Molecular Characterization of MexL, the Transcriptional Repressor of the mexJK Multidrug Efflux Operon in Pseudomonas aeruginosa

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The Pseudomonas aeruginosa mexJK efflux operon is constitutively expressed in mutants with defects in the upstream mexL gene, which encodes a repressor of the TetR family. MexL and a MexLA47D mutant protein were purified from Escherichia coli as fusion proteins with carboxy-terminal hexahistidine tags. Native polyacrylamide gel electrophoresis and size exclusion chromatography revealed that MexL is a tetramer in solution. MexL and MexLA47D oligomerization was confirmed using a genetic approach, and the MexLA47D mutant protein was not impaired in multimerization. Gel mobility shift and footprinting assays demonstrated that MexL, but not MexLA47D, binds specifically to the 94-bp mexL-mexJ intergenic region to sequences located between positions −84 and −20 from the mexJ initiation codon. MexL protected about 60 nucleotides on each strand, and the protected regions overlapped almost perfectly, a finding consistent with MexL regulating the expression of both mexL and mexJK, which was ascertained by gene fusion analyses. The protected region contains predicted −10 and −35 promoter sequences for both mexL and mexJ, with partially overlapping −10 regions. The MexL promoter assignment was verified by mapping the mexL transcription start site, and the mexJ promoter was localized to the predicted regions using lacZ fusions. The MexL-protected region contains two inverted GTATTT repeats, and their location in the protected region and overlap with the mexL and mexJ promoter sequences strongly support a role in MexL binding.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are described in Table 1. Luria-Bertani (LB) medium from Difco (Detroit, MI) was used as a growth medium for all bacterial strains, unless otherwise stated. The minimal medium used was M9 medium (23) supplemented with 0.2% Casamino Acids (Difco) or 0.2% glucose (Sigma, St. Louis, MO). Cultures were routinely incubated at 37°C with shaking (250 rpm). Where necessary, antibiotics were used in growth media as follows: for Escherichia coli, ampicillin (100 to 150 μg/ml) and tetracycline (15 μg/ml); and for Pseudomonas aeruginosa, tetracycline (10 to 15 μg/ml) and carbenicillin (100 to 200 μg/ml). Antibiotics were purchased from Sigma, with the exception of carbenicillin, which was purchased from Fisher Scientific (Pittsburgh, PA).

General DNA methodology. Routine DNA manipulations, including PCR amplifications from genomic DNA templates, were performed as previously described (12, 24). Preparations of chromosomal and plasmid DNAs were performed using the ISOQUICK nucleic acid extraction kit (ORCA Research, Bothell, WA) and QIAprep Mini-spin kit (QIAGEN, Valencia, CA), respectively, and these kits were used according to the manufacturer’s instructions. The lacZ fusion plasmid pTZ120 was derived from pTZ110 (28) by inserting rehybridized Ncol/NsiI linkers 1 and 2 (oligonucleotides used in this study are listed in Table 2) into the BamHI site of pTZ110 using previously described methods (26, 28). The mexL-lacZ fusion plasmid pSI127 was created by inserting the 632-bp XhoI-EcoRI fragment from pPS1176 between the same sites of pTZ110. For complementation experiments, pPS1245 carrying the mexL coding region in the same orientation as Plac was constructed as follows. Plasmid pPS1153 was digested with EcoRI, blunt ended, and then digested with Sall. The mexL fragment was cloned into pRk415 obtained by digesting with HindIII, blunt ending, and then digesting with Sall.

