Expression of the Major Porin Gene \textit{mspA} Is Regulated in Mycobacterium \textit{smegmatis}^{\dagger\ddagger}

Dietmar Hillmann,1,2 Iris Eschenbacher,2‡ Anja Thiel,2§ and Michael Niederweis1,2*

Department of Microbiology, University of Alabama at Birmingham, 609 Bolling Biomedical Research Building, 845 19th Street South, Birmingham, Alabama 35294,1 and Lehrstuhl für Mikrobiologie, Friedrich-Alexander-Universität Erlangen-Nürnberg, Staudtstr. 5, D-91058 Erlangen, Germany2

Received 18 September 2006/Accepted 21 November 2006

\textit{MspA} is the major porin of \textit{Mycobacterium smegmatis} and is important for diffusion of small and hydrophilic solutes across its unique outer membrane. The start point of transcription of the \textit{mspA} gene was mapped by primer extension and S1 nuclease experiments. The main promoter driving transcription of \textit{mspA} was identified by single point mutations in \textit{lacZ} fusions and resembled \textit{\alpha\textsuperscript{oc}} promoters of \textit{M. smegmatis}. However, a 500-bp upstream fragment including \textit{P_{mspA}} in a transcriptional fusion with \textit{lacZ} yielded only low \textit{\beta}-galactosidase activity, whereas activity increased 12-fold with a 700-bp fragment. Activation of \textit{P_{mspA}}, by the 200-bp element was almost eliminated by increasing the distance by 14 bp, indicating binding of an activator protein. The chromosomal \textit{mspA} transcript had a size of 900 bases and was very stable with a half-life of 6 minutes, whereas the stabilities of episomal \textit{mspA} transcripts with three other 5\textsuperscript{' } untranslated region (UTRs) were three- to sixfold reduced, indicating a stabilizing role of the native 5\textsuperscript{' } UTR of \textit{mspA}. Northern blot experiments revealed that the amount of \textit{mspA} mRNA was increased under nitrogen limitation but reduced under carbon and phosphate limitation at 42°C in stationary phase in the presence of 0.5 M sodium chloride, 18 mM hydrogen peroxide, and 10% ethanol and at acidic pH. These results show for the first time that \textit{M. smegmatis} regulates porin gene expression to optimize uptake of certain nutrients and to protect itself from toxic solutes.

\textsuperscript{*} Corresponding author. Mailing address: Department of Microbiology, University of Alabama at Birmingham, 609 Bolling Biomedical Research Building, 845 19th Street South, Birmingham, Alabama 35294, Phone: (205) 996-2711. Fax: (205) 934-9256. E-mail: mnieder@uab.edu.

\textsuperscript{†} Supplemental material for this article may be found at http://jb.asm.org/.

\textsuperscript{‡} Present address: Cenas AG, E.-C.-Baumannstr. 20, D-95326 Kulmbach, Germany.

\textsuperscript{§} Present address: Institut für Klinische Mikrobiologie, Immunologie und Hygiene, Friedrich-Alexander-Universität Erlangen-Nürnberg, Wasserturmstrasse 3-5, D-91054 Erlangen, Germany.

\textsuperscript{??} Published ahead of print on 1 December 2006.
RNA regulator named *ipec* (5) have been discovered to play a role in regulation of *ompF* and *ompC*. Both *ompF* and *ompC* are repressed by the *micF* and *micC* antisense RNAs, respectively, and solely *ompF* is repressed by RseX. These RNAs are complementary to the corresponding 5′ untranslated region (UTRs), inhibit ribosome assembly, and induce mRNA degradation upon binding to their cognate mRNAs (6–8). Transcription of *micF* and *micC* integrates signals from multiple regulatory pathways (6, 7).

Considering the importance of controlling the OM permeability and the extensive and complex regulation of porin gene expression in gram-negative bacteria, it is surprising that nothing is known about the regulation of expression of porin genes in mycobacteria. In this study, we have chosen the major porin gene *mspA* of *M. smegmatis* to examine whether and how porin genes in mycobacteria are regulated. We have identified the major promoter of *mspA* and multiple signals that drastically alter the amount of *mspA* mRNA. Further, the 5′ UTR of *mspA* appears to play an important role in stabilizing the *mspA* transcript and preventing mRNA decay.

**Materials and Methods**

**Bacterial strains and growth conditions.** *Mycobacterium smegmatis* SMR5 (38) was routinely grown at 37°C with shaking in Middlebrook 7H9 liquid medium (Difco Laboratories) supplemented with 0.2% glycerol and 0.05% Tween 80 or on Middlebrook 7H10 agar (Difco Laboratories) supplemented with 0.2% glycerol unless indicated otherwise. *Escherichia coli* DH5α was used for all cloning experiments and was routinely grown in LB medium at 37°C. Hygromycin was used at concentrations of 200 μg/ml for *E. coli* and 50 μg/ml for *M. smegmatis*.

