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The Actinobacterial *mce4* Locus Encodes a Steroid Transporter*[S]

William W. Mohn[‡1], **Robert van der Geize**[§], **Gordon R. Stewart**[‡], **Sachi Okamoto**[‡], **Jie Liu**[‡], **Lubbert Dijkhuizen**[§], **and Lindsay D. Eltis**[‡]

From the ‡*Department of Microbiology and Immunology, Life Sciences Institute, University of British Columbia, Vancouver V6T 1Z3, Canada and the* §*Department of Microbiology, University of Groningen, Kerklaan 30, 9751 NN, Haren, The Netherlands*

Bioinformatic analyses have suggested that Mce proteins in diverse actinobacteria are components of complex ATP-binding cassette transporter systems, comprising more than eight distinct proteins. In *Mycobacterium tuberculosis*, these proteins are implicated in interactions of this deadly pathogen with its human host. Here, we provide direct evidence that the Mce4 system of *Rhodococcus jostii* RHA1 is a steroid uptake system. Transcriptional analyses indicate that the system is encoded by an 11-gene operon, up-regulated 4.0-fold during growth on cholesterol *versus* on pyruvate. Growth of RHA1 on cholesterol and uptake of radiolabeled cholesterol both required expression of genes in the *mce4* operon encoding two permeases plus eight additional proteins of unknown function. Cholesterol uptake was ATP-dependent and exhibited Michaelis-Menten kinetics with a K_m of 0.6 ± 0.1 μM. This uptake system was also essential for growth of RHA1 on β-sitosterol, 5-α-cholestanol, and 5-α-cholestanone. Bioinformatic analysis revealed that all *mce4* loci in sequenced genomes are linked to steroid metabolism genes. Thus, we predict that all Mce4 systems are steroid transporters. The transport function of the Mce4 system is consistent with proposed roles of cholesterol and its metabolism in the pathogenesis of *M. tuberculosis*.

The *mce* genes of *Mycobacterium tuberculosis* have garnered much interest due to their demonstrated role in pathogenesis, although the function of these genes remains unclear. The first *mce* gene, now known as *mce1A*, was discovered when a DNA fragment cloned from *M. tuberculosis* into a noninvasive *Escherichia coli* strain enabled uptake of the latter bacterium by nonphagocytic mammalian epithelial (HeLa) cells and facilitated its phagocytosis by macrophages (1). A subsequent study showed that coating latex beads with the protein encoded by *mce1A* facilitated uptake of the beads by HeLa cells, and so the gene was designated *mce* for mammalian cell entry (2). The

genome sequence of *M. tuberculosis* revealed four loci (*mce1–4*), each containing two *yrbE* genes followed by six *mce* genes (3). The Mce proteins are predicted to have similar structures and to be secreted (4), and Mce1A was localized to the surface of *M. tuberculosis* cells (2). The capacity to facilitate uptake by HeLa cells was further narrowed to a small region of the MceA1 protein (2, 5, 6). Coating beads with either Mce3A or Mce3E also facilitated such uptake (7), whereas coating beads with Mce2A failed to do so (2). Together, these studies suggest that some of the Mce proteins might interact with host cells and play a role in uptake of *M. tuberculosis* by host cells. However, direct evidence for this function of Mce proteins is lacking.

Mutational studies also support the involvement of Mce proteins in pathogenesis of *M. tuberculosis.* One genome-wide screen suggested that disruption of *mce1* and *mce4* genes causes early and late growth defects, respectively, in mice (8), and these phenotypes were confirmed with *mce1D* and *yrbE4A* deletion mutants, respectively (8–10). Another genome-wide screen suggested that disruption of *yrbE4A* impairs macrophage infection (11), and a *yrbE4A* deletion mutant was severely impaired in replication in interferon γ-activated macrophages (10). Disruption of any one of *yrbE1B*, *mce2A*, and *mce3A* genes in *M. tuberculosis* increased survival of intratrachaelly infected mice (12). Similarly, deletion of nearly entire *mce3* or *mce4* operons increased long term survival of mice following low dose aerosol infection, and the latter deletion also decreased bacterial burdens of the mice (13). By contrast, an *mce1A* deletion was hypervirulent in mice (14). Thus, *mce* mutant strains have phenotypes generally supporting the involvement of these genes in pathogenesis, but the mutants exhibited disparate virulence phenotypes in different assays.