Construction of mexL-lacZ transcriptional fusions for mexL promoter mapping. Various portions of the mexL-mexJ intergenic regions were PCR amplified from genomic DNA templates by using different pairs of primers as follows: LJJU-LJ2D, LJJU-LJ5D, LJJU-LJ5D, LJJU-LJ5D, LJJU-LJ5D, and mexLJ-up-mexLJ-down. Each primer contained base mismatch(es) that introduced a restriction site suitable for directional cloning (Table 2). PCR mixtures (50 μl) contained 1 U of Taq DNA polymerase (Invitrogen), 30 pmol of each primer, 5% (vol/vol) dimethyl sulfoxide, 0.2 μM of each deoxynucleotide, 1.5 mM MgCl₂, 0.5 μg chromosomal DNA, and 1 × PCR buffer (Invitrogen). General cycle conditions were 95°C for 5 min, followed by 30 cycles (1 cycle consisting of 95°C for...
TABLE 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant genotype or characteristic</th>
<th>Source or reference</th>
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<tbody>
<tr>
<td>P. aeruginosa</td>
<td></td>
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</tr>
<tr>
<td>PAO1</td>
<td>Prototroph expressing MexAB-OprM</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>PAO238</td>
<td>Δ(mexAB-oprM) Δ(sulA) Δ(mexCD-oprF) derivative of PAO1</td>
<td>Novagen</td>
</tr>
<tr>
<td>PAO238-1</td>
<td>PAO238 mexL</td>
<td></td>
</tr>
<tr>
<td>PAO314</td>
<td>PAO238-1 with Δ(mexJK::FRT)</td>
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<tr>
<td>E. coli</td>
<td></td>
<td></td>
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<tr>
<td>HPS1</td>
<td>F− Δ(lac-proAB) endA1 gmr496 hsdR17 supE44 relA1 recA1 thi1RED recA1 FtrI lacI mini-Tn5 Lac4</td>
<td></td>
</tr>
<tr>
<td>SU101</td>
<td>LexA reporter strain with a wild-type lac operator sequence upstream of lacZ</td>
<td></td>
</tr>
</tbody>
</table>

**Plasmids**

- **pCR2.1**
  - Ap⁺; TA cloning vector
- **pET-21b**
  - Ap⁺; T7 expression vector allowing construction of COOH-terminal His₆ fusion proteins
- **pTZ110**
  - Ch⁺; broad-host-range lacZ transcriptional fusion vector
- **pTZ120**
  - Ch⁺; broad-host-range lacZ transcriptional fusion vector pTZ110 with Ncol and NsiI restriction sites
- **pRK415**
  - Tt⁺; broad-host-range cloning vector containing Pₜₗ₉
- **pSR658**
  - Tt⁺; wild-type LexA-DBD fusion vector
- **pPS1150**
  - Ch⁺; pBSP II SK (+) carrying the mexL-mexK operon on a 6,945-bp NotI fragment from pJ22
- **pJ22**
  - Ch⁺; ~32-kb chromosomal DNA fragment from PAO238-1 cloned into pADD948
- **pPS1153**
  - Ch⁺; pUCP20T with S16-bp mexL fragment. from PAO1
- **pPS1166**
  - Ap⁺; pCR2.1 with a 640-bp PCR fragment from pPS1150 carrying mexL. This study
- **pPS1167**
  - Ap⁺; pET-21b with a 624-bp Ndel-NorI fragment from pPS1166; source of MexL-His6 with A47D change
- **pPS1173**
  - Ap⁺; pCR2.1 with a 205-bp PCR fragment from PAO1 including DNA 165 bp upstream and 36 bp downstream of the mexL start codon
- **pPS1175**
  - Ch⁺; pBluescript SK (+) carrying all of mexL and 466 bp of the 5′ end of mexL
- **pPS1176**
  - Ch⁺; pSS175 with a deletion of a 371-bp Xhol fragment internal to mexL
- **pPS1188**
  - Ap⁺; pCR2.1 with a 173-bp PCR fragment from PAO1 including DNA sequences between −244 and −71 from the mexL start codon
- **pPS1190**
  - Ap⁺; pCR2.1 with a 154-bp PCR fragment from PAO1 including DNA 41 bp upstream and 113 bp downstream of the mexL start codon
- **pPS1191**
  - Ap⁺; pCR2.1 with a 181-bp PCR fragment from PAO1 including DNA 68 bp upstream and 113 bp downstream of the mexL start codon
- **pPS1190**
  - Ap⁺; pCR2.1 with a 154-bp PCR fragment from PAO1 including DNA 41 bp upstream and 113 bp downstream of the mexL start codon
- **pPS1121**
  - Ch⁺; pTZ120 with a 173-bp EcoRI-PsI fragment from pPS1188; carrying DNA sequences located between 244 and 71 bp of the mexL-mexJK intergenic region
- **pPS1122**
  - Ch⁺; pTZ120 with a 154-bp SalI-PstI fragment from pPS1190 containing DNA 41 bp upstream and 113 bp downstream of the mexL start codon
- **pPS1123**
  - Ch⁺; pTZ120 with a 203-bp EcoRI-PstI fragment from pPS1190 carrying DNA 90 bp upstream and 113 bp downstream of the mexL start codon
- **pPS1124**
  - Ch⁺; pTZ120 with a 205-bp Xhol-EcoRV fragment from pPS1173 containing the entire 94-bp mexL-mexJK intergenic region
- **pPS1120**
  - Ch⁺; pTZ120 with a 203-bp EcoRI-PstI fragment from pPS1232 carrying DNA 90 bp upstream and 113 bp downstream of the mexL start codon
- **pPS1121**
  - Ch⁺; pTZ120 with a 184-bp PCR fragment from pPS1233; carrying DNA 71 bp upstream and 113 bp downstream of the mexL start codon
- **pPS1126**
  - Ap⁺; pCR2.1 with a 640-bp PCR fragment containing mexL from PAO1
- **pPS1127**
  - Ap⁺; pET-21b with a 624-bp Ndel-NorI fragment from pPS1216; source of MexL-His6
- **pPS1232**
  - Ch⁺; pTZ120 with a 203-bp PCR fragment from PAO1 including 90 bp upstream and 113 bp downstream of the mexL start codon
- **pPS1233**
  - Ap⁺; pCR2.1 with a 184-bp PCR fragment from PAO1 including 71 bp upstream and 113 bp downstream of the mexL start codon
- **pPS1236**
  - Ch⁺; pTZ120 with a 49-bp DNA fragment including DNA between −87 and −36 of the mexL-mexJK intergenic region
- **pPS1237**
  - Ch⁺; pTZ110 with Xhol-EcoRI fragment from pPS1176; this Xhol-EcoRI fragment containing the 5′ end of mexL and mexJK
- **pPS1245**
  - Ch⁺; pRK415 with a 816-bp mexL fragment from pPS1153
- **pPS1248**
  - Ap⁺; pCR2.1 carrying a 728-bp mexL fragment from pPS1153 (carries wild-type mexL)
- **pPS1249**
  - Ap⁺; pCR2.1 carrying a 728-bp mexL fragment from pPS1175 (carries PAO238-1 mutant mexL)
- **pPS1260**
  - Ch⁺; pSR658 carrying a 718-bp SalI-KpnI fragment containing mexL from pPS1264
- **pPS1287**
  - Ch⁺; pSR658 carrying a 718-bp SalI-KpnI fragment containing mexL from pPS1285
- **pPS1463**
  - Ap⁺; pCR2.1 with a PCR fragment containing 147 bp of mexL, the entire mexL-mexJK intergenic region, and 150 bp of mexL from PAO1

