***migA*, a quorum-responsive gene of *Pseudomonas aeruginosa*, is highly expressed in the cystic fibrosis lung environment and modifies low-molecular-mass lipopolysaccharide**

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***Pseudomonas aeruginosa* is an opportunistic human pathogen which poses a major threat to patients with cystic fibrosis (CF). Excessive amounts of mucus present in the lungs of CF patients promotes the colonization of *P. aeruginosa*. The *migA* gene, encoding a putative glycosyltransferase, has been shown to be highly inducible by respiratory mucus derived from CF patients. In this study, it is further demonstrated by population transcript analysis that the *migA* gene is highly expressed in the CF lung environment. Deletion analysis of the *migA* promoter identified a *las*-box-like sequence commonly found in promoters that are responsive to quorum sensing regulation. Further analysis of *migA* expression in quorum-sensing-defective strains, as well as its expression in response to autoinducer molecules, demonstrated that *migA* is regulated by the RhII/RhlR quorum sensing regulatory system. Functionally, as the *MigA* sequence homology data suggested, the *migA* gene indeed affects the structure of LPS in *P. aeruginosa*. Increased expression of the *migA* gene results in a loss of core-plus-one LPS, while having no obvious effect on the long-chain O-antigen-bearing LPS. Although the exact biological role of the core-plus-one LPS is not clear, these experimental results suggest that *migA* up-regulation in the CF lung environment is part of the adaptive response which confers on *P. aeruginosa* a survival advantage.**

Keywords: *migA*, mucus, LPS and quorum response

INTRODUCTION

Pseudomonas aeruginosa is an opportunistic pathogen which poses a major threat to immunocompromised patients, burn victims and cystic fibrosis (CF) patients (Bodey *et al.*, 1983; Gilligan, 1991; Holder, 1993). More than 90% of mortality among CF patients is caused by lung infection with *P. aeruginosa*. The presence of excessive amounts of viscous mucus in the lungs of CF patients is thought to be a major contributing factor to their susceptibility to infection by *P. aeruginosa*. The mucus not only affects the host defence system by blocking the movement of alveolar macrophages and

inhibiting cilia movement-associated bacterial clearance, but it also provides a unique growth environment, allowing *P. aeruginosa* to persist (Carnoy *et al.*, 1993; Marshall & Carroll, 1991). The host immune response mounted against the infecting bacteria causes lung tissue damage and eventually leads to the failure of lung function (Gilligan, 1991).

As an opportunistic pathogen, *P. aeruginosa* relies on a combination of virulence factors to infect its host. Production and secretion of many of these virulence factors are increased at high cell density through a quorum sensing mechanism. Two such regulatory systems in *P. aeruginosa*, *las* and *rhl*, have been well characterized (Latifi *et al.*, 1996, 1995; Pearson *et al.*, 1994, 1995; Pesci *et al.*, 1997; Pesci & Iglewski, 1997; Winson *et al.*, 1995). Each is composed of two

Abbreviations: CF, cystic fibrosis; C12-HSL, 3-oxo-dodecanoyl homoserine lactone; C4-HSL, butanoyl homoserine lactone.

components, an inducer locus, *lasI* or *rhII*, that controls the synthesis of diffusible autoinducer molecule 3-oxo-dodecanoyl homoserine lactone (C12-HSL; PAI-1) or butanoyl homoserine lactone (C4-HSL; PAI-2), respectively, and a response locus, *lasR* or *rhIR*, that encodes transcriptional factors which become active upon binding to the respective autoinducers (Passador *et al.*, 1993). The PAI-1-bound form of LasR enhances the expression of genes encoding secreted proteases, siderophore, ADP-ribosylating enzymes and genes that control secretion of virulence factors (Passador *et al.* 1993; Seed *et al.*, 1995; Toder *et al.*, 1994, 1991; Winson *et al.*, 1995). PAI-2-bound RhIR controls some genes affected by LasR and, in addition, activates the expression of loci responsible for the production of toxic chemicals, including hydrogen cyanide, rhamnolipids and phenazines (Latifi *et al.*, 1996, 1995; Ochsner & Reiser, 1995; Ochsner *et al.*, 1994; Pearson *et al.*, 1997).