Analysis of genome sequences indicated that diverse actinobacteria, including members of *Mycobacterium, Nocardia, Rhodococcus*, and *Streptomyces*, harbor *mce* loci (15, 16). Moreover, PCR-based assays revealed the existence of *mce4* loci in 11 of 20 *Mycobacterium* spp. screened (17). The actinobacteria with *mce* loci include both pathogens and free-living bacteria, strongly suggesting that the function of these genes is not limited to virulence. Bioinformatic analyses (15, 18) indicated that the *yrbE* genes of all *mce* loci encode transmembrane proteins with similarity to permease components of ATP-binding cassette (ABC)[2] transporters. The hallmark of ABC transporters is

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[2] The abbreviations used are: ABC, ATP-binding cassette; RT-PCR, reverse transcription-PCR; DCCD, dicyclohexylcarbodiimide; BCG, Bacille de Calmette et Guèrin.

an ATPase with highly conserved features. Some *mce* loci include genes encoding such ATPases, which belong to the Mkl family, a subset of ABC transporter ATPases (15). Further, all actinobacteria containing *mce* loci were found to also contain genes encoding Mkl ATPases, usually not proximal to the *mce* loci. Based on genetic interaction mapping and mutagenesis, Joshi *et al.* (9) provided evidence that the Mce1 and Mce4 proteins in *M. tuberculosis* are both functionally linked to a single Mkl ATPase (MceG), encoded by a gene not linked to either the *mce1* or the *mce4* loci. These observations strongly suggest that *mce* loci encode a novel type of ABC transporter. However, direct evidence for this transport function has not been demonstrated.

Many *mce* loci also include two or four genes downstream of the *yrbE-mce* genes (14). The latter genes have been referred to as *mce*-associated (*mas*) genes (15); however, there is already precedent for using Mas to denote mycocerosic acid synthase of *M. tuberculosis* (19), so we will simply refer to the latter genes in *mce* loci as additional *mce* genes (*i.e. mce4HI*).

We previously found that the *mce4* locus of *Rhodococcus jostii* RHA1 was among a large cluster of genes up-regulated during growth on cholesterol (20). Moreover, we found that RHA1 mutants with deletions of either the *supAB* genes (homologous to *yrbE4AB* genes; Δ*sup*) or the *mce4ABCDEF* genes (Δ*mce4AF*) lost the ability to grow on cholesterol. These findings led us to hypothesize that the Mce4 system of RHA1 functions in cholesterol uptake. Because the *mce4* locus of *M. tuberculosis* is also clustered with cholesterol metabolism genes homologous to those in RHA1, we hypothesized the same function for the Mce4 systems in both organisms. Consistent with this, Pandey and Sassetti (10) recently showed that an *M. tuberculosis yrbE4A* disruption mutant is impaired in mineralization of C-4 of cholesterol, assimilation of C-26 of cholesterol, and growth with cholesterol as a substrate.

In the present study, we used mutational analysis and a direct assay of cholesterol uptake to conclusively determine the role of the Mce4 system of RHA1 in steroid uptake. We also used bioinformatic analysis to assess the role of Mce4 systems in other actinobacteria.

EXPERIMENTAL PROCEDURES

Bacterial Growth—*R. jostii* RHA1 was grown at 30 °C on a shaker on defined Goodies medium (21) plus either 10 mM pyruvate or 2 mM of a steroid substrate. Cholesterol was from Sigma-Aldrich, and other steroids were from Steraloids (Newport, RI). Biomass was estimated on the basis of total protein measured by disrupting cells with hot alkaline lysis and using the BCA protein assay (Pierce) with bovine serum albumin as the standard.

PCR—Nucleic acids were extracted, and RNA was purified as described previously (20). cDNA was synthesized with the SuperScript™ III reverse-transcriptase (Invitrogen) and random hexamers (Invitrogen). Quantitative PCR was done using TaqMan probes (Applied Biosystems), and gene expression differences were calculated as described previously (20). Primer and probe sequences are in supplemental Table S1. PCR conditions for reverse-transcription PCR (RT-PCR) and RT-quantitative-PCR assays were: 95 °C for 5 min and then 35 cycles of

95 °C for 40 s, 66 °C (PCR 1) or 59 °C (PCR 2–10) for 40 s, 72 °C for 1 min. A final extension at 72 °C was for 7 min before holding at 4 °C.

