, University of Rochester Department of Chemistry. Growth of the cultures was monitored by measuring the A_{600}.

DNA techniques and nucleotide sequencing. Standard techniques were used for the purification and manipulation of DNA (30). Restriction endonucleases were purchased from Gibco/BRL (Gaithersburg, Md.) or New England Biolabs (Beverly, Mass.). Amplification of inserts for cloning was accomplished by PCR with a GeneAMP kit (Perkin-Elmer Cetus, Norwalk, Conn.) and a Hybaid

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TABLE 1. Strains and plasmids used

Strain or plasmid	Genotype or description[a]	Reference or source
Strains		
E. coli		
MG4	*recA1* (*argF lacIPOZYA*)*205*	28
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′*proAB lacI*^{q}*Z*Δ*M15* Tn*10*(Tet^r)]	Stratagene
P. aeruginosa		
PAO1	Wild type	20
PAO-R1	Δ*lasR*::Tet^r	12
Plasmids		
pBluescript II/KS+	General-purpose cloning vector, Amp^r	Stratagene
pQF50	Broad-host-range transcriptional fusion vector with a promoterless *lacZ*, Amp^r	10
pLJR50	195-bp *Eco*RI-*Hin*dIII *lasB* fragment (with *Eco*RI blunted) inserted into *Sma*I-*Hin*dIII-digested pQF50 to create a *lasBp-lacZ* transcriptional reporter fusion; contains nt −331 to −138 of the *lasB* promoter region	49
pLJR91	Same as pLJR50 but *lasBp-lacZ* has *lasB* promoter region deleted of nt −331 to −241	This study
pLJR50.404	Same as pLJR50 but *lasBp-lacZ* has *lasB* promoter region deleted of nt −277 to −211	This study
pLJR33	Same as pLJR50 but *lasBp-lacZ* has *lasB* promoter region deleted of nt −331 to −299	This study
pLJR141	Derived from pLJR50; *lasBp*[nt −191:C→T]-*lacZ*	This study
pLJR143	Derived from pLJR50; *lasBp*[nt −189:G→C]-*lacZ*	This study
pLJR118	Derived from pLJR50; *lasBp*[nt −176:G→A]-*lacZ*	This study
pLJR156	Derived from pLJR50; *lasBp*[nt −191:C→T; nt −176:G→A]-*lacZ*	This study
pLJR501.10	Derived from pLJR50; *lasBp*[nt −191:C→T; nt −189:G→C]-*lacZ*	This study
pLJR502.7	Derived from pLJR50; *lasBp*[nt −249:G→C; nt −251:C→T]-*lacZ*	This study
pQF50/B	Reversion of pLJR501.10 to create wt *lasBp-lacZ* (same as pLJR50)	This study
pACYC184	General-purpose cloning vector, Tet^r Chlor^r	4
pPCS11	*tacp-lasR* on pACYC184	This study
pKDT37	*tacp-lasR lacI*^{q} *lasB′-lacZ* Amp^r	40

[a] Nucleotides are numbered relative to the start codon of *lasB*.

thermal reactor (National Labnet Co., Woodbridge, N.J.). Oligonucleotides used for PCR were synthesized by G. Kampo and J. Maniloff at the Core Nucleic Acid Laboratory at the University of Rochester Medical Center. The introduction of plasmids into *E. coli* and *P. aeruginosa* was accomplished by transformation (30) and electroporation (46), respectively. *E. coli* and *P. aeruginosa* containing plasmid DNA were selected for on L-agar (30) plates containing ampicillin (100 μg/ml) and carbenicillin (200 μg/ml), respectively. Plasmid DNA was isolated from selected colonies and analyzed by mapping relevant restriction endonuclease sites. Nucleotide sequencing was accomplished by using a Sequenase kit (U.S. Biochemical Corp., Cleveland, Ohio) and [α-^{35}S]dATP (NEN Research Products, Boston, Mass.).

Transcriptional start site mapping. RNA isolation and S1 nuclease analysis were performed as previously described (6, 7). Cultures of *P. aeruginosa* PAO1 and PAO-R1 were grown in LB at 37°C to an A_{540} of 0.4. Primer extension analysis was carried out as described by Kingston (24). Two different oligonucleotide primers, 5′-CCTTCTTCATCTTGTTCAGTTCTCCTGG-3′ and 5′-CCTTGTGGGCTGATCGGAACAG-3′, corresponding to nucleotides (nt) +10 to −18 and nt −79 to −58, respectively, relative to the *lasB* start codon, were used in primer extension reactions. For each reaction, 100 ng of primer was end labeled with [γ-^{32}P]ATP (NEN Research Products) and polynucleotide kinase (Boehringer Mannheim, Indianapolis, Ind.) as described by Maniatis et al. (30). Labeled primers were added to 20 μg of RNA, and extension was completed by using avian myeloblastosis virus reverse transcriptase (Promega). Extended primers were loaded onto a sequencing gel next to sequencing reactions performed with the same primer. For S1 nuclease analysis, uniformly labeled, single-stranded DNA probes were made by using plasmid M13mp18 containing the 1.04-kb *Eco*RI-*Sal*I fragment of the *P. aeruginosa* PAO1 *lasB* gene. Two different oligonucleotides, 5′-AAGCGTAGAAACCTTC-3′ and 5′-CTTGTTCAGTTCTCCT-3′, covering nt +21 to +6 and nt −1 to −16, respectively, relative to the *lasB* start codon, were used to map the transcriptional start of *lasB*, using S1 nuclease analysis. Single-stranded probes produced by using these primers were hybridized to total *P. aeruginosa* RNA and subjected to S1 nuclease digestion (7). Mobilities of the resulting protected fragments were compared against a sequencing ladder generated by using each respective primer.