**Identification of growth conditions that alter transcription of the *mspA* gene of *M. smegmatis*.** Cultures of *M. smegmatis* SMR5 for RNA preparation were grown to an optical density at 600 nm (OD 600) of 0.8 to 1.0 at 37°C in *M. smegmatis* Tris-HCl, 5 mM MgCl2, 20 mM Na3). The cell suspension was incubated on ice for 5 min. Cells were harvested by centrifugation, resuspended in RA1 buffer and by adding denaturing buffer (50 mM NaOH, 10 mM NaCl) and neutralizing positively charged nylon membrane by using a vacuum blot apparatus at 6,000 Pa for 10 min and 20 °C. cDNA was synthesized using avian myeloblastosis virus reverse transcriptase and the corresponding reverse transcription buffer (250 mM Tris HCl, pH 8, 130 mM MgCl2, 75 mM dithiothreitol, 2 mM each dcyctonucleoside triphosphate). The reaction mixture was incubated at 42°C for 1 h.

**RNA preparation.** After incubation at the above mentioned conditions, cultures were mixed with half of the volume of precooled buffer (20 mM Tris-Cl, 5 mM MgCl2, 20 mM NaCl). The cell suspension was incubated on ice for 5 min. Cells were harvested by centrifugation, resuspended in RAI buffer with β-mercaptoethanol (Nucleospin RNAII kit), and lysed by agitation with glass beads (FastRNA Tubes-Blue) in a FastPrep FP120 bead beater apparatus (Bio-101) by three 20-second pulses at level 6.5. Suspensions were cooled on ice for 5 min between agitation steps. Further processing of the sample to purify the RNA was performed using the Nucleospin RNAII kit (Machery-Nagel) following the manufacturer's instructions.

**Northern blot analysis.** One to three micrograms of total RNA from *M. smegmatis* was loaded on a denaturing RNA gel (1.2% agarose, 1× morpholinepropanesulfonic acid [MOPS] [1× MOPS is 200 mM MOPS, 50 mM sodium acetate, and 10 mM EDTA, pH 7], 3.15% formaldehyde). Gel electrophoresis was done at 70 V for 3 to 4 h in 1× MOPS. The RNA was transferred to a positively charged nylon membrane by using a vacuum blot apparatus at 6,000 Pa and by adding denaturing buffer (50 mM NaOH, 10 mM NaCl) and neutralizing buffer (0.1 M Tris HCl, pH 7.4) for 10 min and 20 °C. The hybridization buffer (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7) for 3 h. After the transfer, the RNA was cross-linked twice to the membrane with UV light at 1,200 kJ. The membrane was washed twice for 5 min each in 2× SSC-0.1% SDS at room temperature and three times for 15 min each time in 0.2× SSC-0.1% SDS at 60°C. For detection, the membrane was equilibrated in 1× maleic acid buffer (1 M maleic acid, 0.3 M Na citrate, pH 7.5) for 1 minute, then in blocking buffer (1× blocking reagent in 1× maleic acid buffer) for 30 min, and finally in blocking buffer with alkaline phosphatase-conjugated antibody against digoxigenin for 30 min. Then, the membrane was washed twice for 15 min each time in 1× maleic acid buffer and for 5 min in detection buffer (0.1 M diethanolamine). All incubation steps were performed with gentle shaking at room temperature. The membrane was completely covered with CDP* (DIG Northern starter kit; Roche) and incubated in darkness for 5 min. Chemiluminescent signals on the membrane were photographed, and detected bands were densitometrically measured and quantified in a UVP EpiChem® Darkroom using LabWorks software. Detection of the 16S rRNA was performed as a control, and the amounts of *mspA* transcripts were normalized to that of 16S rRNA in the same sample.

**Dot blot analysis.** One to three micrograms of total RNA from *M. smegmatis* was loaded directly on a positively charged nylon membrane. To avoid differences in dot size when using the same amounts of total RNA, the RNA concentrations of all samples were adjusted to 500 to 600 ng/μl. The membrane was dried and cross-linked as described above. Hybridization and detection were performed by methods similar to those of Northern blot analysis.

**RNA probe construction.** Probes were amplified from chromosomal DNA from *M. smegmatis* SMR5 by PCR using specific primers (see Table S4 in the supplemental material) for the sigA gene and the 16S rRNA gene. Probes for the *mspA* and lacZ genes were amplified from the plasmids pPOR6 and pMlacZsd (see Table S1 in the supplemental material), respectively. A recognition site for T7 RNA polymerase was added to the 5′ ends of the reverse primers. The PCR products were purified by elution from agarose gels and quantified. Two hundred nanograms was used for in vitro transcription using the Roche DIG RNA labeling mix according to the manufacturer’s instructions. The RNA probes were purified by elution from agarose gels, precipitated, and resuspended in 50 μl of RNase-free water. For hybridization overnight, 10 μl of an RNA probe was added to 10 μl prehybridization solution.

**RNA stability and half-life.** Cultures of *M. smegmatis* were grown to an OD600 of 0.8 to 1.0, and then 10 ml of the cell suspension was used for RNA preparation as described above for time zero. Simultaneously, rifampin was added to the incubating cultures to a final concentration of 200 μg/ml to inhibit further transcription. Another 10 ml of each culture was used for RNA preparation 1, 2, and 5 min after the addition of rifampin to monitor time-dependent RNA decay (see above).