Gene Deletion and Complementation—The *mce4H-mce4I-ro04706* genes of *R. jostii* RHA1 were deleted (Δ*mce4HI*) using the *sacB* counter selection system essentially as described (22). Sequences of all oligonucleotides used are in supplemental Table S1. Oligonucleotides ro04706-F and ro04706-R were used to amplify and clone a 1.8-kb region downstream of the three genes of RHA1, comprised of the last 13 codons and the stop codon of ro04706, into SmaI-digested pK18mobsacB, yielding pK18-Ro04706. Oligonucleotides ro04704-F and ro04704-R were used to amplify the upstream region of the three genes. The obtained 1.8-kb PCR product, composed of the first 20 codons including the start codon of ro04704, was cloned into pGEM-T. A double digestion with NcoI (blunt-ended with Klenow) and SpeI was performed on the resulting plasmid, and the liberated fragment was cloned into BamHI (blunt-ended with Klenow) and SpeI double-digested pK18-Ro04706, resulting in pK18-Ro04704-06 used for *mce4H-mce4I-ro04706* gene deletion. The deletion was verified by PCR using oligonucleotides ro04704-06-F with ro04704-06-R and ro04704contr-F with ro04706contr-R, matching sequences flanking the targeted three genes.

The *supA-supB* gene deletion (Δ*sup*) and the *mceH-mceI* gene deletion (Δ*mce4HI*) were complemented, resulting in the strains Δ*sup-C* and Δ*mce4HI-C*, respectively, as follows. The *supA-supB* genes were amplified using primers ro04696-F and ro04797-R. The resulting 1636-bp amplicon was digested with NdeI and HindIII and cloned in pTip-QC2 (23), yielding pTip-supAB. Similarly, the *mce4H* and *mce4I* genes were amplified using primers ro04704ex-F and ro04705ex-R. The resulting 1267-bp amplicon was subcloned, digested with BspHI and HindIII, and ligated into NcoI/HindIII-digested pTip-QC1 (23), yielding pTip-mce4HI. The pTip-supAB and pTip-mceHI plasmids were introduced into the Δ*sup* strain and the Δ*mce4HI* strain, respectively, by electroporation. A "vector control" strain without the *supA-supB* genes (Δ*supV*) was also produced by electroporating Δ*sup* with pTip-QC2.