**a** Abbreviations: Ap⁺, ampicillin resistance; Ch⁺, carbenicillin resistance; Gm⁺, gentamicin resistance; Rif⁺, rifampin resistance; Sm⁺/Sp⁺, streptomycin/spectinomycin resistance.

**b** mexL, E. coli lac operon promoter.

45 ± 0.5°C for 45 s, and 72°C for 10 min per kb of DNA fragment to be amplified, and a final extension at 72°C for 10 min. Following the manufacturer’s instructions, the PCR fragments were cloned into pCR2.1 to yield pSS1188, pPS1190, pPS1191, pPS1232, pPS1233, and pPS1173, respectively. An additional fragment was obtained by hybridizing two oligonucleotides, L91Uinker and L91Dinker, encompassing positions −36 to −87 relative to the first nucleotide of the mexL start codon.

Next, the various portions of the mexL-mexJK intergenic region were fused to lacZ in pTZ120. To do this, pSS1188, pPS1190, pPS1173, pPS1232, and pPS1233 were digested with EcoRI-PstI, SalI-PstI, XhoI-EcoRV, EcoRI-PstI, and EcoRI-PstI, respectively. The digested DNA fragments were purified from the agarose gel and then ligated to pTZ120 digested with EcoRI-NsiI, SalI-NsiI, XhoI-SmaI, EcoRI-SalI, and EcoRI-NsiI to produce pPS1201, pPS1202, pPS1204, pPS1209, and pPS1210, respectively. For construction of pPS1236, the L91U and L91D oligonucleotide linkers with EcoRI and HindIII overhangs was ligated between the same sites of pTZ120. The inserts were PCR amplified from each plasmid and their nucleotide sequences determined. The fusion plasmids were then transformed into Δ(mexJK) strain PAO314 by electroporation (6). β-Galactosidase (β-Gal) activity was measured, and activity units were determined by the method of Miller (18). For complementation experiments, pPS1245 (mexL⁺) was transformed into strain PAO314 carrying the respective fusion plasmids.