Plasmid construction and mutagenesis. (i) Construction of pLJR50. Construction of the transcriptional fusion plasmid, pLJR50, has been previously described (49). In this plasmid, the *lacZ* reporter gene is under the regulatory control of the *lasB* promoter region (nt −331 to −137 relative to the *lasB* translational start codon).

(ii) Site-directed mutagenesis of the *lasB* promoter region. Point mutations in the *lasB* promoter region were constructed either by the method of Kunkel (27) (pLJR501.10) or by the method of Ho et al. (17) (pLJR502.7, pLJR141, pLJR143, and pLJR156). Oligonucleotides used in the construction of *lasBp-lacZ* fusion plasmids are listed in Table 2. Site-directed mutagenesis and sequencing were carried out with pBluescript II/KS+ containing the *lasB* promoter region (nt −331 to −137 relative to the *lasB* translational start site). *E. coli* XL1-Blue was used as the host for these experiments. Mutated (and wild-type) DNA sequences were amplified by using PCR in which the oligonucleotide primers incorporated unique restriction enzyme sites on each end of the product. Purified, digested PCR products were then ligated into pQF50 to create transcriptional *lasBp-lacZ* fusions. Inserts in recombinant plasmids were resequenced to ensure that unwanted mutations were not introduced during DNA manipulations.

During the process of revising this report, we noticed that our *lasB* promoter region sequence disagreed with the previously published sequence (3) of this region. There was a T nucleotide that was supposed to be 144 nt upstream from the *lasB* start codon that was not present on our sequencing gels during the mapping of the *lasB* transcriptional start site. To determine if this nucleotide was present in the original *lasB* promoter region, we resequenced this region from the original *lasB* DNA cloned by Bever and Iglewski (3). We discovered that this nucleotide was not present in the original sequence and therefore concluded that the mistake probably arose as a typographical error reported by Bever and Iglewski (3). Unfortunately, this region was used to design the oligonucleotide primer, spanning nt −137 to −154 (Table 2), that was used to construct pLJR50. This plasmid was then used in all subsequent plasmid constructions. Therefore, we have unknowingly added a nucleotide at position −144 of the *lasB* promoter. However, we feel that this mutation in no way affects the results or conclusions that we have presented. This is evident when one compares previously reported induction levels from *lasB-lacZ* fusions (41, 48) with that from our fusion. The 100-fold or greater induction that we present for the pLJR50 *lasBp-lacZ* fusion in the presence of LasR and PAI is well within the expected range for *lasB-lacZ* fusions. Furthermore, this base does not fall within the *lasB* −10 or −35 region or within either of the two putative *lasB* operators. We therefore feel that our results produced with use of the described fusion are valid and sound. This error has been reported to and corrected by GenBank (accession number M19472).

(iii) Construction of deletion mutations in the *lasB* promoter region. To construct pLJR91, the *Bam*HI-*Nhe*I fragment of pLJR50 was deleted. The ends of the restriction enzyme-digested vector were filled in with the Klenow fragment and religated to create a deletion of 91 bp (positions −331 to −241 relative to the *lasB* translational start codon) in the *lasB* promoter region.

To construct pLJR50.404, the *lasB* promoter region was digested with *Nhe*I and subjected to *Bal* 31S exonuclease digestion (International Biotechnologies, Inc., New Haven, Conn.). Digested plasmids were religated and screened for the absence of a *Mae*I restriction site (centered at nt −261 relative to the *lasB* translational start site). DNA sequencing of a recombinant plasmid that had lost the *Mae*I site revealed a 67-bp deletion from nt −277 to −211 of the *lasB* promoter region. This mutated promoter was subsequently amplified by PCR using oligonucleotides that incorporate *Eco*RI and *Hin*dIII recognition sites

TABLE 2. Oligonucleotides used for construction of mutants

Plasmid[a]	Strand[b]	Oligonucleotide sequence[c] (5′→3′)	Location[d]
Standard[e]	−	GCATCCATG**AAGCTT**CGGTGCTTTTCGTGTACC	−137 to −154
Standard[e]	+	TACGC**GGATCC**AGGAAAGCGTGCAACTGA	−331 to −310
pLJR50, pLJR50.404	+	ACCGAGCTC**GAATTC**CGCGG	Vector sequence
pLJR33	+	GCATCCATC**GGATCC**GGCCCCTCGCTGAGCGCGTCCC	−299 to −278
pLJR141	+	GCGAAATCAAGGCTACTTGCCAGTTCTGGC	−207 to −178
pLJR141	−	GCCAGAACTGGCAAGTAGCCTTGATTTCGC	−178 to −207
pLJR143	+	GCGAAATCAAGGCTACCTCCCAGTTCTGGC	−207 to −178
pLJR143	−	GCCAGAACTGGGAGGTAGCCTTGATTTCGC	−178 to −207
pLJR156	+	GTTCTGGCAAGTTTGGCC	−175 to −168
pLJR156	−	GGCCAAACTTGCCAGAAC	−168 to −175
pLJR501.10	+	GCGAAATCAAGGCTACTTCCCAGTTCTGGC	−207 to −178
pLJR502.7	+	CCTAGCTGCCACTTCCTTTTCTGCTAGC	−252 to −236
pLJR502.7	−	GCTAGCAGAAAAGGAAGTGGCAGCTAGG	−236 to −252
pQF50/B	+	GCTACCTGCCAGTTCTGGC	−196 to −178
pQF50/B	−	GCCAGAACTGGCAGGTAGC	−178 to −196

[a] Plasmid that was constructed by using the respective oligonucleotide.
[b] DNA strand containing the respective oligonucleotide sequence. +, *lasB* coding strand; −, *lasB* noncoding strand.
[c] Nucleotide changes are underlined, and relevant restriction endonuclease sites are in boldface.
[d] Location of the corresponding sequence within the *lasB* promoter region relative to the *lasB* ATG start codon.
[e] The oligonucleotide was used to construct all *lasBp-lacZ* transcriptional fusion plasmids used in this study except pLJR91.

upstream and downstream from the *lasB* promoter, respectively. The PCR product was digested with *Eco*RI, and the overhanging ends were filled in with the Klenow fragment. The product was then digested with *Hin*dIII and ligated into pQF50 previously digested with *Sma*I and *Hin*dIII, to generate pLJR50.404.