Cholesterol Uptake Assay—Cholesterol uptake was measured in resting cell suspensions. Cells were grown to mid-log phase on pyruvate, washed twice, and suspended at a cell density of 900 mg of protein/liter (A_{600} = 13.3) in Goodies buffer. Aliquots of 0.05 ml Goodies medium were placed in 4-ml vials, and [4-^{14}C]cholesterol (53 mCi/mmol, PerkinElmer Life Sciences) was added from an ethanol stock solution. Aliquots of 0.05 ml of cell suspensions were added to the vials for a final cell density of 450 mg of protein/liter. The vials were incubated with shaking for 5 min at room temperature. Cholesterol uptake was stopped by diluting the suspensions with 1.0 ml of ice-cold buffer, collecting the cells on a 0.22-μm Millipore nitrocellulose filter (Fisher Scientific, Mississauga, Ontario), washing the cells with 10 ml of 5% Tween 20 (Fisher) and then with 10 ml of 50% ethanol, and finally, with 10 ml of 5% Tween 20. The filters were placed in Beckman Ready-Safe scintillation mixture (Beckman Coulter) and counted in a Beckman LS-600IC scintillation counter to determine the amount of cholesterol taken up by the cells. The rate of uptake was found to be
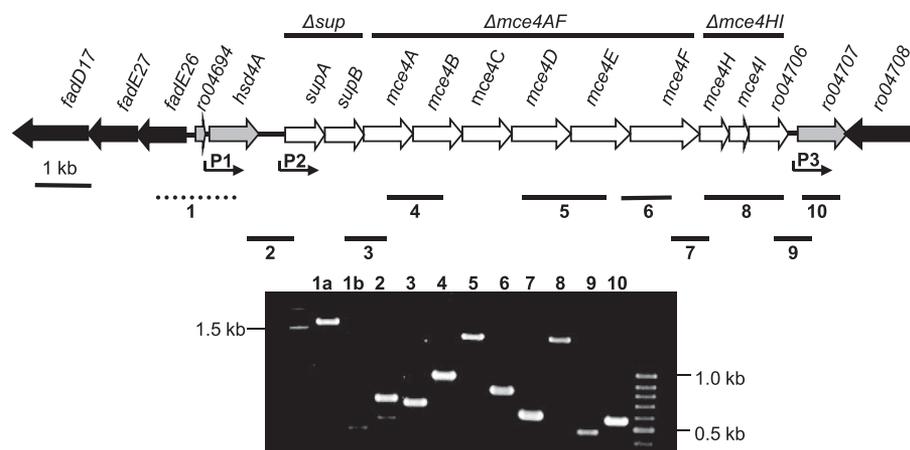
FIGURE 1. **The *mce4* locus of RHA1.** Three predicted transcriptional promoters are indicated (*P1*, *P2*, *P3*). *Numbered solid bars* (*2–10*) indicate transcribed regions detected in RT-PCR assays, and those numbers correspond to the electrophoresis gel lane numbers, showing RT-PCR products. All positive RT-PCR reactions were negative in controls without reverse-transcriptase (not shown). The *dashed bar* (*1*) indicates a nontranscribed control, with *lane 1a* showing the corresponding PCR product from genomic DNA and *lane 1b* showing the result of the RT-PCR assay. *Bars* at the *top* show regions deleted in three mutant strains (Δ*sup,* Δ*mce4AF,* Δ*mce4HI*).

constant for greater than 5 min, and there was a linear relationship between uptake rate and cell biomass over a cell density range of 69–1375 mg of protein/liter. Where indicated, 60 mM sodium azide, 2.0 mM dicyclohexylcarbodiimide (DCCD), or 10 mM sodium orthovanadate was added to cell suspensions 10 min prior to the addition of the labeled cholesterol. Steady-state kinetic parameters were analyzed using the least squares and dynamic weighting options of LEONORA (24).

RESULTS

The mce4 Locus—The RHA1 *mce4* locus has the typical gene arrangement of other *mce* loci and appears to be an operon (Fig. 1). As reported previously (15), orthologous *mce4* loci occur in *Nocardia farcinica* (IFM 10152), *Mycobacterium bovis* (AF2122/97), *Mycobacterium avium subsp. paratuberculosis* (k-10), *Mycobacterium smegmatis* (MC2 155), and *M. tuberculosis* (H37Rv and other strains). We also found *mce4* loci in *M. avium* (104), *M. bovis* BCG (Bacille de Calmette et Guèrin), *Mycobacterium marinum* (M), *Mycobacterium ulcerans* (Agy99), and *Mycobacterium vanbaalenii* (PYR-1). The *mce4* gene products of RHA1 have the greatest similarity to their respective orthologs in *N. farcinica.* The *sup* genes of the RHA1 *mce4* locus are orthologs of *yrbE4* genes in other *mce4* loci.

The RHA1 *mce4* locus has the conserved configuration of two *yrbE* (*sup*) plus eight *mce* genes found in all known *mce4* loci. However, the *mce4* locus of RHA1 is unique in that *mce4I* is followed by two additional genes. The first of these genes, ro04706, is only two nucleotides downstream of *mce4I* and encodes a hypothetical protein with an N-terminal region similar to the conserved domain of the substrate-binding site of ketosteroid isomerases (Pfam family PF02136). The second of these genes, ro04707, encodes a protein with similarity to the conserved domain of the 3-β-hydroxysteroid dehydrogenase/isomerase family (Pfam family PF01073). Both of these genes appear to have orthologs in all of the above strains, but none of those orthologs is closely linked to *mce4* loci. The orthologs in H37Rv are Rv2042c and Rv0139, respectively, which are not closely linked to the large steroid metabolism gene cluster of H37Rv. However, Rv0139 is part of a large regulon that also includes the steroid metabolism genes and *mce4* genes of H37Rv (25). The four genes upstream of *supA* (*yrbE4A*) are conserved in all of the above organisms and putatively encode a 3-ketoacyl-(acyl-carrier-protein) reductase, a ferredoxin and two acyl-CoA dehydrogenases. The fifth gene upstream of *supA* (*yrbE4A*) is conserved in all the above organisms, except *N. farcinica,* and putatively encodes an acyl-CoA synthetase.