Chronic CF lung infections by *P. aeruginosa* start from acute infections early in the life of CF patients. The chronic infection has interspersed episodes of acute lower respiratory tract symptoms. As the chronic stage of the infection develops, there is a gradual transition of the *P. aeruginosa* isolates from a predominately non-mucoid, smooth LPS, piliated and motile phenotype to a predominately mucoid, rough LPS, non-piliated and non-motile phenotype (Govan, 1988; Luzar & Montie, 1985; Mahenthiralingam *et al.*, 1994; Hancock *et al.*, 1983; Woods *et al.*, 1986). These morphological changes reflect bacterial adaptation to the CF lung environment, resulting from changes in gene regulation as well as mutation and selection. The mechanism for conversion to the mucoid phenotype was shown to be due to mutations in the anti- σ factor gene, *mucA*, which alleviate repression of a 22 kDa σ factor encoded by *algU* (or *algT*) that is required for the expression of the alginate biosynthetic pathway genes, in combination with environmental signals that activate genes under the control of two transcriptional regulators, AlgR and AlgB (Deretic *et al.*, 1995; Govan & Deretic, 1996; Ma *et al.*, 1998). Alginate is the predominant exopolysaccharide moiety of the mucoid substance in CF isolates. In the case of pili and motility losses, mutation in the *rpoN* gene, which is required for pilus and flagellum assembly, accounts for the majority of the cases (Mahenthiralingam *et al.*, 1994). However, the molecular mechanism for the conversion from smooth LPS to rough LPS is not as clear. *P. aeruginosa* LPS consists of distinct A and B bands; the high-molecular-mass B-band determines the O-antigenic specificity of the bacterium, whereas the antigenically conserved A-band is a common antigen consisting of α -D-rhamnose trisaccharide repeating units. Wild-type *P. aeruginosa* strains express a proportion of their LPS as long-chain molecules, containing up to 50 repeating units (smooth LPS). In addition, these strains will have LPS that is devoid of O-polysaccharide (rough LPS) and LPS containing only one O chain repeating unit (core-plus-one LPS) (Rocchetta *et al.*, 1999).

To understand the molecular mechanism of bacterial

adaptation to the CF lung environment, we have previously isolated and characterized *P. aeruginosa* genes that are specifically inducible by respiratory mucus derived from CF patients (Wang *et al.*, 1996). One of these encodes a putative glycosyltransferase which was predicted to be involved in LPS or exopolysaccharide modification. In this report, we show that the *migA* gene product indeed modifies bacterial LPS structure, predominantly the core-plus-one LPS. Furthermore, we demonstrate that the *migA* gene is regulated by the RhII/RhIR quorum sensing regulatory system and is highly expressed in the CF lung environment. The significance of these observations is discussed.

METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* and *P. aeruginosa* were grown in L-agar or L-broth at 37 °C. Minimal medium A (Min A; Davis & Mingioli, 1950) was also used for the growth of *P. aeruginosa*. Antibiotics were used at the following final concentrations ($\mu\text{g ml}^{-1}$): for *E. coli*, ampicillin, 100; spectinomycin, 50; streptomycin, 25; tetracycline, 20; for *P. aeruginosa*, carbenicillin, 150; spectinomycin, 200; streptomycin, 200; tetracycline, 100. *P. aeruginosa* isolates from soil, blood of infected individuals and CF sputa were designated as environmental, non-CF and CF isolates, respectively. Both CF and non-CF clinical isolates were obtained from the Medical Center of the University of Arkansas for Medical Sciences, USA.

To generate the *migA* insertional mutant, a 2 kb Ω fragment was inserted into the *SalI* site of the 5' *migA* coding region in pMSB3A (Wang *et al.*, 1996). The resulting plasmid, pMSB3A Ω , was linearized by *EcoRI* digestion and electroporated into PAK as described previously (Jin *et al.* 1994). Colonies with resistance to spectinomycin/streptomycin were selected, followed by screening for sensitivity to carbenicillin. Chromosomal double crossovers were further confirmed by Southern hybridization.

To generate *migA* promoter deletion constructs, either available restriction sites upstream of the *migA* promoter or *EcoRI* sites generated in desired positions by site-directed mutagenesis (Kunkel *et al.*, 1987) were used in combination with the *BamHI* site in pMS18 (Wang *et al.*, 1996). Isolated *migA* promoter fragments were fused to a promoterless *lacZ* by cloning into the *EcoRI/BamHI* sites of pDN19lac Ω (Totten & Lory, 1990). To introduce an *EcoRI* site at positions -200, -89 and -38 relative to the ATG translational start codon of *migA* in pMS18, the following three oligonucleotides were used: *migA*-200, 5'-GGCCTACCTCTAGAATTCCGACGGTATACTG-3'; *migA*-100, 5'-CCAGCCCCGCGCGAATTCGATCGCCGGAACG-3'; and *migA*-50, 5'-GACGGCCCCCTGGAATTCCTACCGGGCAAGACG-3', respectively.