To construct pLJR33, bp −298 to −137 of the *lasB* promoter region were PCR amplified by using pLJR50 as a template and oligonucleotides that incorporated *Bam*HI and *Hin*dIII restriction sites upstream and downstream from the promoter, respectively. This resulted in a PCR product that lacked bp −331 to −299 from the *lasB* promoter region. The PCR product was digested with *Bam*HI and *Hin*dIII and ligated into pQF50 that was previously digested with *Bam*HI and *Hin*dIII, to generate pLJR33.

Assay of β-galactosidase activity. β-Galactosidase activity was assayed in triplicate as described by Miller (31), and the mean ± 1 standard deviation was determined.

RESULTS

Determination of the *lasB* transcriptional start site. The transcriptional start site of *lasB* was determined by both primer extension and S1 nuclease analysis. For each transcriptional start mapping experiment, two different primers were used (described in Materials and Methods). The results of all four mapping experiments showed that the *lasB* mRNA transcript starts at nt −141 relative to the *lasB* start codon (Fig. 1). The result of one primer extension is shown in Fig. 2. In this experiment, total RNA from *P. aeruginosa* PAO1 and PAO-R1 was extended. The only product that resulted from these extensions is indicated by an arrowhead in Fig. 2. Strain PAO-R1 is a *lasR* mutant that produces minimal or no *lasB* message (12), and as expected, no extended product was produced when PAO-R1 RNA was used. A second primer extension experiment with a different primer (data not shown) and two different S1 nuclease analysis experiments (data not shown) also clearly indicated that the *lasB* mRNA started at nt −141. Examination of the DNA sequence upstream from the transcriptional start site did not reveal any region with obvious high homology to a known consensus sequence for a sigma factor.

The *lasB* promoter region contains two potential operator sequences. The *lasB* promoter region contains two sequences that are similar to the *lux* operator of *V. fischeri* (Fig. 3). The most similar region (OP1; Fig. 1) is a 20-bp sequence previously described by Fuqua et al. (11) that is centered 42 nt upstream from the *lasB* transcriptional start site. This sequence is in a position that could easily overlap the −35 region of the *lasB* promoter. It contains an inverted repeat and matches 13 of 20 nt of the *V. fischeri lux* operator sequence (Fig. 3). The second *lux* operator-like sequence (operator 2 [OP2]; Fig. 1) in the *lasB* promoter region is centered 102 nt upstream from the *lasB* transcriptional start site. This sequence does not contain an inverted repeat but does match 10 of 20 nt of the *lux* operator sequence (Fig. 3). This sequence also matches 14 of 20 nt of OP1 of *lasB* (Fig. 3). To explore the influence of these potential operator sequences on *lasB* activation, a series of *lasBp* site-directed and deletion mutants was constructed and transcriptionally fused to a *lacZ* reporter gene.

Site-directed mutagenesis of the *lasB* promoter region. Single-point mutations at positions 3 and 5 in the *lux* operator abolished or diminished by 97%, respectively, *lux* operon induction in *V. fischeri* (1). To determine if OP1 had a similar effect on *lasB*, a *lasBp-lacZ* fusion containing two point mutations, one at position 3 (nt −191; C to T) and another at position 5 (nt −189; G to C) of OP1, was constructed (pLJR501.10). This plasmid was transformed into *P. aeruginosa* PAO1, and β-galactosidase activity was measured (Fig. 4). The expression of *lasB* from pLJR501.10 was reduced to levels obtained from the vector control (Fig. 4). Furthermore, reversion of the point mutations to the original *lasB* promoter sequence restored the level of β-galactosidase expression to that of PAO1(pLJR50) (data not shown). These data indicate

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-331  CCAGAAAGCG TGCAACTGAT GATCGTCCAC ATGGCCCCTC GCTGAGCGCG TCCCGGAGCT
                                    OP2
-271  GGGGGCAACC TAGCTGCCAC CTGCTTTTCT GCTAGCTATT CCAGCGAAAA CATACAGATT
                                    OP1
-211  TCCGGCGAAA TCAAGGCTAC CTGCCAGTTC TGGCAGGTTT GGCCGCGGGT TCTTTTTGGT
                  mRNA
-151  ACACGAAAGC ACCGTCGAAA ACGGGACCGA GCCAGGGGAG TGCAGTTCCT TCTACCCGAA

 -91  GGACTGATAC GGCTGTTCCG ATCAGCCCAC AAGGCGGCGG TAAGCGTCGG CCGAGTACTT

 -31  CGGCCTGAAA AAACCAGGAG AACTGAACAA GATGAAGAAG GTTTCTACGC TTGACCTGTT
                                      -1
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FIG. 1. Nucleotide sequence of the *lasB* promoter region. Numbering is relative to the *lasB* ATG start codon, which is indicated by boldface type. The mRNA start site, confirmed by both multiple primer extension and S1 nuclease analysis experiments, is indicated by an arrow pointing in the direction of transcription (see Fig. 2). The putative operator sequences in the *lasB* promoter region, OP1 and OP2, are underlined (see Fig. 3).