**Construction of LexA-DBD-MexL fusions.** Wild-type mexL was PCR amplified from pPS1153 by using primer MexL-FL (including a SacI site and engineered to allow in-frame fusion of the MexL start codon to LexA) and primer MexL-FL.
Oligonucleotide Sequence

mexL-pET21b-up ............................................ 5'-TAAGCaATGTCCAGAATTCACCCTCC3- (PstI)
mexL-pET21down ........................................... 5'-CTCGGCGGCGGCGGAGGC3- (NotI)
LJ11 .......................................................... 5'-CTGGGATATCCGGCTCCAGGGC-3' (EcoRI)
LJ12 .......................................................... 5'-GATCCCATGGTTATGCATG-3 (NcoI)
LJ4U .......................................................... 5'-GATCCCATGGTTATGCATG-3' (NcoI)
LJ6U .......................................................... 5'-TGGACccATGGTTCGATTTTACT3- (NcoI)
LJ7U .......................................................... 5'-GCCGcaATTCATAGTATGAAATACTGGACT-3' (EcoRI)
LJ8U .......................................................... 5'-GCCGgaATTCATAGTATGAAATACTGGACT-3' (EcoRI)
LJ9U.......................................................... 5'-ATTTCCATGGGAATTACGTATGGTCTTCGTTTAATCTACGCGGCAAG-3'
LJ10.......................................................... 5'-AGGTCTTGGCCTATGATAGTTATGAAATACTGGACT-3' (EcoRI)
LJ11 .......................................................... 5'-GCGGCGCTCGGAGGAGGTCG-3' (PstI)
LJ12D .......................................................... 5'-GCGGCGCTCGGAGGAGGTCG-3' (PstI)
LJ13 .......................................................... 5'-GGGGAAGGCGCGCTCACCCTCCC3- (SacI)
LJ14 .......................................................... 5'-TGGACccATGGTTCGATTTTACT3- (NcoI)
LJ16D .......................................................... 5'-CTAGATGCACTGCAGCGGCCG-3'
NcoI/NsiI linker 1 ........................................ 5'-GATCCCATGGTTATGCATG-3' (NcoI, NsiI)
NcoI/NsiI linker 2 ........................................ 5'-GATCCCATGGTTATGCATG-3' (NcoI, NsiI)
MexLF .......................................................... 5'-AGGCGcaATTCATAGTATGAAATACTGGACT-3' (SacI)
MexLR .......................................................... 5'-CGAGCGGATACCTCGGCGCGGC-3' (KpnI)

* Some oligonucleotides were designed to contain mismatches (indicated by lowercase letters) that introduced new restriction sites (underlined and listed in parentheses) after PCR amplification.

Nondenaturing gradient PAGE. The native molecular size of MexL-His6 was estimated by nondenaturing gradient polyacrylamide gel electrophoresis (PAGE) (8). The gel contained a 5 to 20% linear gradient of polyacrylamide stabilized with a 0 to 20% linear glycerol gradient. Ten microliters of each sample was mixed with an equal volume of loading buffer (100 mM Tris-HCl [pH 7.5], 40% glycerol, 2 mg/ml bromophenol blue) and loaded on the gel. The electrophoresis was carried out at 80 V for 16 to 20 h at room temperature. The native molecular size of MexL-His6 was estimated by comparison of its migration distance to those of standard proteins (Sigma).