Sputum collection and extraction of total RNA. Sputum samples from nine paediatric patients were utilized in this study. These patients were part of a larger cohort of patients currently being followed (Storey *et al.*, 1992, 1997, 1998). The patients in this study all attend the Alberta Children's Hospital Cystic Fibrosis Clinic, Canada. Voluntary consent was obtained from all patients and their guardians. Ethical approval of the study design was given by the Conjoint Research Ethics Board of the University of Calgary, Canada. All samples used in this study were collected from patients who were in the

Table 1. Strains and plasmids used in this study

Strain/plasmid	Description	Source
<i>E. coli</i>		
DH5 α	F ⁻ ϕ 80 δ lacZ Δ M15 <i>endA1 recA1</i> <i>hsdR17(m⁺m⁻) supE44 thi-1 relA1</i> Δ (<i>lacZYA-argF</i>) <i>gyrA96 deoR</i>	Hanahan (1983)
CJ236	<i>ung⁻/dut⁻</i> mutant strain	Kunkel <i>et al.</i> (1987)
<i>P. aeruginosa</i>		
PAK	Laboratory strain	David Bradley, Newfoundland, Canada
PAK <i>migA</i> :: Ω	<i>migA</i> insertional mutant of PAK	This study
PAK <i>wbpL</i> ::Gm ^R	<i>wbpL</i> mutant strain of PAK, deficient in A- band and B-band LPS	Belanger <i>et al.</i> (1999)
PAO1	Laboratory strain	Holloway <i>et al.</i> (1979)
PAO1 <i>algR</i> ::Gm ^R	<i>algR</i> mutant derivative of PAO1	Wozniak & Ohman (1994)
PDO100	<i>rhII</i> mutant of PAO1	Brint & Ohman (1995)
PAO-JP1	<i>lasI</i> mutant strain of PAO1	Passador <i>et al.</i> (1993)
PAO-JP2	<i>lasI</i> and <i>rhII</i> double mutant of PAO1	Pearson <i>et al.</i> (1997)
Plasmids		
pDN18	Broad-host-range plasmid, IncP; Tc ^r	Nunn <i>et al.</i> (1990)
pSJ9610	<i>migA</i> gene clone under the control of <i>lac</i> promoter in pDN18	This study
pDN19lac Ω	A promoterless <i>lacZ</i> transcriptional fusion vector; Sp ^r Sm ^r Tc ^r	Totten <i>et al.</i> (1990)
pMS18	5' <i>migA</i> clone in pTZ18R	Wang <i>et al.</i> (1996)
pMSB3A	3 kb <i>EcoRI</i> fragment containing <i>migA</i> cloned into pTZ18R	Wang <i>et al.</i> (1996)
pMSB3A Ω	<i>migA</i> gene in pMSB3A disrupted by Ω insertion	This study
pMS18lac Ω	<i>migA</i> :: <i>lacZ</i> fusion in pDN19lac Ω	Wang <i>et al.</i> (1996)
pSJ9717	<i>migA</i> :: <i>lacZ</i> fusion in pDN19lac Ω	This study
pSJ9718	<i>migA</i> :: <i>lacZ</i> fusion in pDN19lac Ω	This study
pSJ9729	<i>migA</i> :: <i>lacZ</i> fusion in pDN19lac Ω	This study
pSJ9728	<i>migA</i> :: <i>lacZ</i> fusion in pDN19lac Ω	This study
pSJ9727	<i>migA</i> :: <i>lacZ</i> fusion in pDN19lac Ω	This study
pTS400	<i>lasB-lacZ</i> translational fusion construct	Passador <i>et al.</i> (1993)
PSJ9445	2 kb <i>KpnI-BamHI</i> fragment containing <i>migA</i> gene of PAK in pTZ18R	This study
pIL17	<i>migA</i> gene of PA103 cloned into pUC18	This study
pCC27	<i>algD</i> -containing clone	Chitinis & Ohman (1990)
pPS1816	<i>lasA</i> -containing clone	Schad & Iglewski (1988)

moderate to severe disease categories. Also, the selected patients had acquired solely or predominantly *P. aeruginosa* infections.