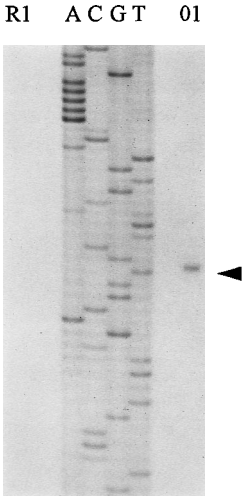

FIG. 2. Primer extension analysis of the 5′ end of the *lasB* transcript. Sequencing reaction lanes are labeled according to nucleotide (A, C, G, or T). Lanes containing extended primers are labeled according to the strain from which the total RNA was purified: R1, primer extension experiment using RNA from *P. aeruginosa* PAO-R1 (negative control); and 01, primer extension experiment using RNA from *P. aeruginosa* PAO1. The arrowhead points to the extension product.

that positions 3 and/or 5 of OP1 are involved in the regulation of *lasB* expression.

To examine the effects of both point mutations present in pLJR501.10 independently, fusions with single-point mutations were constructed. pLJR141 and pLJR143 incorporate point mutations at position 3 (nt −191; C to T) and position 5 (nt −189; G to C) of the OP1 sequence, respectively. The mutations at positions 3 and 5 in OP1 reduced *lasB* expression by 98 and 27%, respectively (Fig. 4). Relative to the vector control [PAO1(pQF50)], PAO1(pLJR141) had only a 3.5-fold induction of *lasB*, compared with 144-fold in PAO1(pLJR50) (Fig. 4).

To determine the relative importance of the 3′ portion of OP1, pLJR118 was constructed. This plasmid contains *lasBp-lacZ* with a mutation in position 18 of OP1 (nt −176; G to A). The G at position 18 of OP1 would pair with the C at position 3 relative to the inverted repeat structure of OP1. The mutation at position 18 abolished *lasB* promoter activity (pLJR118; Fig. 4), thus indicating that position 18 of OP1 is important for operator or promoter recognition. The negative effect of changing position 3 or 18 of OP1 may be a result of either disruption of the dyad symmetry of the sequence or alteration of nucleotides involved in operator or promoter recognition. To determine whether restoration of the dyad symmetry would also restore *lasB* expression, pLJR141 was mutagenized at position 18 (G to A) to construct pLJR156, thus restoring dyad symmetry to the mutated sequence. This restoration did not increase *lasB* promoter activity from the level of pLJR141 (Fig. 4). These data suggest that both positions 3 (C·G) and 18 (G·C) of OP1 are critical for operator or promoter recognition (depending on the exact location of the −35 region of the *lasB* promoter). This indicates that these positions are crucial for *lasB* transcription and reconfirms that OP1 is involved in *lasB* regulation.

To determine if OP2 was involved in the activation of *lasB*, a double site-directed mutant was constructed. To create pLJR502.7, nt −251 was changed from C to T and nt −249 was changed from G to C, resulting in a *lasB* promoter that contained two mutations in OP2. Double-point mutagenesis of OP2 resulted in a 20% decrease in *lasB* expression (pLJR 502.7; Fig. 4), indicating that this sequence may be involved in, but is not as critical as OP1 for, *lasB* activation. This suggests that *lasB* is potentially controlled by two separate, homologous operators within its promoter region.

Mutation of OP1 renders the *lasB* promoter unresponsive to activation by LasR and PAI. Passador et al. (39) have previously demonstrated that LasR and PAI are necessary and sufficient for activation of *lasB* in *E. coli*. We examined whether the OP1 double-point mutation on pLJR501.10 prevented LasR and PAI induction of *lasB* expression in *E. coli*. To do this, a pBR322-compatible plasmid carrying *lasR* was required. The 1.7-kb *Bam*HI-*Sca*I fragment coding for *tacp-lasR* from pKDT37 was ligated into the *Bam*HI-*Hin*cII sites of pACYC184, thus creating pPCS11 (Table 1). Either pPCS11 or the control vector, pACYC184, was transformed into *E. coli* MG4 containing pLJR50, pLJR501.10, pQF50/B, or pQF50. Transformed cells were cultured in the presence of PAI as described in Materials and Methods. β-Galactosidase expression from MG4(pPCS11)(pLJR501.10) was minimally induced in the presence of LasR and PAI, indicating that the OP1 mutations render *lasB* unresponsive to LasR and PAI (Table 3). Reversion of these nucleotides to the original sequence (pQF50/B) restored the ability of LasR and PAI to activate the *lasB* promoter. These data confirm the importance of these bases for LasR- and PAI-mediated activation of *lasB* transcription. In addition, these data support the utility of using the *E. coli* system to explore components of *P. aeruginosa* gene regulation.

Deletion mutagenesis of the *lasB* promoter region. We decided to determine the effect of deleting OP2 and other sequences upstream from OP1 on *lasB* expression. Limited site-directed mutations in OP2 indicated that this sequence may affect *lasB* expression (pLJR502.7; Fig. 4). To further explore this and other regions, three deletions were constructed in the *lasB* promoter region upstream from OP1. Deleted *lasB* promoters were transcriptionally fused to *lacZ* to create pLJR33, which lacks nt −331 to −299, pLJR91, which lacks nt −331 to −241, and pLJR50.404, which lacks nt −277 to −211 relative to the *lasB* start codon. β-Galactosidase expressed from *P. aeruginosa* PAO1 containing each deletion mutant plasmid was assayed and compared with that of PAO1 (pLJR50; Fig. 5). Expression of *lasB* from pLJR33 was unchanged relative to that from the full-length promoter region (Fig. 5). However, expression of *lasB* from pLJR91 and pLJR50.404 was reduced to 30 and 6%, respectively, of that from pLJR50 (Fig. 5). While the precise extent of the expression reductions varied between experiments (Fig. 5; Table 4), these data indicate that the region between nt −298 and −210 relative to the *lasB* start codon contains elements important for *lasB* promoter activation. These data also suggest that OP2 may be important for *lasB* expression and that this potential operator is worthy of further study.