Size exclusion chromatography. Gel filtration chromatography of MexL-His6 was performed at 4°C as previously described (16) with some modifications. Sephadex G200 (Bio-Rad) was packed into a column (1.5 cm by 50 cm) (Bio-Rad) at a flow rate of 10.5 ml/h using an ECONO gradient pump (Bio-Rad). The packed column was equilibrated with 0.02 M Tris-HCl [pH 7.5], 1 mM EDTA, and 0.1 mM dithiothreitol (DTT) at the packing flow rate. Aliquots (500 μl) containing 1 mg/ml of protein, 25 μg/ml of pUCP20F DNA (20), and 1 mM ATP were loaded and chromatographed with 0.02 M Tris-HCl (pH 7.5), 1 mM EDTA, 0.1 mM DTT, and 15% glycerol using a flow rate of 7 ml/h. The absorbance (280 nm) of the effluent was monitored using an ECONO UV monitor (Bio-Rad) and recorded utilizing a Bio-Rad chart recorder. The standard proteins included β-amylase, BSA, chicken ovalbumin, alcohol dehydrogenase, and carbonic anhydrase (Sigma). The elution volume (V0) of each protein was determined using the time at which the sample peak (absorbance at 280 nm) was detected and the operating flow rate. The voided volume (Vv) and total volume (Vt) were determined using plasmid DNA and ATP, respectively. The average partition coefficient (K0) was determined as follows: K0 = (Vv - V0)/(Vt - V0).

DNA binding assay and measurement of binding affinity. To obtain templates for MexL binding assays, pPS1173 and pS1232 were digested with EcoRI-Xho1 and EcoRI-PstI, respectively. The EcoRI-Xho1 fragment of pPS1173 is a 205 bp DNA fragment containing the 94 bp mex-mex intergenic region. The fragments were gel purified, end labeled with [32P]dATP (3,000 Ci mmol−1; Perkin-Elmer Life and Analytical Sciences, Boston, MA) using Klenow fragment (Invitrogen), and purified by using the QIAquick nucleotide removal kit (QIA-GEN) (23). The end-labeled DNA fragments (~10,000 cpm) were incubated with purified MexL-His6 (~4.2 μM) in a binding reaction mixture containing 1× binding buffer (10 mM Tris-HCl [pH 7.5], 150 mM KCl, 20% glycerol, 2 mM EDTA, 2 mM DTT) and BSA (66 ng/ml) for 15 min at room temperature (21). The mixtures were immediately loaded on a 4% nondenaturing polyacrylamide gel in 1× TBE buffer (25 mM Tris-HCl [pH 8.3], 192 mM glycine) and electrophoresed at 160 to 165 V for 1 to 2 h using the same buffer. The gels were dried under vacuum, and labeled fragments were visualized by autoradiography. To determine the specificity of MexL, an excess amount of the same unlabeled 205 bp fragment from pPS1173 was included as competitor DNA.

To estimate MexL binding affinity, the labeled 205 bp DNA fragment from pPS1173 and the same experimental conditions described above were used except that the binding reactions were performed with increasing concentrations of MexL (0.5, 1.0, 2.0, 3.1, 4.1, 5.2, 6.2, 7.2, and 8.3 μM). After autoradiography, the
FIG. 1. Purification and characterization of MexL-His<sub>6</sub>. A) MexL was overexpressed with a carboxy-terminal hexahistidine tag in E. coli BL21(DE3). The expressed MexL-His<sub>6</sub> fusion protein was purified by Ni<sup>2+</sup> nitrilotriacetic acid affinity chromatography and analyzed by SDS-PAGE. The gel was stained with Coomassie blue. Lanes: M, marker proteins (from top to bottom, myosin, phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor); 1, uninduced cells containing pET-21b; 2, induced cells containing pET-21b; 3, uninduced cells containing pPS1216 expressing MexL-His<sub>6</sub>; 4, induced cells containing pPS1216; 5, purified MexL-His<sub>6</sub>. B) Native polyacrylamide gradient gel electrophoresis. The standard proteins (in kilodaltons) were as follows: 1, urease (11,000); 2, serum albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor; 3, chicken ovalbumin (45 kDa). The relative migration of MexL is indicated by the black diamond. C) Size exclusion chromatography on Sephadex G-200. The numbers and symbols refer to some of the same proteins employed in panel B. The additional standard proteins were as follows: 4, β-amylase (202 kDa); 5, alcohol dehydrogenase (167 kDa); and 6, carbonic anhydrase (29 kDa). The black diamond marks the K<sub>av</sub> of MexL-His<sub>6</sub>.