Sputum from the CF patients was collected as described previously (Storey *et al.*, 1998). RNA was extracted from the sputum samples, blotted onto Nylon membrane, hybridized and signal intensity was measured as described by Storey *et al.* (1992). A 613 bp internal *HincII* fragment of *migA* from pIL17 was used as a probe to measure *migA*-specific transcripts. A 700 bp internal *EcoRI* fragment of *algD* from plasmid pCC27 (Chitinis & Ohman, 1990) and a 450 bp *PstI* fragment of *lasA* from pPS1816 (Schad & Iglewski, 1988) were used to examine *algD*-specific and *lasA*-specific transcripts, respectively.

Primer extension. Total bacterial RNA was isolated from strain PAK that was grown in minimal medium A with or

without 10 % respiratory mucus from CF patients. For primer extension experiments, an oligonucleotide, *migA-down* (5'-CTTCTTCCAGGTACTTTTCCGCGTTG-3') was used as primer and according to the protocol described by Maniatis *et al.* (1982). The same primer was also used to sequence the *migA* promoter region to identify the transcription initiation site.

LPS preparation, SDS-PAGE and Western blot analysis. LPS was prepared from whole-cell lysates of *P. aeruginosa* by the method of Hitchcock & Brown (1983). LPS samples were run on standard discontinuous 12.5 % glycine SDS-PAGE gels (Hancock & Carey, 1979) or by commercially prepared 16 % Tricine SDS-PAGE gels (Novex). LPS was visualized by the silver-staining methods of Dubray & Bezard (1982) for glycine SDS-PAGE and Tsai & Frasch (1982) for Tricine gels. For Western immunoblots, the procedure was essentially as

described by de Kievit & Lam (1994). LPS separated by SDS-PAGE was transferred onto nitrocellulose and then blocked with 3 % skim milk. For detection of *P. aeruginosa* LPS, blots were immersed in hybridoma-culture supernatants containing mAb O25G3D6 (specific for B-band LPS serotype O6; Emara *et al.*, 1995), mAb N1F10 (specific for A-band LPS; Lam *et al.*, 1989) or mAb 7-4 (specific for LPS inner core; de Kievit & Lam, 1994). After incubation with the corresponding first antibodies, blots were washed and then incubated with the appropriate second antibody. The second antibody for anti-*P. aeruginosa* LPS mAbs was goat anti-mouse F(ab')₂-alkaline phosphatase conjugate (Jackson ImmunoResearch Laboratories) at the dilutions suggested by the manufacturer. Following a series of washes, the blots were developed using a substrate consisting of 30 mg nitro blue tetrazolium and 15 mg 5-bromo-4-chloro-3-indolyl phosphate (toluidine salt; Sigma) in 100 ml 0.1 M sodium bicarbonate buffer (pH 9.8).

β -Galactosidase assay. *P. aeruginosa* strains harbouring various *lacZ* fusion constructs were grown in MinA with or without 10 % CF respiratory mucus and measured for β -galactosidase activities as described by Wang *et al.* (1996). To induce *migA* and *lasB*, 100 nM of their respective auto-inducers, C12-HSL (PAI-1) or C4-HSL (PAI-2) was added at the start of culture and β -galactosidase activities were measured at various cell densities.

RESULTS

migA of *P. aeruginosa* is highly expressed in the CF lung environment

The *migA* gene was identified by virtue of its inducibility *in vitro* by respiratory mucus derived from CF patients (Wang *et al.*, 1996). To test if this gene is indeed expressed *in vivo* during infection of CF lungs, we conducted population transcript analysis. Sputum samples were collected from CF patients who had confirmed *P. aeruginosa* infections. Total RNA was isolated from 25 sputum samples obtained from nine CF patients over a period of 3 years and blotted onto a nitrocellulose membrane. When the membrane was hybridized with the *migA* probe, all 25 samples showed a hybridization signal above background levels of approximately 100- to 400-fold relative intensity (Fig. 1). In the samples that we selected, *P. aeruginosa* was either the sole or the predominant pathogenic organism in the sample. Thus, hybridization of the *migA* probe is not likely to be due to hybridization of the probe to RNA from other species. This suggested that in the lungs of patients with CF, *migA* was commonly expressed. We have also probed these RNA samples with *algD*, encoding a key enzyme in the biosynthesis of alginate, which is known to hybridize strongly to these RNA samples (Storey *et al.*, 1997). Our second probe, *lasA*, encoding an elastase enzyme, usually hybridizes at an intermediate level to these RNA samples (Storey *et al.*, 1997, 1998). The hybridization signals with the *migA* probe appear to be at a level nearer to the *algD* probe, suggesting abundant transcript accumulation in the bacterial populations found in the sputa from CF patients (Fig. 1).