**Primer extension experiments.** For primer extensions, the *mspA* gene-specific reverse primer MP-P2 was designed (see Table S2 in the supplemental material; its position is depicted in Fig. 2). End-labeled primer (labeled with γ-32P) (0.4 pmol) was annealed to 10 μg total RNA from exponential-phase cultures of *M. smegmatis* SMR5 in 1× hybridization buffer (1× hybridization buffer is 0.15 M KCl, 0.01 M Tris HCl, pH 8, and 1 mM EDTA). Samples were denatured for 5 min at 95°C and hybridized for 2 h at 50°C. cDNA was synthesized using avian myeloblastosis virus reverse transcriptase and the corresponding reverse transcription buffer (250 mM Tris HCl, pH 8, 130 mM MgCl2, 75 mM dithiothreitol, 2 mM each dcyctonucleoside triphosphate). The reaction mixture was incubated at 42°C for 1 h.

Five micrograms of plasmid DNA from pPOR6 (see Table S1 in the supplemental material) and the primer MP-P2 were used for sequencing reaction. Plasmid DNA was denatured in 0.2 M NaOH and 0.2 mM EDTA for 30 min at 37°C, precipitated, and redissolved in 7 μl H2O. The end-labeled MP-P2 (labeled with γ-32P) was annealed, and sequencing was performed using the USB Sequenase version 2.0 sequencing kit following the manufacturer’s guidelines. The sequencing reactions together with the corresponding primer extension reactions were analyzed on a 8% denaturing polyacrylamide gel (7 M urea).

**Nuclease S1 mapping.** RNA from *M. smegmatis* cultures during exponential growth phase was prepared as described above. The primer MP-P2 (see Table S2 in the supplemental material) was radioactively labeled (see “Primer extension experiments” above), precipitated, and resuspended in 10 μl TE (Tris-EDTA) buffer. PCR was performed using 2 μg of StyI-linearized plasmid pPOR6 (see Table S1 in the supplemental material) and 2 pmol of the labeled primer MP-P2, which yielded a radioactive DNA probe of 320 bp. The PCR product was purified by elution from a 5% polyacrylamide gel (7 M urea). The probe was precipitated together with 40 μg RNA from *M. smegmatis* and resuspended in 40 μl hybridization buffer [40 mM piperazine-N,N′-bis(2-ethanesulfonic acid) (PIPES), pH 6.4, 400 mM NaCl, 1 mM EDTA, 80% formamide]. Hybridization was carried out at 50°C for 90 min, at 45°C for 90 min, and at 40°C for 60 min. Nonhybridized RNA was digested by the addition of 30 U nuclease S1 and 300 μl S1 reaction buffer (50 mM sodium acetate, pH 4.6, 28 mM NaCl, 4.5 mM ZnSO4, 20 μg/ml sheared DNA) and incubated at 37°C for 1 h. After addition of 50 μl stop solution (4 M NaH acetate, 0.1 M EDTA), the sample was precipitated with ethanol, resuspended in 3 μl TE buffer with 0.4 μg/ml RNase A, mixed with 1 μl denaturing loading buffer, and run on a 0.1-mm-thick 8% denaturing polyacrylamide gel (7 M urea).

**Expression of *mspA* is regulated in *M. smegmatis*.**
Construction of β-galactosidase-expressing vectors. To reduce background activity, the transcriptional terminator of gene 32 of phage T4, tT4-lacZ, which has been shown to efficiently terminate transcription in mycobacteria (45), was cloned upstream of the lacZ gene. Therefore, primers T4g32T_Pme_Sph_fwd and T4g32T_Pme_Sph_rev were used (see Table S2 in the supplemental material). They were phosphorylated using T4 polynucleotide kinase (New England BioLabs) following the recommendations of the manufacturer. Five hundred picomoles of each phosphorylated primer was mixed and annealed by heating to 90°C and cooling down to 65°C over 20 min. Hybridization resulted in a tT4g32 fragment, which was inserted via restriction sites for PmlI and SphI into the pMacZ2d plasmid (kindly provided by Sabine Ehrt, Cornell University, NY) to yield pML163.

To obtain vectors harboring transcriptional fusions of the mspa promoter fragments to the lacZ gene, the mspa promoter fragments were inserted upstream of the lacZ gene into pML163 via the PmlI and SphI restriction sites. The mspa promoter fragments were amplified by PCR from the template plasmid pPOR6 (29) using the following primers: mpp4 and mpp1-fwd for Pspa-500 bp (resulting in pML164), mpp4 and mpp10 for Pspa-600 bp (pML808), mpp4 and mpp11 for Pspa-700 bp (pML809), mpp4 and mpp12 for Pspa-800 bp (pML810), mpp4 and mpp13 for Pspa-900 bp (pML811), mpp4 and mpp14 for Pspa-1000 bp (pML812), mpp4 and mpp7 for Pspa-1100 bp (pML817), and mpp5 and mpp9 for Pspa-600 bp up (pML810). To obtain the plasmids with constant 500-bp proximal fragments but different 200-bp fragments distal to mspa, pML164 was digested with PmlI and ligated with similarly cut PCR fragments obtained with oligonucleotide pairs mpp15/mpp16 (pML823; 200 bp of mspa), mpp17/mpp18 (pML824; 200 bp of −700 to −900), and mpp19/mpp20 (pML825; 200 bp of −900 to −1100). The entire mspa promoter fragments were sequenced to ensure the right orientation and the absence of secondary site mutations. The verified plasmids were electroporated in M. smegmatis SMR5.