In RHA1, the 11 genes from *supA* through ro04706 are closely spaced (Fig. 1), with maximum intergenic regions of 20 bases and with six of the predicted genes overlapping the following ones by four nucleotides. Putative transcriptional promoters were found upstream of *hsd4A, supA,* and ro04707, but none were found within the 11-gene *mce4* cluster, suggesting co-transcription of that cluster. This conclusion was further supported by RT-PCR analysis, which detected transcripts including intergenic regions within this cluster. Seven of the 10 intergenic regions were assayed, and all were detected. Transcripts including the intergenic regions on either side of the 11-gene cluster were also detected, suggesting some level of transcription through those regions. We used expression of the *supA* gene as a proxy for expression of *mce4* locus genes in an RT-quantitative-PCR assay. The *supA* gene was up-regulated 4.0-fold during growth on cholesterol, relative to during growth on pyruvate. Together, the above results strongly suggest that the 11-gene *mce4* cluster is co-transcribed and regulated as an operon. The results do not preclude the existence of additional smaller transcripts within the region nor larger transcripts including additional flanking genes.

Like other *mce4* loci, that of RHA1 is not proximal to a gene encoding an Mlk family ATPase. However, the RHA1 genome includes two genes, ro01974 and ro02744, encoding ATPases orthologous to MceG, the Mlk ATPase gene of *M. tuberculosis.* Both of these RHA1 genes are associated with other *mce* loci of RHA1. One or both of these ATPases may function in the RHA1 Mce4 system.

Growth Phenotypes of Mutants—We made an unmarked, inframe deletion mutant of the *mce4HI*-ro04706 genes (Δ*mce4HI*) in RHA1 (Fig. 1). Like the previous Δ*sup* and Δ*mce4AF* deletion mutants (20), the Δ*mce4HI* mutant completely lost the ability to grow on cholesterol (Fig. 2*A*). We constructed two complementation strains to confirm that deleted genes were the cause of the mutant phenotypes.

The Δ*sup-C* complementation strain consists of the Δ*sup* mutant with plasmid pTip (23) containing *supAB.* The Δ*sup*-C strain partially regained the ability to grow on cholesterol. An "empty vector" control strain (Δ*sup*-V), consisting of the Δ*sup* mutant with plasmid pTip, could not grow on cholesterol.
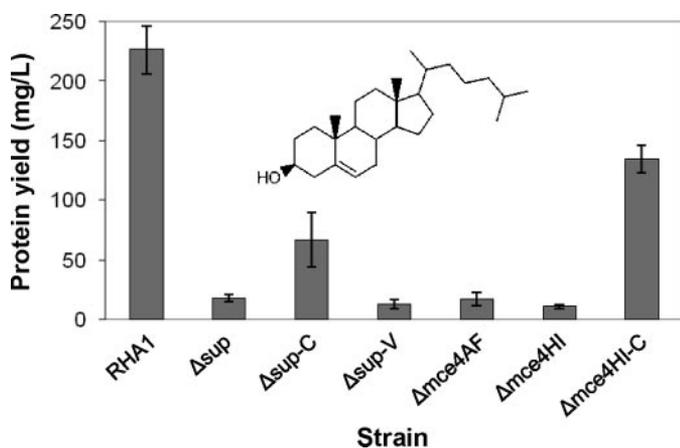
FIGURE 2. **Growth of RHA1 and mutant strains on cholesterol (*n* ≥ three independent cultures, *bars* indicate standard deviation).**

$$V_{max} = 15 \pm 2 \; \mu mol/min/mg$$

$$K_m = 0.6 \pm 0.1 \; \mu M$$

FIGURE 3. **Cholesterol uptake by RHA1 and mutant strains.** *A*, kinetics of uptake by RHA1 (*n* = three independent experiments using different cultures). The curve represents the fitted Michaelis-Menten equation. *B*, specific uptake rates of RHA1 and mutants as well as effects of inhibitors (*n* ≥ three suspensions of cells from independent cultures, except *n* = two for DCCD treatment, *bars* indicate range).