The data in Fig. 1 indicate that *migA* transcript levels

positively correlate to that of *algD*. This suggests that these genes might be controlled by the same regulatory system. This possibility seems more likely when we consider the fact that both genes are possibly involved in polysaccharide biosynthesis or modification. To test this, we examined the effect of *algR*, a known regulator of *algD*, on *migA* expression. A *migA::lacZ* fusion construct, pMS19lac Ω , was introduced into wild-type PAO1 and its *algR* mutant derivative, and the levels of β -galactosidase activity were compared. Results showed that mucus-mediated *migA* gene activation was not altered in the *algR* mutant background compared to the wild-type strain (data not shown), indicating that mucus-mediated expression of *migA* is independent of *algR*.

Deletion analysis of the *migA* promoter

To identify functional promoter elements of the *migA* gene, a series of promoter deletion constructs were generated using both existing restriction sites as well as sites generated by site-directed mutagenesis. The promoter deletion fragments were fused to the promoterless *lacZ* gene in pDN19lac Ω , and PAK cells harbouring the resulting constructs were tested for β -galactosidase activities with or without the addition of CF respiratory mucus. As shown in Fig. 2, promoter deletion up to -200 (relative to the putative ATG start codon) did not affect wild-type promoter activity but deletion to -89 totally abolished the inducibility by mucus. These results indicate that important *migA* promoter elements reside within the 111 bp region between -200 and -89.

The *migA* transcription initiation site was further determined. Based on the above promoter deletion analysis, an oligonucleotide that hybridizes downstream of the ATG start codon was used as a primer to conduct a primer extension experiment. Total RNA was prepared from the same number of PAK cells grown in MinA medium with or without 10 % CF respiratory mucus. Under both growth conditions, a single transcription initiation site corresponding to the A at position -23 upstream of the ATG start codon was identified (Fig. 3). A higher intensity of signal was detected in the RNA sample prepared from cells grown in the presence of CF mucus, representing a higher transcript level. An identical size transcript was detected under non-inducing conditions after a longer exposure (data not shown). Comparison of sequences between -200 and -23 to binding sites of known transcriptional factors revealed two *las*-box-like sequences (Fig. 3) that are found among quorum-responsive gene promoters (Whiteley *et al.*, 1999). The consensus *las* box consists of invariant CT and AG that are separated by 12 variable nucleotides CT-(N₁₂)-AG and the two *las*-box-like sequences of *migA* have CT-(N₁₁)-AG sequences. This finding suggests that the *migA* gene might be regulated by a quorum sensing system. However, no obvious recognition sequence for σ^{70} or σ^{54} was found in the sequence upstream of the transcription initiation site. Since pSJ9729 contains the smallest functional *migA*

Fig. 1. Comparison of population transcript accumulation of *migA* (white bars), *algD* (hatched bars) and *lasA* (black bars) in *P. aeruginosa* cells chronically colonizing the lungs of CF patients. Total RNA was isolated from sputum samples of CF patients and blotted onto a nitrocellulose membrane. Relative intensities of radioactive signals after hybridization with individual probes are shown (see Methods).

Fig. 2. Promoter deletion analysis of the *migA* gene. DNA fragments containing various lengths of the *migA* promoter region were fused with the promoterless *lacZ* reporter gene and introduced into the wild-type PAK strain. β -Galactosidase activities (Miller units) were measured after overnight growth in MinA with (+) or without (-) 10% CF respiratory mucus. B, *Bam*HI; K, *Kpn*I; R, *Eco*RI site introduced by site-directed mutagenesis (see Fig. 3 for sequence). A chloramphenicol acetyltransferase homologue (*cat*) and two exotoxin A regulatory genes (*regA* and *regB*) reside upstream of *migA*.