Effect of the *lasB* promoter region deletions on uninduced

Operator	Sequence	Location
OP2	5'-ACCTGCTT-TTCTGCTAGCT-3'	-253 to -235
OP1	5'-ACCTGCCAGTTCTGGCAGGT-3'	-193 to -174
lux	5'-ACCTGTAGGATCGTACAGGT-3'	

FIG. 3. Putative operator sequences in the *lasB* promoter region. The nucleotide sequences of the two potential *lasB* operators and the *V. fischeri lux* operator are aligned. The locations of the *lasB* operator sequences are relative to the *lasB* start codon (see Fig. 1). The *lasB* OP2 and *V. fischeri lux* operator sequences are aligned with the *lasB* OP1 sequence. Vertical lines indicate identical nucleotides.

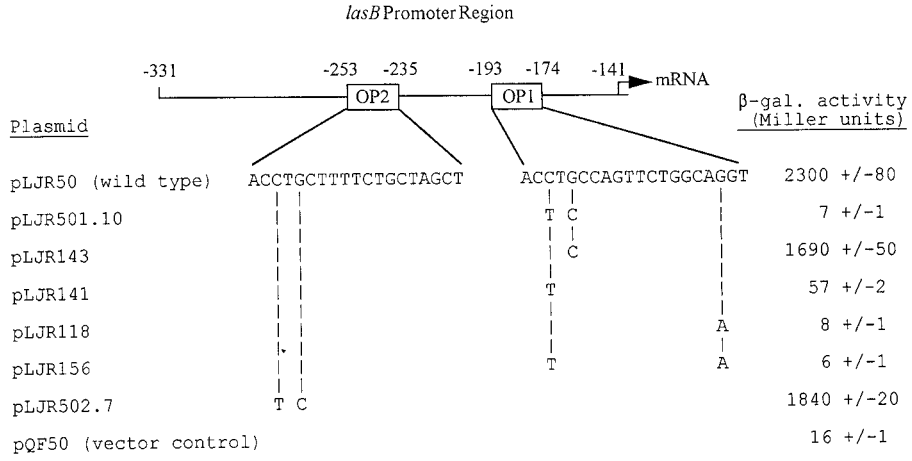

FIG. 4. Effects of site-directed mutations in OP1 and OP2 on *lasB* promoter activity. *P. aeruginosa* PAO1 containing *lasBp-lacZ* fusion plasmids with the indicated nucleotide changes was cultured and assayed for β-galactosidase (β-gal.) activity as described in Materials and Methods. β-Galactosidase activity expressed in Miller units is provided along with the standard deviation. Nucleotides are numbered relative to the *lasB* start codon.

***lasB* expression.** In *V. fischeri*, deletion of sequences upstream from the *lux* operator resulted in enhanced *lux* promoter activity in the absence of LuxR and VAI, indicating that a repressive element had been deleted (1). To determine the effect of deleting regions upstream from OP1 on uninduced *lasB* promoter activity, β-galactosidase activities expressed from *P. aeruginosa* PAO1 and PAO-R1 containing pLJR91, pLJR 50.404, or pLJR50 were measured. The results of these experiments showed that deletion of sequences upstream from OP1 did not result in increased *lasB* expression in the absence of LasR (strain PAO-R1; Table 4). This result indicates that a *lasB* repressor element is not present in the region from nt −331 to −211 relative to the *lasB* start codon.

DISCUSSION

We have mapped the transcriptional start site of the *lasB* gene, which led to the discovery that two interesting, homologous regions were near the *lasB* promoter. Mutational analysis of these regions, OP1 and OP2, suggested that they are involved in *lasB* activation by LasR and PAI. The OP1 sequence is an inverted repeat that is centered 42 nt upstream from the *lasB* transcriptional start site, where it could overlap with the −35 region of the *lasB* promoter. No potential *lasB* promoter has been identified because the *lasB* promoter region does not contain an apparent sequence that is similar to a known promoter consensus sequence. The OP2 sequence of *lasB* is centered 102 nt upstream from the *lasB* transcriptional start site.

TABLE 3. Effect of an OP1 double mutation on *lasB* expression in the presence and absence of LasR and PAI[a]

Plasmid	Mean β-galactosidase activity (Miller units) ± SEM	
	pPCS11 (*tacp-lasR*)	pACYC184 (*lasR* mutant control)
pLJR50	410 ± 30	3 ± 0
pLJR501.10	10 ± 0	0
pQF50/B	389 ± 7	3 ± 0
pQF50	0	0

[a] 68 nM PAI was added to mid-log-phase cultures of *E. coli* MG4 containing the indicated compatible plasmids, and β-galactosidase was assayed after 90 min of growth.

It is interesting that these sequences are very similar to each other and are reasonably similar to the *lux* operator of *V. fischeri*. Fuqua et al. (11) originally pointed out the OP1 sequence as a *lux* operator-homologous region and reported a consensus sequence for other genes controlled by an autoinduction system. Both OP1 and OP2 are similar to the reported consensus sequence, but OP2 is not an inverted repeat like other identified autoinduction operators (11).

We have shown that mutations in both halves of OP1 caused a downregulation of *lasB* activation (Fig. 4; Table 3). Individual mutations at positions 3 and/or 18 of OP1 caused *lasB* promoter activity to be reduced by at least 98%. These data demonstrate the importance of both halves of the OP1 inverted repeat for *lasB* activation. A double mutant (positions 3 and 5 of OP1) was also shown to be inactivatable by LasR and PAI in *E. coli*, suggesting that LasR may interact with OP1 to activate *lasB*. It is possible that OP1 either overlaps with or is contiguous to the −35 region of the *lasB* promoter. Stevens et al. (47) have recently shown that the DNA-binding regions of LuxR and RNA polymerase holoenzyme are required to protect the *lux* operator from DNase I digestion. This finding suggests a synergistic mechanism of activation in this system. The location of OP1 in relation to the *lasB* transcriptional start site and its obvious influence on *lasB* activation lead us to speculate that LasR may interact with both OP1 and RNA polymerase to activate *lasB* transcription. Future experiments involving gel shift and DNase I protection assays are planned to determine whether LasR interacts directly with OP1 in the presence of *P. aeruginosa* RNA polymerase.