TABLE 3. Genetic evidence for multimerization of MexL<sup>a</sup>

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Protein expressed</th>
<th>β-Gal activity (Miller units)&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>pSR658 LexA-DBD</td>
<td></td>
<td>2.360 ± 172</td>
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<tr>
<td>pSR658NeuD</td>
<td></td>
<td>726 ± 36</td>
</tr>
<tr>
<td>pPS1256 LexA-DBD-MexL</td>
<td></td>
<td>445 ± 34</td>
</tr>
<tr>
<td>pPS1287 LexA-DBD-MexL&lt;sub&gt;47D&lt;/sub&gt;</td>
<td></td>
<td>248 ± 14</td>
</tr>
</tbody>
</table>

<sup>a</sup> Plasmids were transformed into E. coli SU101 carrying ald-lacZ on the chromosome, and β-Gal activity was measured in IPTG-induced cells. The negative control was pSR658 expressing LexA-DBD, and the positive control was pSR658NeuD expressing LexA-DBD-NeuD. <sup>b</sup> β-Gal activities were measured in triplicate samples. The averages ± standard deviations from a representative experiment are shown.

RESULTS AND DISCUSSION

Purified MexL is a tetrameric protein. To study repressor action at the molecular level, MexL-His<sub>6</sub> was overexpressed in E. coli and purified to near homogeneity by Ni<sup>2+</sup> affinity chromatography (Fig. 1A). The apparent molecular mass of purified MexL-His<sub>6</sub> was ~25,000 Da, which is close to the 24,242 Da calculated for the 220-amino-acid fusion protein monomer.

The molecular mass of MexL-His<sub>6</sub> in solution was determined by native PAGE and size exclusion chromatography (Fig. 1B and C). By native PAGE, the mass of MexL-His<sub>6</sub> was estimated at 97,600 Da, and by gel filtration, its mass was indicated...
as 83,500 Da. Both of these values are consistent with native MexL-His₆, being a tetrameric protein, with a calculated molecular mass of 96,968 Da.

MexL multimerization was genetically verified using the LexA-based system devised for this purpose (5, 7). To this end, the entire MexL protein-coding sequence was fused in frame to the LexA-DBD. The resulting plasmid construct, pPS1286, along with the vector control encoding only LexA-DBD, was transformed into E. coli strain SU101, which contains a chromosomally integrated sulA/H₁₁₀₃₂-/lacZ fusion, whose expression is under LexA control. As a positive control, a LexA-DBD-NeuD construct was used, since NeuD, a protein involved in polysialic acid synthesis in E. coli, is known to multimerize in this system (5, 7). IPTG-induced cells expressing only LexA-DBD (pSR658) exhibited high levels of /H₉₂₅₂-/Gal activity (Table 3), since LexA-DBD cannot multimerize and thus does not bind to the sulA promoter region and repress sulA'-lacZ transcription. In contrast, cells containing the positive-control pSR658/neuD expressing LexA-DBD-NeuD expressed lower (repressed) levels of β-Gal activity. Levels of β-Gal in IPTG-induced SU101 cells containing either pPS1286 (LexA-DBD-MexL) or pPS1287 (LexA-DBD-MexL₄₇D) were both greatly reduced, indicating that both forms of MexL can assist in LexA-DBD multimerization. Thus, the inability of MexL₄₇D to repress mexJK transcription is not due to lack of oligomerization of the mutant protein. β-Gal activity levels in uninduced cells containing the various plasmids were similar to those of uninduced cells containing pSR658 (data not shown).

MexL binds specifically to the mexL-mexJ intergenic region. To demonstrate that MexL binds to the mexL-mexJ intergenic region in a specific manner, gel mobility shift assays were performed. Purified MexL-His₆ band shifted an end-labeled
and MexL operator(s) to a region located between positions 81 to 20 region on the mexL coding strand that is protected by MexL, and the white bar indicates the −81 to −20 region on the mexL coding strand that is protected by MexL. All coordinates are relative to +1, which was arbitrarily chosen as the first nucleotide in the mexL start codon. The boundaries of a double-stranded synthetic oligonucleotide used for band shift assays are indicated by large brackets.