Fig. 3. Determination of the transcription initiation site of the *migA* gene by primer extension. (a) DNA sequence of the *migA* promoter region. EcoRI sites introduced by site-directed mutagenesis which were used to construct plasmids pSJ9727, pSJ9728 and pSJ9729 are shown. Lower case letters represent mismatch mutations introduced to generate EcoRI sites. The transcription initiation site 'A' is shown in bold and underlined. (b) Autoradiogram of primer extension experiment. Lanes marked with - and + contain RNA samples prepared from PAK grown in the absence or presence, respectively, of 10% CF mucus. Equal amounts of the two RNA samples were used. DNA sequencing termination reactions for A, C, G and T are marked. The arrow indicates the extended primer and the DNA sequence of the anti-sense strand is shown next to it.

promoter, we utilized this plasmid construct for further analysis of *migA* promoter activity.

migA is regulated by the RhII/RhIR quorum sensing system

To investigate whether *migA* is indeed regulated by the quorum sensing system, cell-density-dependent expression of the *migA*::*lacZ* fusion was first tested in a PAK background. PAK(pSJ9729) was grown in L-broth with

Fig. 4. RhII/RhIR-mediated activation of *migA*::*lacZ* expression. (a) Cell-density-dependent expression of the *migA* gene. Strain PAK containing the *migA*::*lacZ* fusion construct pSJ9729 (■) or vector pDN19lacZ (▲) was cultured in L-broth at 37 °C and β -galactosidase activity was plotted against cell density. (b) *rhII* is required for the cell-density-dependent *migA* expression. Plasmid pSJ9729 was introduced into wild-type PAO1 as well as isogenic *lasI*, *rhII* and *lasI/rhII* mutant derivatives PDO100, PAO-JP1 and PAO-JP2, respectively. β -Galactosidase activities were measured at low (OD_{600} 1.0; black bars) or high (OD_{600} 4.5; white bars) cell density after growth in L-broth. (c) Effect of PAI-1 and PAI-2 autoinducers on the expression of *migA*. β -Galactosidase activities of the *lasI/rhII* double mutant (PAO-JP2) harbouring *migA*::*lacZ* and *lasB*::*lacZ* fusion constructs pSJ9729 and pTS400, respectively, were grown to an OD_{600} of 3.5 in L-broth in the absence (white bars) or presence of 100 nM C12-HSL (shaded bars), 100 nM C4-HSL (black bars) or 100 nM each of C12-HSL and C4-HSL (hatched bars).

appropriate antibiotics at 37 °C. As shown in Fig. 4(a), β -galactosidase activity increased proportional to cell density and more than fourfold greater β -galactosidase activity was observed at high cell density than that at

Fig. 5. Effect of *migA* on the production of high-molecular-mass LPS. (a) Silver-stained SDS-PAGE. (b, c) Western immunoblots reacted with the A-band-specific mAb N1F10 and B-band-specific mAb O25G3D6, respectively. The *migA* gene was constitutively expressed by a *lac* promoter in pSJ9610. Lanes: 1, wild-type PAK; 2, PAK(pSJ9610); 3, PAK(*migA*:: Ω).

low cell density (OD_{600} 4.5 vs 1.0), demonstrating cell-density-dependent expression, a characteristic of genes under the control of quorum sensing systems. We further examined whether *migA* is regulated by either one or both of the two well characterized quorum sensing systems in *P. aeruginosa*, *lasI*/R or *rhII*/R. Expression of *migA*::*lacZ* in *lasI* and *rhII* single mutants, as well as in a *lasI*/*rhII* double mutant background was compared to that in the wild-type PAO1 background. At high cell density (OD_{600} 4.5), *migA* expression levels were much lower in either the *rhII* or *lasI*/*rhII* double mutant background than in wild-type strain. However, the *migA* expression level in the *lasI* mutant background was not different from that in the wild-type PAO1 background (Fig. 4b), suggesting that *migA* is regulated by the RhII/R system. Furthermore, *migA* expression in a *lasI*/*rhII* double mutant background is stimulated by the addition of C4-HSL (PAI-2) but not by C12-HSL (PAI-1). Interestingly, addition of both PAI-1 and PAI-2 did not induce *migA* expression (Fig. 4c), which is similar to other RhII/RhIR-regulated genes described by Pesci *et al.* (1997). It was postulated in these cases that PAI-1 inhibits the binding of PAI-2 to RhIR. As a control, the expression of *lasB*, which is known to be regulated by LasR, was stimulated by PAI-1 but not by PAI-2 in the same mutant background (Fig. 4c).