We have also shown, by deletion analysis, that a sequence(s) upstream from OP1 is critical for *lasB* activation. Deletion of sequences upstream of OP1, between nt −299 and −210 relative to the translational start codon, negatively affected *lasB* induction (Fig. 5). This region contains the second putative *lasB* operator, OP2, identified in this study (Fig. 3). Since LasR and PAI are necessary and sufficient to activate *lasB* expression in *E. coli* to the level observed in *P. aeruginosa* (39, 41), it is likely that a sequence(s) within the −299 to −210 region, as well as OP1, responds to the presence of LasR and PAI. Stevens et al. (47) have also shown that the DNA-binding region of LuxR specifically binds to sequences upstream from the *lux* operator. That discovery accompanied by the results of our deletion experiments entices us to hypothesize that LasR

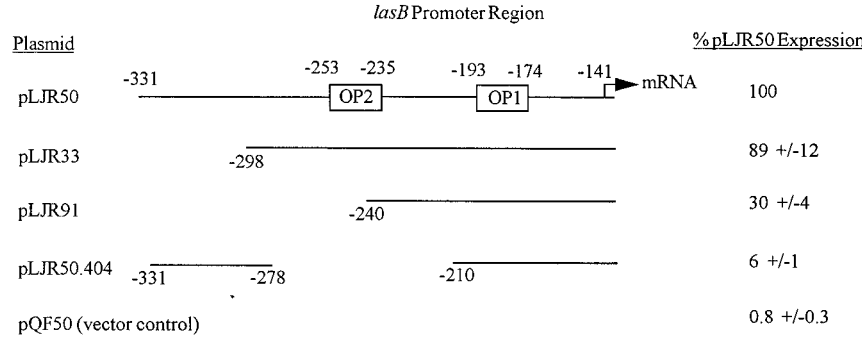

FIG. 5. Effects of deletions in the *lasB* promoter region on *lasB* expression. *P. aeruginosa* PAO1 containing *lasBp-lacZ* fusion plasmids with the indicated deletions was cultured and assayed for β-galactosidase activity as described in Materials and Methods. Values represent average β-galactosidase activities of six independent experiments expressed as a percentage of that obtained from the fusion with the wild-type *lasB* promoter (pLJR50). Nucleotides are numbered relative to the *lasB* start codon.

interacts with a *lasB* promoter region DNA sequence or sequences, in addition to OP1, to activate *lasB* transcription. This theory is also supported by our limited site-directed mutagenesis of OP2, which suggested that nucleotides within OP2 were involved in *lasB* activation. These mutations caused a 20% decrease in *lasB* expression (Fig. 4). However, this decrease is not as great as the 66% decrease in *lasB* expression caused by deleting sequences within the region from nt −299 to −211 (Fig. 4 and 5). This finding indicates that this region contains nucleotides, other than those mutated, which are involved in *lasB* expression. We hypothesized that this region contains a sequence that interacts with LasR. Alternatively, this region may serve to reduce the randomness of an activator-operator encounter. The *lac* repressor, for example, has been shown to slide along the DNA molecule until the operator is recognized (2, 51). Finally, this region could be required for proper conformation of the operator, as proposed for catabolite gene activator protein operator recognition (44).

Despite numerous similarities in structure, function, and sequence, there is only limited interchangeability of regulatory components and target gene operators between the *P. aeruginosa las* and *V. fischeri lux* systems (13). The presence of LuxR and VAI cannot fully complement the absence of LasR and PAI for *lasB* activation (13). LuxR and VAI were able to stimulate *lasB* expression by only a factor of 4, indicating the importance of specific operator DNA sequences for gene activation in autoinduction systems. LasR and PAI, and LuxR and VAI, may recognize corresponding positions of their respective operators with different affinities. This view is supported by our finding that a mutation at position 5 of *lasB* OP1 resulted in only a 27% loss in *lasB* induction (Fig. 4), while the corresponding mutation in the *lux* operator results in a 97% loss in LuxR- and VAI-mediated *lux* operon induction (1). This indicates that while specific nucleotides of the *lasB* OP1 and *lux* operators are important in gene activation, they are involved to different degrees in the two systems.

TABLE 4. Effects of *lasB* promoter region deletions on *lasB* expression from PAO1 and PAO-R1

Strain (phenotype)	Plasmid	Mean β-Galactosidase activity (Miller units) ± SEM[a]
PAO1 (LasR$^+$)	pLJR50	2,020 ± 60
PAO-R1 (LasR$^-$)	pLJR50	26 ± 1
PAO1 (LasR$^+$)	pLJR91	163 ± 7
PAO-R1 (LasR$^-$)	pLJR91	23 ± 0
PAO1 (LasR$^+$)	pLJR50.404	50 ± 1
PAO-R1 (LasR$^-$)	pLJR50.404	7 ± 0
PAO1 (LasR$^+$)	pQF50	61 ± 0
PAO-R1 (LasR$^-$)	pQF50	34 ± 1

[a] β-Galactosidase activity assays were performed on late-log-phase *P. aeruginosa* cultures.

Our analysis of the *lasB* promoter region has added much insight to the control of *lasB* expression by *P. aeruginosa*. We have located regions that are important for *lasB* activation by LasR and PAI, and our results have suggested potential mechanisms through which LasR and PAI may act. Future experiments are planned to determine both the exact sequence(s) recognized by LasR and the mechanism through which this sequence(s) and LasR act to control *lasB* expression.