Mutation of the mspa promoter. Promoter point mutations in the Pspa-1100 bp fragment were obtained by the combined chain reaction as described earlier (21) using the pPOR vector as the template. The end primers were mpp4 and mpp7, and the mutation primers were PromT1C, PromA2C, and PromT16C (see Table S2 in the supplemental material). The mutation primers were phosphorylated using T4 polynucleotide kinase (New England BioLabs) following the manufacturer’s manual. The resulting plasmids had the following mutations: T to C at position −147 (pML820), A to C at −146 (pML821), and T to C at −142 (pML822) relative to the mspa start codon. The entire mspa promoter fragments were sequenced to verify the mutations and to ensure the absence of secondary site mutations. Verified plasmids were electroporated in M. smegmatis SMR5.

β-Galactosidase activity measurements. To determine β-galactosidase activity of recombinant M. smegmatis, cells were grown in Middlebrook 7H9 to an OD600 of 0.8 to 1. For measuring activity at different pH, cells were grown in HdB medium as described above. After incubation at different conditions for 3 h, 1 ml samples were taken, harvested, and resuspended in the same volume of neutral HdB medium. OD600 was determined with 100-μl portions of these samples. The remaining 900 μl was sonicated using a Misonix sonicator 3000 with the following settings: two complete intervals of 20 seconds total pulse time consisting of pulses for 0.9 s and breaks for 0.5 s at strength 3. Between intervals, the samples were kept on ice. Two hundred microliters of sonicated cells was mixed with 800 μl of freshly prepared LacZ medium consisting of 1× LacZ buffer (1× LacZ buffer is 60 mM Na2HPO4, 40 mM NaH2PO4, 3 mM MgCl2, 10 mM KCl, and 50 mM β-mercaptoethanol) and 600 μl of Middlebrook 7H9 neutral HdB medium, depending on the growth medium. The samples were prewarmed at 28°C for 15 min, and 200 μl α-nitrophenyl-β-D-galactopyranoside (ONPG) (4 mg ml−1 in 1× LacZ buffer) was added. Samples were incubated at 28°C until they turned yellow. Then, β-galactosidase was inactivated by adding 500 μl 1 M Na2CO3 and the time was determined. Absorption was measured at 420 and 550 nm, and Miller units were calculated.

RESULTS

Identification of the mspa promoter. In a first step to identify the promoter of the mspa gene, the transcriptional start point (TSP) of mspa was determined. To this end, M. smegmatis was grown to the exponential growth phase and RNA was prepared. Primer extension experiments revealed a strong signal for mspa transcripts starting with a G at position −135 relative to the start codon (Fig. 1A). S1 nuclease mapping was performed with the same RNA and confirmed this TSP (Fig. 1B). A much weaker signal corresponding to longer transcripts with a TSP at −153 bp upstream of mspa was also observed (Fig. 1B). A search within the 5′ upstream region (5′ UTR) of the mspa gene for sequences with similarities to the consensus sequence for promoters in M. smegmatis (TATAAT with 100% conservation for T, 93% for A, 50% for T, 57% for A, 43% for G, and 71% for T [26]) revealed a potential promoter sequence TATGTG upstream of the main TSP at position −135 (Fig. 2). To examine whether the sequence TATGTG at position −147 represents the mspa promoter, a transcriptional fusion of a 1,100-bp fragment upstream of mspa to the E. coli lacZ gene was constructed (pML167 [see Table S1 in the supplemental material].