***migA* affects low-molecular-mass LPS production**

The *migA*-encoded protein shows homology to glycosyltransferases associated with LPS biosynthesis in other Gram-negative bacteria (Wang *et al.*, 1996), suggesting a

possible role of *migA* in LPS core synthesis. To determine the role of *migA* in LPS biosynthesis, LPS banding patterns were compared among strains of wild-type PAK, a *migA*:: Ω mutant and PAK harbouring a plasmid that constitutively expresses the *migA* gene (pSJ9610). Two gel systems were used to examine the LPS banding patterns, Tricine SDS-PAGE and the standard glycine discontinuous SDS-PAGE system followed by silver-staining and Western immunoblotting with appropriate mAbs. The Tricine SDS-PAGE system allows for significantly improved resolution of low-molecular-mass LPS of the core region (Lesse *et al.*, 1990) while standard SDS-PAGE provides good resolution of high-molecular-mass LPS, such as O antigen. High-molecular-mass LPS of wild-type *P. aeruginosa* strain PAK, which includes A-band and B-band LPS, was analysed by silver-stained SDS-PAGE gels and Western immunoblots using B-band-specific mAb O25G3D6 and A-band-specific mAb N1F10. No significant difference in the production of high-molecular-mass LPS was observed between the parent strain PAK and the *migA*:: Ω mutant (Fig. 5). Results from the Tricine gel analysis showed, however, that the expression of *migA* clearly affected core LPS production. Wild-type PAK exhibits two LPS bands corresponding to 'complete-core' (band 2) and 'core-plus-one O antigen repeat' (band 1) (Fig. 6, lane 1). For comparison, LPS from a PAK *wbpL* mutant, deficient in the initial glycosyltransferase required for both A-band and B-band LPS production, was included as a control in lane 4. As expected, only band 2 (complete core) was detected. PAK(pSJ9610) produced only band 2 (Fig. 6,

Fig. 6. Visualization of low-molecular-mass LPS by Tricine SDS-PAGE analysis. LPS was purified from the four bacterial strains and subjected to separation by Tricine SDS-PAGE. (a) Silver-stained Tricine SDS-PAGE gel. (b) Western immunoblot reacted with LPS inner-core-specific mAb 7-4 which reacts with low-molecular-mass LPS. Lanes: 1, wild-type PAK; 2, PAK(pSJ9610); 3, PAK(*migA*:: Ω); 4, PAK(*wbpL*⁻). Band 1, core-plus-core; band 2, complete core.

lane 2) which co-migrated with the complete core band of the *wbpL* mutant (Fig. 6, lane 4). Thus high-level expression of *migA* in PAK(pSJ9610) affects core biosynthesis and resulted in the loss of the core-plus-one LPS molecules. The inhibitory role of *migA* in the formation of core-plus-one LPS correlates with the observation that the *migA* mutant strain produces an increased amount of the core-plus-one LPS (Fig. 6, lane 3).

DISCUSSION

The initial screen for *migA* used mucus from CF sputa to induce the expression of various genes (Wang *et al.*, 1996). Although the *migA* gene was shown to be inducible by CF respiratory mucus *in vitro*, it was difficult to demonstrate its expression *in vivo* due to the lack of an animal model which mimics the CF lung infection. Population transcript analysis, first described by Storey *et al.*, (1992) can best predict the *in vivo* situation and was therefore adopted in this study. We detected a high level of transcript accumulation in all 25 RNA samples extracted from the bacterial population found in CF sputum samples. The level of *migA* transcript accumulation was almost as abundant as *algD* transcript accumulation (Fig. 1). These results argue that *migA* is commonly transcribed at high levels by the *P. aeruginosa* populations in the lungs of patients with CF.

Quorum sensing gene regulation plays an important role in the pathogenesis of *P. aeruginosa*. In response to an

increased bacterial cell density, the quorum sensing system not only mediates an increased synthesis and secretion of extracellular enzymes to meet the nutritional requirement of the pathogen, but also mediates biofilm formation to overcome adverse environmental conditions (Pesci & Iglewski, 1997; Fuqua & Greenberg, 1998). Whiteley *et al.* (1999) have recently reported on the identification of a large number of genes that are under the control of quorum sensing systems. By comparing their promoter sequences, a consensus *las* box promoter element (CT-N₁₂-AG), potentially the LasR and/or RhIR recognition sequence, has been deduced. However, the *migA* gene was not included in that study. Our experimental data clearly demonstrate that *migA* expression is dependent on the *rhII/rhlR* regulatory system and its promoter also contains two *las*-box-like sequences (CT-N₁₁-AG) that fall into the essential promoter region identified by the promoter deletion analysis. Whether one or both of the *las*-box-like sequences are required for *migA* expression is not known at this point.