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REFERENCES

1. **Baldwin, T. O., J. H. Devine, R. C. Heckel, J.-W. Lin, and G. S. Shadel.** 1989. The complete nucleotide sequence of the *lux* regulon of *Vibrio fischeri* and the *luxABN* region of *Photobacterium leiognathi* and the mechanism of control of bacterial bioluminescence. J. Biolumin. Chemilumin. **4:**326–341.
2. **Barkley, M. D.** 1981. Salt dependence of the kinetics of the *lac* repressor-operator interaction: role of non-operator deoxyribonucleic acid in the association reaction. Biochemistry **20:**3833–3842.
3. **Bever, R. A., and B. H. Iglewski.** 1988. Molecular characterization and nucleotide sequence of the *Pseudomonas aeruginosa* elastase structural gene. J. Bacteriol. **170:**4309–4314.
4. **Chang, A. C. Y., and S. N. Cohen.** 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. J. Bacteriol. **134:**1141–1156.
5. **Cicmanec, J. F., and I. A. Holder.** 1979. Growth of *Pseudomonas aeruginosa* in normal and burned skin extract: role of extracellular proteases. Infect. Immun. **25:**477–483.
6. **Deretic, V., J. F. Gill, and A. M. Chakrabarty.** 1987. Gene *algD* coding for GDPmannose dehydrogenase is transcriptionally activated in mucoid *Pseudomonas aeruginosa*. J. Bacteriol. **169:**351–358.
7. **Deretic, V., J. F. Gill, and A. M. Chakrabarty.** 1987. *Pseudomonas aeruginosa* infection in cystic fibrosis: nucleotide sequence and transcriptional regulation of the *algD* gene. Nucleic Acids Res. **15:**4567–4581.
8. **Doring, G., W. Goldstein, A. Roll, P. O. Schiotz, N. Hoiby, and K. Botzen-**