205-bp DNA fragment containing the 94-bp mexL-mexJ intergenic region (Fig. 2A). MexL-His6, binding to this fragment was specific, since the band shift was not observed in the presence of excess competitor DNA (the same unlabeled 205-bp fragment). When the same labeled DNA fragment was incubated with MexL-His6 mutant protein, no band shift was observed (Fig. 2B), demonstrating that the single A47D change in MexL was responsible for the band shift pattern as that observed with the wild-type MexL. The observed shift was not as complete as those passed the two inverted repeats, as well as the predicted protected regions encompassed the two inverted repeats, as well as the predicted.

Identification of the MexL operator sites. A closer analysis of the mexL-mexJ intergenic region (Fig. 3) revealed two inverted GTATTT repeats that are separated by 16 nucleotides. To more precisely map the MexL operator sites with respect to these inverted repeats, the mexL-mexJ intergenic region was end labeled on either the mexL or mexJ coding strand and subjected to DNase I footprinting. The footprinting profiles revealed a single protected region on either strand (Fig. 4A and B), and these two protected regions overlapped almost perfectly. Whereas the protected region on the mexJ coding strand was located between positions −22 and −84 upstream of the mexJ start codon, that of the mexL coding strand was situated between −20 and −81. The protected regions encompassed the two inverted repeats, as well as the predicted −35 and −10 regions of the mexL and mexJ promoters.

Several attempts were made to map the mexL transcription start sites using either primer extension, rapid amplification of cDNA ends, or RNase protection, but all attempts failed. However, the mexL transcription start was successfully mapped using RNase protection (data not shown). The labeled riboprobe covered the mexL-mexJ intergenic region and started 147 bp downstream of the mexL initiation codon. The protected area was 176 bp in length, which corresponded to a transcriptional start at a T residue located 29 bp upstream of the mexL start codon. This transcription start site was verified by reverse transcription-PCR on P. aeruginosa PAO238 total RNA using a common primer annealing within the expected mexL transcript and a second primer either annealing inside (3') or outside (5') of the start of the expected mexL transcript. The common primer yielded a PCR product only in combination with the inside primer (data not shown).

Autoregulation of mexL was ascertained by gene fusion analysis (Table 4). To do this, pPS1237 carrying a mexL-lacZ transcriptional fusion and pPS1245 containing the wild-type mexL gene under PmexL control were electroporated either alone or in combination into Δ(mexLJK) strain PAO314. β-Gal activity measurements revealed that mexL-lacZ transcription was reduced approximately sixfold in the presence of MexL. Similar results were obtained when the same plasmids were analyzed in E. coli HPS1, but the repression by MexL was only approximately twofold (data not shown). These data verified that MexL negatively autoregulates its own expression.

The predicted mexL promoter contains a −35 TTGAAA

TABLE 4. Autoregulation of mexL expression

<table>
<thead>
<tr>
<th>Plasmid (relevant genotype)</th>
<th>β-Gal activity (Miller units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pTZ110 (lacZ)</td>
<td>58 ± 25</td>
</tr>
<tr>
<td>pPS1245 (mexL-)</td>
<td>30 ± 4</td>
</tr>
<tr>
<td>pPS1237 (mexL-lacZ)</td>
<td>2,244 ± 287</td>
</tr>
<tr>
<td>pPS1237 (mexL-lacZ) + pPS1245 (mexL-)</td>
<td>374 ± 13</td>
</tr>
</tbody>
</table>

β-Gal expression was measured in cells of P. aeruginosa Δ(mexLJK) strain PAO314 containing empty fusion vector (lacZ) or plasmids expressing mexL from the lac promoter (mexL-) or a mexL-lacZ transcriptional fusion. Cells were grown in M9 medium plus glucose.

β-Gal activities were measured in triplicate samples. The averages ± standard deviations from a representative experiment are shown.

optimized. (ii) The techniques used for determining Kd and K0 were not optimal. (iii) An “assisting factor” was missing from the in vitro binding reactions, which is less likely.