FIG. 1. Start points of transcription of the mspa gene and dependence of mspa mRNA levels on growth phase. (A) The primer extension experiment was performed using RNA from M. smegmatis SMR5 and the primer MP-PE2 labeled by phosphorylation of the 5′-OH with [γ-32P]ATP. The sequencing reaction was carried out using the same oligonucleotide with plasmid pPOR6 as the template. PE, primer extension. The transcription start point of mspa at position −135 is marked with an asterisk. (B) The S1 nuclease mapping experiment was done using RNA from M. smegmatis SMR5 and a radioactive 320-bp DNA probe covering 300 bp of the upstream region of mspa. S1, RNA/probe hybrid after digestion with nuclease S1; C, undigested DNA probe as a control; PE, primer extension product (Fig. 1A). TSPs at positions −135 and −153 were determined and are marked by an asterisk. (C) Dependence of mspa transcription on the growth phase. RNA was prepared from M. smegmatis SMR5 harvested at the indicated optical densities at 600 nm and was used for extension experiments with the labeled primer MP-PE2. For all experiments, the sequencing reactions were used as a reference and are labeled with A, C, G, and T according to the dideoxynucleotide triphosphate in the termination mix.
To identify whether an additional promoter caused this increased expression of the lacZ gene, a 600-bp fragment ranging from -500 to -1100 with respect to mspa ("600 bp up") was fused to lacZ. This upstream sequence had promoter activity as indicated by a β-galactosidase activity fivefold above background. However, the activities of both individual fragments of 500 bp and "600 bp up" were 11-fold below the activity of the complete 1,100-bp construct (Fig. 4B). Thus, it was concluded that both fragments alone are not able to drive high expression of the lacZ gene. We further investigated the "600 bp up" fragment by dividing it into three 200-bp pieces. These 200-bp fragments (-500 to -700, -700 to -900, and -900 to -1100 upstream of mspa) were inserted upstream of the original 500-bp fragment of the mspa UTR, resulting in three additional 700-bp constructs (Fig. 4C). The β-galactosidase activities of the original 700-bp and 1100-bp fragments were high and almost identical as observed earlier (Fig. 4A). In contrast, the other three 700-bp constructs, consisting of the 200-bp fragments fused to the 500-bp promoter fragment via a restriction site, which added a 14-bp spacer between the two DNA fragments, only slightly increased the activity (Fig. 4C). This included the 200-bp fragment immediately upstream of the 500-bp promoter fragment and indicated a dependence of the activation of PmspA on the phasing of the DNA helix.

### Size and half-life of the mspa transcript
To further characterize expression of the mspa gene in *M. smegmatis*, the size and half-life of the mspa transcript were determined by Northern blot experiments. An RNA probe that covered 355 bp of the mspa gene was initially fused to the lacZ gene to examine regulation of the mspa promoter. However, transcription from the mspa promoter in this fragment was very low compared to other mycobacterial promoters (not shown). To test whether this fragment may not contain all functions for full promoter activity, longer fragments of the DNA upstream of mspa were fused to lacZ. The β-galactosidase activity rose by 7- and 12-fold as a result of increasing the length of the promoter fragment to 600 bp and 700 bp, respectively. This activity did not increase further with longer fragments (Fig. 4A).

---

**Figure 2.** Upstream region of the mspa gene. Genes with annotations are msmeg0951 (transcriptional regulator of the TetR family), msmg0958 (cytochrome P450), and hemL (glutamate-1-semialdehyde-2,1-aminoimidase). Other genes represent open reading frames of unknown functions (43). The region -450 to +22 relative to the mspa start codon is shown enlarged. The large arrow depicts the mspa promoter. Italic underlined letters indicate palindromes that may act as potential binding sites for regulator proteins. Italic letters that are not underlined represent mismatches in the palindromes.

**Figure 3.** Mutational analysis of the mspa promoter. The activity of β-galactosidase of *M. smegmatis* SMR5 with plasmids harboring fusions of lacZ with different fragments upstream of mspa was measured and is indicated in Miller units (MU). Mutational analysis of the putative -10 region (TATGTT at position -147) of the mspa promoter within the fusion of a 1,100-bp fragment with lacZ. The T nucleotide at position 1, A nucleotide at position 2, and T nucleotide at position 6 were changed to C. The β-galactosidase activity was compared to the β-galactosidase activities of the constructs with an unmutated PmspA, 1,100-bp fragment or with a promoterless lacZ construct.

---

**Legend:**
- **T1C** and **T6C** mutations reduced expression of the mspa promoter.
- **T2C** mutation completely eliminated expression of the mspa promoter.
- **T1A** mutation decreased expression of the mspa promoter.
- **A2C** mutation completely eliminated expression of the mspa promoter.
- **T** nucleotide at position 1, **A** nucleotide at position 2, and **T** nucleotide at position 6 were changed to **C**.
- The **bold letter with an asterisk** indicates the main TSP. Bold underline letters denote the putative Shine-Dalgarno sequence.
- The **underlined letters** denote the potential binding sites for regulator proteins.
- **Italic underlined letters** indicate palindromes that may act as potential binding sites for regulator proteins.
- **Italic letters that are not underlined** represent mismatches in the palindromes.

---

**References:**
1. [Journal of Bacteriology](http://jb.asm.org/)
2. [Expression of mspa is regulated in M. smegmatis](http://jb.asm.org/)
3. [Northern blot experiments](http://jb.asm.org/)
4. [RNA probe that covered 355 bp of the mspa gene](http://jb.asm.org/)
5. [β-galactosidase activity](http://jb.asm.org/)
6. [Promoter activity](http://jb.asm.org/)
7. [Promoterless lacZ construct](http://jb.asm.org/)
tant, demonstrating that the RNA detected with the probe in wild-type *M. smegmatis* was indeed the *mspA* mRNA (Fig. 5A).

The length of the *mspA* transcript was determined to be approximately 900 bases by comparison to digoxigenin-labeled RNAs of known lengths. Considering the 636-bp length of the *mspA* gene and the 135-bp length of the 5'UTR, it is concluded that the transcription of the *mspA* gene terminates 130 bp after the stop codon. However, no intrinsic terminator was found in this region.