hart. 1985. Role of *Pseudomonas aeruginosa* exoenzymes in lung infections of patients with cystic fibrosis. Infect. Immun. **49:**557–562.
9. **Doring, G., H.-J. Obernesser, and K. Botzenhart.** 1981. Extracellular toxins of *Pseudomonas aeruginosa*. II. Effect of two proteases on human immunoglobulins IgG, IgA and secretory IgA. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. Reihe A **249:**89–98.
10. **Farinha, M. A., and A. M. Kropinski.** 1990. Construction of broad-host-range plasmid vectors for easy visible selection and analysis of promoters. J. Bacteriol. **172:**3496–3499.
11. **Fuqua, W. C., S. C. Winans, and E. P. Greenberg.** 1994. Quorum sensing in bacteria: the LuxR-LuxI family of cell density-responsive transcriptional regulators. J. Bacteriol. **176:**269–275.
12. **Gambello, M. J., and B. H. Iglewski.** 1991. Cloning and characterization of the *Pseudomonas aeruginosa lasR* gene: a transcriptional activator of elastase expression. J. Bacteriol. **173:**3000–3009.
13. **Gray, K. M., L. Passador, B. H. Iglewski, and E. P. Greenberg.** 1994. Interchangeability and specificity of components from the quorum-sensing regulatory systems of *Vibrio fischeri* and *Pseudomonas aeruginosa*. J. Bacteriol. **176:**3076–3080.
14. **Grimwood, K., R. A. Semple, H. R. Rabin, P. A. Sokol, and D. E. Woods.** 1993. Elevated exoenzyme expression by *Pseudomonas aeruginosa* is correlated with exacerbations of lung disease in cystic fibrosis. Pediatr. Pulmonol. **15:**135–139.
15. **Heck, L. W., P. G. Alarcon, R. M. Kulhavy, K. Morihara, M. W. Russell, and J. F. Mestecky.** 1990. Degradation of IgA proteins by *Pseudomonas aeruginosa* elastase. J. Immunol. **144:**2253–2257.
16. **Heck, L. W., K. Morihara, W. B. McRae, and E. J. Miller.** 1986. Specific cleavage of human type III and IV collagens by *Pseudomonas aeruginosa* elastase. Infect. Immun. **51:**115–118.
17. **Ho, S. N., H. D. Hunt, R. M. Horton, J. K. Pullen, and L. R. Pease.** 1988. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene **77:**51–59.
18. **Holder, I. A., and A. N. Neely.** 1989. *Pseudomonas* elastase acts as a virulence factor in burned hosts by Hageman factor-dependent activation of the host kinin cascade. Infect. Immun. **57:**3345–3348.
19. **Holder, I. A., and A. N. Neely.** 1991. The role of proteases in *Pseudomonas* infections in burns: a current hypothesis. Antibiot. Chemother. **44:**99–105.
20. **Holloway, B. W., V. Krishnapillai, and A. F. Morgan.** 1979. Chromosomal genetics of *Pseudomonas*. Microbiol. Rev. **43:**73–102.
21. **Hong, Y., and B. Ghebrehiwet.** 1992. Effect of *Pseudomonas aeruginosa* elastase and alkaline protease on serum complement and isolated components Clq and C3. Clin. Immunol. Immunopathol. **62:**133–138.
22. **Iglewski, B. H., and T. I. Nicas.** 1985. Extracellular factors in the virulence of *Pseudomonas aeruginosa*, p. 257–66. *In* Bayer-Symposium VIII: the pathogenesis of bacterial infections. Springer-Verlag, Berlin.
23. **Kawaharajo, K., J. Homma, Y. Aoyama, K. Okada, and K. Morihara.** 1975. Effects of protease and elastase from *Pseudomonas aeruginosa* on skin. Jpn. J. Exp. Med. **45:**79–88.
24. **Kingston, R. E.** 1989. Primer extension, p. 4.8.1–4.8.3. *In* F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.), Current protocols in molecular biology. John Wiley & Sons, Inc., New York.
25. **Klinger, J. D., D. C. Strauss, C. B. Hilton, and J. A. Bass.** 1978. Antibodies to proteases and exotoxin A of *Pseudomonas aeruginosa* in patients with cystic fibrosis: demonstration by radioimmunoassay. J. Infect. Dis. **138:**49–58.
26. **Kreger, A. S., and L. D. Gray.** 1978. Purification of *Pseudomonas aeruginosa* proteases and microscopic characterization of *Pseudomonas* protease-induced rabbit corneal damage. Infect. Immun. **19:**630–648.
27. **Kunkel, T. A.** 1985. Rapid and efficient site-specific mutagenesis without phenotypic selection. Proc. Natl. Acad. Sci. USA **82:**488–492.
28. **Linn, T., and G. Ralling.** 1985. A versatile multiple- and single-copy vector system for the *in vitro* construction of transcriptional fusions to *lacZ*. Plasmid **14:**134–142.
29. **Liu, P. V.** 1973. Exotoxins of *Pseudomonas aeruginosa*. I. Factors that influence the production of exotoxin A. J. Infect. Dis. **128:**506–513.
30. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
31. **Miller, J.** 1972. Experiments in molecular genetics, p. 352–355. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
32. **Morihara, K.** 1964. Production of elastase and proteinase by *Pseudomonas aeruginosa*. J. Bacteriol. **88:**745–757.
33. **Morihara, K., and J. Y. Homma.** 1985. *Pseudomonas* proteases, p. 49–79. *In* I. A. Holder (ed.), Bacterial enzymes and virulence. CRC Press, Inc., Boca Raton, Fla.
34. **Morihara, K., H. Tsuzuki, M. Harada, and T. Iwata.** 1984. Purification of human plasma α_1-proteinase inhibitor and its inactivation by *Pseudomonas aeruginosa* elastase. J. Biochem. **95:**795–804.
35. **Morihara, K., H. Tsuzuki, and K. Oda.** 1979. Protease and elastase of *Pseudomonas aeruginosa*: inactivation of human plasma α_1-proteinase inhibitor. Infect. Immun. **24:**188–193.
36. **Nicas, T. I., and B. H. Iglewski.** 1985. The contribution of exoproducts to virulence of *Pseudomonas aeruginosa*. Can. J. Microbiol. **31:**387–392.
37. **Ochsner, U. A., A. K. Koch, A. Fiechter, and J. Reiser.** 1994. Isolation and characterization of a regulatory gene affecting rhamnolipid biosurfactant synthesis in *Pseudomonas aeruginosa*. J. Bacteriol. **176:**2044–2054.
38. **Parmely, M., A. Gale, M. Clabaugh, R. Horvat, and W. W. Zhou.** 1990. Proteolytic inactivation of cytokines by *Pseudomonas aeruginosa*. Infect. Immun. **58:**3009–3014.
39. **Passador, L., J. M. Cook, M. J. Gambello, L. Rust, and B. H. Iglewski.** 1993. Expression of *Pseudomonas aeruginosa* virulence genes requires cell-to-cell communication. Science **260:**1127–1130.
40. **Passador, L., A. S. Kende, K. D. Tucker, and B. H. Iglewski.** Unpublished data.
41. **Pearson, J. P., K. M. Gray, L. Passador, K. D. Tucker, A. Eberhard, B. H. Iglewski, and E. P. Greenberg.** 1994. Structure of the autoinducer required for expression of *Pseudomonas aeruginosa* virulence genes. Proc. Natl. Acad. Sci. USA **91:**197–201.
42. **Pearson, J. P., L. Passador, B. H. Iglewski, and E. P. Greenberg.** 1995. A second *N*-acylhomoserine lactone signal produced by *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. USA **92:**1490–1494.
43. **Schultz, D. R., and K. D. Miller.** 1974. Elastase of *Pseudomonas aeruginosa*: inactivation of complement components and complement-derived chemotactic and phagocytic factors. Infect. Immun. **10:**128–135.
44. **Schultz, S. C., G. C. Shields, and T. A. Steitz.** 1991. Crystal structure of a CAP-DNA complex: the DNA is bent by 90 degrees. Science **253:**1001–1007.
45. **Seed, P. C., L. Passador, and B. H. Iglewski.** 1995. Activation of the *Pseudomonas aeruginosa lasI* gene by LasR and the *Pseudomonas* autoinducer PAI: an autoinduction regulatory hierarchy. J. Bacteriol. **177:**654–659.
46. **Smith, A. W., and B. H. Iglewski.** 1989. Transformation of *Pseudomonas aeruginosa* by electroporation. Nucleic Acids Res. **17:**10509.
47. **Stevens, A. M., K. M. Dolan, and E. P. Greenberg.** 1994. Synergistic binding of the *Vibrio fischeri* LuxR transcriptional activator domain and RNA polymerase to the *lux* promoter region. Proc. Natl. Acad. Sci. USA **91:**12619–12623.
48. **Swift, S., N. J. Bainton, and M. K. Winson.** 1994. Gram-negative bacterial communication by N-acyl homoserine lactones: a universal language? Trends Microbiol. **2:**193–198.
49. **Toder, D. S., S. J. Ferrell, J. L. Nezezon, L. Rust, and B. H. Iglewski.** 1994. *lasA* and *lasB* genes of *Pseudomonas aeruginosa*: analysis of transcription and gene product activity. Infect. Immun. **62:**1320–1327.
50. **Twining, S. S., S. E. Kirschner, L. A. Mahnke, and D. W. Frank.** 1993. Effect of *Pseudomonas aeruginosa* elastase, alkaline protease, and exotoxin A on corneal proteinases and proteins. Invest. Ophthalmol. Visual Sci. **34:**2699–2712.
51. **Winter, R. B., O. G. Berg, and P. H. von Hippel.** 1981. Diffusion-driven mechanisms of protein translocation on nucleic acids. 3. The *Escherichia coli lac* repressor-operator interaction: kinetic measurements and conclusions. Biochemistry **20:**6961–6977.
52. **Wretlind, B., and T. Wadstrom.** 1977. Purification and properties of a protease with elastase activity from *Pseudomonas aeruginosa*. J. Gen. Microbiol. **103:**319–327.