Since the CF respiratory mucus that we used was derived from CF patients who had acquired *P. aeruginosa* infection, the mucus should contain high concentrations of autoinducers. However, the mucus was subjected to dialysis against distilled water before use and therefore it is unlikely that residual autoinducer molecules are responsible for the activation of *migA* gene expression. Recently, our group has observed that addition of CF respiratory mucus into minimal medium actually promotes *P. aeruginosa* growth which results in a higher cell density (unpublished results). It is therefore possible that this higher cell density triggers the quorum sensing response which activates *migA* expression. The growth promoting activity of the CF mucus is consistent with the reported observations of high bacterial cell densities in CF lungs (Potts *et al.*, 1995). The exact components of the mucus that promote the growth of *P. aeruginosa* deserve further study, since they represent important host factors that promote *P. aeruginosa* colonization and the bacterial genes mediating the utilization of these mucus components could potentially serve as targets in antimicrobial strategy.

The observation that MigA shares amino acid sequence homology with glycosyltransferases, implies a potential role of this protein in LPS modification. We have shown here that *migA* indeed affects the modification of low-molecular-mass LPS. High-level expression of *migA* leads to the loss of the core-plus-one LPS band, whereas *migA* mutation results in an increase in this band. We hypothesize that MigA is a glycosyltransferase that transfers a sugar residue onto the core region and consequently modifies the attachment point for O antigen on the outer-core oligosaccharide. The MigA modified outer-core molecule would therefore be less able to serve as an acceptor for O antigen. A ligase enzyme, WaaL, joins the O antigen polysaccharide to the lipid A core and there is a strong evidence that the nature of the acceptor molecule is important for this ligation reaction. Evidence to support this hypothesis is

as follows. (i) In *P. aeruginosa*, the structure of the outer-core region in a fully assembled O chain containing LPS differs from that of the O-chain-deficient rough LPS. The point of attachment of the O chain to the outer core is at the 1,3-linked rhamnose (Sadovskaya *et al.*, 2000). When this residue is missing, the core molecule is less able to serve as an acceptor for O antigen attachment. Genetic evidence suggests that the rhamnose residue in the outer-core region is necessary for O antigen attachment (Rahim *et al.*, 2000). (ii) A rhamnosyltransferase, required for the addition of the 1,3-linked rhamnose which serves as the attachment point for O antigen to the outer core, shares amino acid sequence homology with MigA (Rahim *et al.*, 2000). (iii) MigA is homologous to glycosyltransferases, including WaaV of *E. coli*, possessing a type R1 core (Heinrichs *et al.*, 1998). The proposed function of WaaV is that of a UDP-glucosyltransferase required for the addition of a 1,3-linked glucose, which is a side-branch substituent in the outer core and acts as the attachment site for long-chain O antigen to an R1-type core.

Recently, Ernst *et al.* (1999) have demonstrated that the lipid A portion of the *P. aeruginosa* LPS undergoes specific modification during chronic colonization of CF lungs. The CF-specific lipid A apparently contains palmitate and aminoarabinose which are associated with bacterial resistance to cationic antimicrobial peptides and increased inflammatory responses, indicating that they are likely to be involved in airway disease. Since the *migA* gene is highly expressed in the CF lung environment and interferes with the formation of core-plus-one LPS, it is possible that suppression of such low-molecular-mass LPS renders *P. aeruginosa* a survival advantage in the CF lung environment. If indeed this is the case, a prolonged colonization in the CF lung environment could result in enrichment of adaptive mutants in which *migA* gene expression become constitutive, similar to the enrichment of mucoid isolates of *P. aeruginosa*. Our survey of CF- and non-CF-derived *P. aeruginosa* strains indicates that a high proportion of CF isolates lost core-plus-one LPS, whereas all of the non-CF isolates retained it (unpublished results). However, it is not clear if this is due to *migA* up-regulation or a defect in the ligase which mediates core-plus-one LPS synthesis. Further studies are underway to understand this as well as the role of *migA* up-regulation in the adaptation of *P. aeruginosa* to the CF lung environment.

ACKNOWLEDGEMENTS

We would like to thank Dr R. Ramphal for providing respiratory mucus from CF patients, Dr B. Iglewski and Dr P. Greenberg for providing *lasI*, *rhlI* and *lasI/rhlI* mutant strains and Dr D. Wozniak for providing the *algR* mutant. We would also like to acknowledge the helpful comments of two anonymous referees. This work was supported by NIH grant R29AI39524 (to S.J.) and Canadian Cystic Fibrosis Foundation (CCFF) operating grants (to D.S. and J.L., respectively). M.M. is a recipient of a CCFF studentship.

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- Received 2 June 2000; revised 21 July 2000; accepted 24 July 2000.