Given this complementation and the complete inability of the Δ*mce4AF* and Δ*mce4HI* deletion mutants to grow on cholesterol, it is clear that the Δ*sup* deletion did not have a polar effect preventing expression of the downstream genes. The unmarked, in-frame Δ*mce4AF* and Δ*mce4HI* deletion mutants are also very unlikely to have polar effects.

The Δ*mce4HI-C* complementation strain consists of the Δ*mce4HI* mutant with plasmid pTip (23) containing *mce4HI*, but not ro04706. The Δ*mce4HI-C* strain also partially regained the ability to grow on cholesterol. This indicates that the last gene of the *mce4* operon, ro04706, is not essential for growth on cholesterol. Overall, the above results demonstrate that each group of genes, *supAB*, *mce4ABCDEF*, and *mce4HI*, encode one or more proteins essential for growth on cholesterol.

Cholesterol Uptake—The role of the Mce4 system in cholesterol uptake was directly examined by measuring cholesterol uptake by wild-type RHA1 and the mutant strains. We assayed uptake of ^{14}C-labeled cholesterol by resting cell suspensions. Attempts to use cholesterol-induced cells were unsuccessful as cholesterol used for induction could not be washed from the cells, making it impossible to control the concentration and specific activity of cholesterol in the assay. However, uninduced RHA1 cells grown on pyruvate had measurable cholesterol uptake activity. Uptake followed Michaelis-Menten kinetics and exhibited a low K_m (0.6 ± 0.1 μM) for cholesterol (Fig. 3*A*). Uptake activity was abolished by preincubation of cells with the respiratory inhibitor, sodium azide, or either of two ATPase inhibitors, DCCD and vanadate, indicating that uptake is energy-dependent, and more specifically, ATP-dependent (Fig. 3*B*). The intended effect of DCCD in RHA1 is supported by previous studies (26, 27) that have shown that DCCD inhibits proton-driven ATPases of mycobacteria that are phylogenetically related, and physiologically similar, to RHA1. Further, the consistent effect of these two, mechanistically distinct ATPase inhibitors strongly indicates that they indeed inhibited the ATPase. The maximum specific uptake activity for the uninduced cells was 15 ± 2 μm/min/mg of protein, but we predict a higher rate for induced cells.

The Δ*sup*, Δ*mce4AF*, and Δ*mce4HI* strains had no measurable cholesterol uptake activity (Fig. 3*B*). These uptake phenotypes are entirely consistent with the growth phenotypes of the
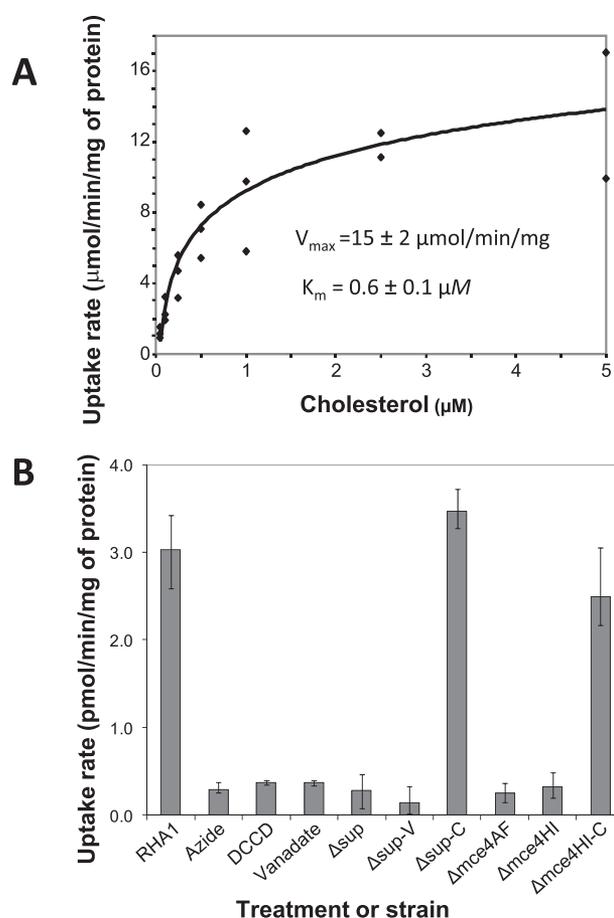
mutants. Moreover, the Δ*sup-C* and Δ*mce4HI-C* complementation strains had wild-type cholesterol uptake activity. These phenotypes are consistent with the growth phenotypes, but it is interesting that complementation is more complete in the uptake assay. The explanation for this difference in degree of complementation may be the use of uninduced cells for the uptake assay. Thus, expression of the *supAB* and *mce4HI* genes from pTip may be sufficient for uninduced uptake activity but may not be sufficient for maximal growth on cholesterol. These results clearly indicate that products of the *mce4* genes are part of a cholesterol uptake system.