FIG. 3. Regulatory elements within the mexL-mexJ intergenic region. The mexL and MexL coding regions are shown on the respective strands and indicated by light grey shading. The regions containing the inverted GTATTT repeat are boxed and shaded. The putative −10 and −35 regions of the mexL and mexJ promoters, as predicted by the promoter prediction program www.fruitfly.org/seq_tools/promoter.html, are boxed. The mexL transcription start site is marked with an asterisk. The black bar indicates the −84 to −22 region on the mexL coding strand that is protected by MexL, and the white bar indicates the −81 to −20 region on the mexL coding strand that is protected by MexL. All coordinates are relative to +1, which was arbitrarily chosen as the first nucleotide in the mexL start codon. The boundaries of a double-stranded synthetic oligonucleotide used for band shift assays are indicated by large brackets.

Several attempts were made to map the mexL transcription start sites using either primer extension, rapid amplification of cDNA ends, or RNase protection, but all attempts failed. However, the mexL transcription start was successfully mapped using RNase protection (data not shown). The labeled riboprobe covered the mexL-mexJ intergenic region and started 147 bp downstream of the mexL initiation codon. The protected area was 176 bp in length, which corresponded to a transcriptional start at a T residue located 29 bp upstream of the mexL start codon. This transcription start site was verified by reverse transcription-PCR on P. aeruginosa PAO238 total RNA using a common primer annealing within the expected mexL transcript and a second primer either annealing inside (3') or outside (5') of the start of the expected mexL transcript. The common primer yielded a PCR product only in combination with the inside primer (data not shown).

Autoregulation of mexL was ascertained by gene fusion analysis (Table 4). To do this, pPS1237 carrying a mexL-lacZ transcriptional fusion and pPS1245 containing the wild-type mexL gene under PmexL control were electroporated either alone or in combination into Δ(mexLJK) strain PAO314. β-Gal activity measurements revealed that mexL-lacZ transcription was reduced approximately sixfold in the presence of MexL. Similar results were obtained when the same plasmids were analyzed in E. coli HPS1, but the repression by MexL was only approximately twofold (data not shown). These data verified that MexL negatively autoregulates its own expression.

The predicted mexL promoter contains a −35 TTGAAA

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region, which is close to the TTGACA consensus sequence, but its −10 region TGTATT shares only 3 of 6 bases of the TATAAT consensus. The −10 and −35 regions of the mexL promoter are far from those of the consensus sequence, but this promoter location was confirmed by mapping the mexL transcript start site and regulators are often weakly expressed. Although the involvement of the GTATTT hexanucleotide sequences in MexL binding has not yet been directly proven, their central localizations within the protected regions and overlap with the mexL and mexJ promoter regions strongly supports their involvement in MexL binding and thus regulation of mexJ and mexL transcription.

Mapping of the mexJ promoter. Since we failed to obtain the mexJ transcriptional start site(s) using conventional mapping technologies, transcriptional lacZ gene fusions carrying various portions of the mexL-mexJ intergenic regions were constructed and used to approximate the location of the mexJ promoter. The various portions of the mexL-mexJ intergenic region contained in the mexL-lacZ fusion plasmids are shown in Fig. 5. The fusion plasmids were electroporated into Δ(mexLJK) strain PAO314, and β-Gal activities were measured. Cells containing either vector pTZ120 or pPS1201, which contains the −244 to −71 region devoid of obvious promoter sequences, did not show any detectable β-Gal activity. In contrast, cells containing pPS1204, which carries the entire intergenic region from positions −165 to +36, showed the highest activity, followed by cells containing pPS1236 (−86 to −37) and pPS1209 (−90 to +113), which exhibited significant levels of β-Gal activities. MexL, coexpressed from a compatible plasmid, repressed mexJ-lacZ transcription from pPS1204, pPS1209, and pPS1236. Plasmids pPS1202 (−41 to +133) and pPS1210 (−71 to +113) directed expression of lower levels of β-galactosidase activity; however, expression of this activity was not repressed by MexL. In summary, these experiments narrowed the mexJ promoter and its
FIG. 5. Localization of the mexP promoter within the mexL-mexJ intergenic region using lacZ transcriptional fusions. PCR fragments containing the portions of mexL-mexJ intergenic region indicated by the boxes were cloned in front of a promoterless lacZ to form the respective mexL-lacZ fusion plasmids pPS1201 to pPS1210; pPS1236 was obtained by ligating a synthetic oligonucleotide encompassing the sequences between positions −86 and −37 into pTZ120. The plasmids were transformed into Δ(mexLJ) strain PAO314, and the results are consistent with the results of footprinting experiments.

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REFERENCES