To determine the half-lives of the *mspA* transcripts, RNA was isolated from wild-type *M. smegmatis* at different times after inhibition of transcription by rifampin. Northern hybridization was used to analyze the RNA (Fig. 5B). The intensities of the *mspA* mRNA bands were quantified by image analysis, and the half-life of the *mspA* transcript was determined to be 6 minutes (Fig. 5C). Surprisingly, the stability of the *mspA* transcript is dependent on the *mspA* expression construct.

When the original promoter fragment of *mspA* was replaced by the constitutive mycobacterial promoters *P_\text{imyc}* and *P_\text{smyc}* (16), episomal expression decreased the half-lives of these *mspA* transcripts to 1 min and 2.3 min, respectively (Fig. 5D). Thus, no *mspA* mRNA was detected after 5 min for the plasmid-based *mspA* expression cassettes in contrast to wild-type *mspA* transcripts, which were still detectable after 10 min (Fig. 5B and D). Faster RNA degradation was also observed on Northern blots with RNA isolated from strains containing *mspA*.
expression plasmids compared to the distinct bands for mspA mRNA from wild-type M. smegmatis (not shown).

Expression of mspA depends on the growth phase. To examine whether transcription from the corresponding promoters was dependent on the growth phase of M. smegmatis, RNA was prepared from cells harvested at optical densities ranging from 0.1 to 3.0. Primer extension experiments revealed that transcription of mspA is maintained throughout early and exponential growth but decreased sharply after the cells entered stationary phase (Fig. 1C). Quantitative image analysis showed that the amount of the shorter mspA mRNA was reduced 25-fold in stationary phase, indicating that mspA expression is dependent on the growth phase of M. smegmatis. To examine whether the weaker promoter using the TSP at position −153 had any regulatory function, we compared the amounts of the two transcripts in M. smegmatis grown in Middlebrook 7H9 medium under different conditions. Neither a shift in temperature from 37°C to 28°C or to 45°C nor an increased osmolarity (0.5 M NaCl) resulted in any detectable change of the ratio in the two mRNAs (data not shown).

Different environmental signals regulate transcription of the mspA gene. To identify signals that regulate the expression of porin genes in M. smegmatis, we analyzed mspA expression under conditions that are known to modify porin gene expression in E. coli. Since nutrient limitation is among those signals (19), Hartmans-de Bont medium, which contains single compounds, such as carbon, nitrogen, and phosphorous sources, was used and reduced the amounts of glycerol, ammonium sulfate, and potassium phosphate to 11 mM, 150 μM, and 96 μM, respectively. These concentrations were shown to limit the growth of M. smegmatis in HdB medium significantly (data not shown), consistent with previous results (41). RNA was prepared from M. smegmatis grown at the indicated conditions. Both mspA mRNA and 16S rRNA were detected in the same blot using a mixture of specific probes (see Table S4 in the supplemental material). The intensities of the mspA mRNA bands were normalized to those of the corresponding 16S rRNA to eliminate differences in the total amount of RNA loaded on the gel. Northern blots with RNA from M. smegmatis revealed that mspA mRNA levels are decreased under carbon and phosphate limitation, while limitation of nitrogen el-
DISCUSSION

Identification and activity of the mspA promoter. Here, we present the first analysis of porin gene expression in mycobacteria. Primer extension analysis revealed a strong signal for a TSP at position –135 upstream of the mspA gene. Single point mutations identified the –10 sequence of the mspA promoter, which is similar to σ^A promoters of M. smegmatis (26). A potential –35 region was found 17 bp upstream of the –10 region (Fig. 2). However, we did not examine the importance of this element for activity of PmspA. The observation that the A2C mutation in the –10 region of PmspA completely eliminated the β-galactosidase activity of transcriptional fusions of the 1,100-bp DNA fragment upstream of mspA with lacZ (Fig. 3) demonstrates that there is no promoter in addition to PmspA. Surprisingly, a 500-bp fragment of the mspA 5′ region yielded only low expression of lacZ in a transcriptional fusion, whereas lacZ expression increased 12-fold with a 700-bp fragment. Longer fragments did not increase expression of lacZ further. Thus, it is concluded that approximately 700-bp DNA upstream of mspA are required for full activity of the PmspA promoter. Importantly, activity of a 700-bp DNA upstream of mspA was only twice as high as the basal activity of the 500-bp fragment with PmspA when the 200-bp activating element (–500 to –700) was separated by a 14-bp spacer from the 500-bp basal promoter (Fig. 4C). Thus, activation of PmspA by the 200-bp element was dependent on the phasing of the DNA helix, an indicator of binding of an activator protein (13, 24). The requirement for rather long upstream regions for full activity has been observed for other mycobacterial promoters. For example, a region between 436 bp to 559 bp upstream of katG of Mycobacterium tuberculosis is required for full transcription in M. smegmatis, although no additional promoter was detected in this fragment. However, in this case the phasing between the UTR and the promoter was not critical for promoter activity (27). Another enhancer-like element was identified between 670 bp and 760 bp upstream of the mas gene of M. tuberculosis, but it is unknown whether proteins bind to this DNA (40). Further experiments are required to identify the putative activator protein for transcription of mspA.

The 5′ UTR contributes to the stability of the mspA transcript. The mspA transcript has a defined size of approximately 900 bases (Fig. 5A). This means that the mRNA ends approximately 130 bases after the mspA gene, considering the lengths of the gene (636 bp) and the 5′ UTR (135 bp). This is within the intergenic region between mspA and msmeg0956 (43) and indicates that the mspA gene is transcribed independently and is not part of an operon. A U trail following a hairpin structure is an essential component of intrinsic transcription terminators in other bacteria (47) but was not detected within this region. It was proposed that terminators of transcription in mycobacteria do not need a U trail when they have RNA hairpins with a stem length exceeding 27 bp (47). However, the 3′ end of the mspA transcript contains only sequences capable of forming hairpins with a stem length shorter than 5 bp. The half-life of the mspA transcript is 6 minutes during exponential growth at 37°C. For E. coli, an average messenger stability was reported to be 2.4 min at 37°C (37), whereas to our knowledge, nothing is known about mRNA stability in mycobacteria. An altered 5′ end in transcriptional fusions of mspA with other promoters increased mRNA degradation three- to sixfold (Fig. 5A), con-

FIG. 7. Dependence of mspA mRNA levels on the pH of the culture. (A) RNA was prepared from M. smegmatis SMR5 grown in modified HdB medium including 50 mM citrate buffer at pH 6.8, 5.5, 5.0, and 4.5. Detection of the RNA on positively charged nylon membranes using Northern blotting was done using mixed specific RNA probes for mspA mRNA and 16S rRNA. Ten milliliters of hybridization solution contained 20 μl of the mspA probe and 10 μl of the 16S rRNA probe. (B) Quantitative analysis. The amounts of mspA transcripts in blot shown in Fig. 7A and two further blots were determined by quantitative image analysis using UVP LabWorks. The values were averaged, and the error bars represent standard deviations. The intensities of the mspA bands were normalized to the intensities of the corresponding 16S rRNA bands. The amount of mspA transcripts at pH 6.8 was set at 100%.
**EXPRESS OF mspA IS REGULATED IN M. SMEGMATIS**

**Fig. 8.** mspA mRNA levels are specifically reduced at pH 4.5. Dot blot analysis was performed. (A) RNA was prepared from M. smegmatis SMR5 cultures grown at pH 6.8, 5.5, and 4.5 in HdB medium and was detected on dot blots using specific RNA probes for mspA mRNA, sigA mRNA, and the 16S rRNA. (B) RNA was prepared from M. smegmatis SMR5 containing no plasmids (wild type [wt]) and from M. smegmatis MN01 (ΔmspA) cultures containing the mspA expression plasmids pPOR6 (P_mspA), pMN012 (P_mspA), pMN013 (P_mspA), and pMN016 (P_mspA) grown at pH 6.8 and 4.5. Dot blot analysis was done as described for panel A. (C) RNA was prepared from M. smegmatis SMR5 harboring either no plasmids (no lacZ), a fusion of a 1,100-bp P_mspA fragment with lacZ (pML167) or lacZ without a promoter (pML163) growing at pH 6.8 or 4.5. Dot blot analysis was done using specific RNA probes for lacZ and 16S rRNA transcripts.

Confirming a pivotal role of the mspA 5' UTR in stabilizing mspA transcripts. Secondary structures, such as stem-loops at the 3' and 5' ends of the transcript, often protect mRNA from degradation by ribonucleases (36). Analysis of the mspA 5' UTR with the RNAstructure software (23) revealed a secondary structure at position -70 to -120 (Fig. 9), which may be involved in stabilizing the mspA transcript in a manner similar to that of the stem-loops at the 5' end of the exceptionally stable ompA transcript of E. coli (12). In fact, the mspA hairpin is more stable (calculated ΔG = -13.4 kcal mol⁻¹) than a modified ompA stem-loop with a stem length of 6 bp (calculated ΔG = -9.9 kcal mol⁻¹), which was sufficient to increase the half-life of the ompA transcript in E. coli from 3.9 min to 20 min (2). An alternative explanation for the stability of the mspA mRNA might be protection from degradation by RNases upon binding of antisense RNA. Indeed, antiparallel transcripts were observed for the 5' end of the mspA transcript (data not shown). However, experimental evidence for the role of antisense RNA in regulating the stability of the mspA mRNA is lacking.

Many environmental signals alter mspA expression. Porins are important for nutrient uptake both in gram-negative bacteria (30) and in M. smegmatis (42, 43). In E. coli, the OM permeability is elevated by increasing the amount of the larger porin OmpF and decreasing the level of the smaller porin OmpC under conditions of nutrient limitation and vice versa under adverse conditions (33). Therefore, we compared regulation of expression of mspA with that of ompF, because MspA and OmpF are the main porins that determine OM permeability of M. smegmatis and E. coli, respectively, under many conditions. Glucose limitation leads to 20-fold-higher ompF mRNA levels compared to the level in medium containing excess glucose (20). In contrast, the amount of mspA mRNA was reduced threefold even after a slight reduction of the glycerol concentration from 22 mM to 11 mM and increased by 40% under nitrogen limitation (Fig. 6). Interestingly, M. smegmatis did not grow in HdB medium at glycerol concentrations lower than 11 mM in striking difference to E. coli, which grows with its maximal rate in a medium containing as little as 10 μM glucose (11). Thus, M. smegmatis does not only appear to experience C limitation at concentrations 1,000-fold higher than E. coli but also responds in an opposite manner by shutting down porin synthesis. Phosphate limitation had the largest effect and caused an almost complete loss of mspA mRNA. In E. coli, expression of another porin, PhoE, with specificity for anions is induced under low phosphate conditions (35), while to our knowledge, the effects of these conditions on expression of general porins such as OmpF have not been examined. Thus, M. smegmatis may induce expression of an unknown phosphate-specific porin and reduce expression of MspA, which does not appear to be well suited for diffusion of anions through the highly negatively charged constriction zone (10). However, alternative explanations such as a transition of M. smegmatis to a state of low metabolic activity under unfavorable conditions cannot be excluded.

As M. smegmatis enters stationary phase, mspA mRNA is barely detectable anymore (Fig. 1C). This is similar to the reduced synthesis of OmpF by E. coli in response to depleted nutrient sources (33). The amount of ompF mRNA in E. coli decreases threefold during growth at temperatures of 37°C and above compared to growth at 24°C (1). Physiologically, the reduced expression of ompF makes sense because diffusion of small solutes through water-filled channels is accelerated at higher temperatures. A similar effect was observed for M. smegmatis. mspA mRNA levels were reduced fivefold at 42°C compared to those at 37°C (Fig. 6). The osmolarity of the medium is high in the intestine, the natural environment of E. coli. Under these conditions OmpC is the predominant protein and ompF expression is repressed (33). Similarly, both 0.56 M glucose (10%) and 0.5 M NaCl reduce mspA mRNA levels in M. smegmatis. Oxidative stress activates the micF promoter via SoxS and therefore represses ompF expression in E. coli (33). A similar effect was observed for M. smegmatis, which reduced mspA mRNA levels by threefold in the presence of 18 mM...
H$_2$O$_2$. _M. smegmatis_ responds to the presence of another toxic agent, ethanol, by down-regulation of porin gene expression (Fig. 6) in a manner similar to that of _E. coli_ (33). The _mspA_ mRNA gradually declined with decreasing pH and was completely absent at pH 4.5 (Fig. 7). It may be argued that pH 4.5 is not physiologically relevant for _M. smegmatis_. However, _M. smegmatis_ is able to maintain an intracellular pH of 6.1 to 7.2 during growth at an extracellular pH of 4.5 (34) and is highly abundant in brook sediments and forest soils at pH 3.5 to 4.3 (14, 15). Repression of _ompF_ expression as a result of low pH was also reported for _E. coli_ (7, 18, 39, 44, 46). A progressive reduction of the OM permeability over a range of pH 6.8 to 4 would reduce proton influx and support an adaptation of _M. smegmatis_ to acidic habitats (32). The mechanism by which _M. smegmatis_ reduced _mspA_ mRNA levels at low pH is specific and is mediated by the 5' UTR of the _mspA_ gene as demonstrated by the unchanged levels of three _mspA_ transcripts with different UTRs at pH 4.5 (Fig. 8B).

In conclusion, we have identified environmental signals that alter porin gene expression in _M. smegmatis_. The results show that _M. smegmatis_ protects itself by reducing the OM permeability via down-regulation of porin gene expression similar to the response of _E. coli_ to toxic agents. This is indicative of a general protection mechanism of bacteria with a second membrane and probably holds true for other mycobacteria as well. The response of porin gene expression to nutrient limitation by _M. smegmatis_ is different from that by _E. coli_ and reflects the fact that these bacteria have evolved different metabolisms to thrive in their habitats. We further discovered that _mspA_ transcripts are stabilized by their original 5' UTR, probably due to the formation of protective secondary structures, and that an enhancing fragment between 500 and 700 bp upstream of _mspA_ is required to fully activate _P$_{mspA}$. Thus, the regulation of _mspA_ expression by environmental signals includes both transcriptional and posttranscriptional mechanisms.

ACKNOWLEDGMENTS

We thank Chuck Turnbough for invaluable advice; Sabine Ehrt for the pMacZsd vector; and Chuck Turnbough, Jason Huff, and Olga Danilchanka for critically reading the manuscript. Preliminary sequence data for _Mycobacterium smegmatis_ were obtained from The Institute for Genomic Research website at http://www.tigr.org.

This work was supported by the Deutsche Forschungsgemeinschaft by grants to M.N. (NI 412) and fellowships to D.H. (Graduiertenkolleg 805) and I.E. (Graduiertenkolleg 40).

REFERENCES