Uptake of Other Steroids—Wild-type RHA1 and the three deletion mutants were additionally tested for growth on a range of steroids and related compounds. RHA1 was able to grow on 4-androstene-3,17-dione, 5-α-cholestanol, 5-α-cholestanone, cholic acid, progesterone, and β-sitosterol, but not on cholcalciferol, cholestane, ergocalciferol, ergosterol, estradiol, or hydrocortisone. The Δ*sup*, Δ*mce4AF*, and Δ*mce4HI* mutants also lost the ability to grow on β-sitosterol (Fig. 4), 5-α-cholestanol, and 5-α-cholestanone (not shown). Cholesterol, 5-α-cholestanol, 5-α-cholestanone, and β-sitosterol have very similar structures. The deletion mutants were unaffected in growth on 4-androstene-3,17-dione, cholic acid, and progesterone,
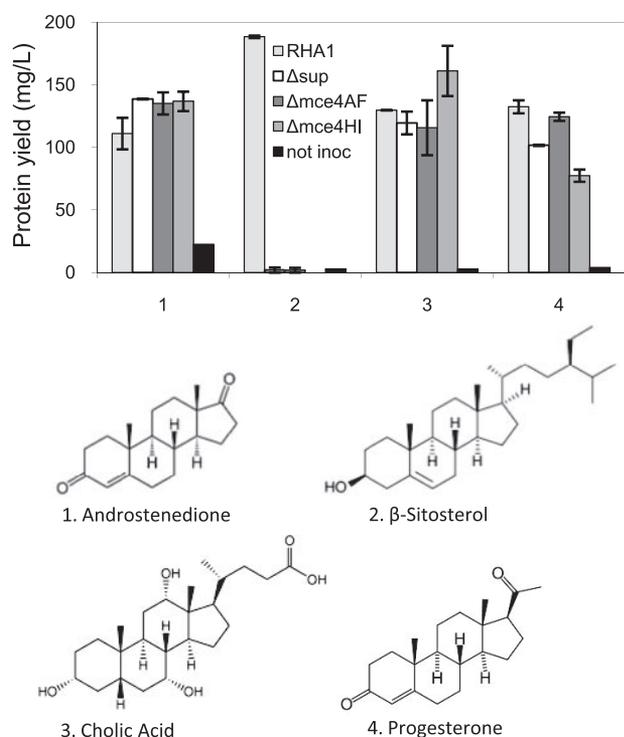
FIGURE 4. **Growth of RHA1 and mutant strains on various steroids ($n \geq 2$ independent cultures, *bars* indicate range).**

which differ notably from the above four compounds in having shorter, polar side chains. These results indicate that the Mce4 system takes up cholesterol, 5-α-cholestanol, 5-α-cholestanone, and β-sitosterol. One or more other systems likely transport 4-androstene-3,17-dione, cholic acid, and progesterone, but we cannot exclude the possibility that the Mce4 system also transports the latter three compounds.

Association of mce4 Genes with Steroid Metabolism Genes— To obtain evidence that Mce4 systems are involved in steroid uptake in other bacteria, we searched available genome sequences with *mce4* loci for genetically linked steroid metabolism genes. We searched for homologs of the RHA1 *kshA* gene, *hsaADCB*, and *hsaEGF* gene clusters. These genes encode most of the steps involved in degradation of rings A and B of the steroid nucleus to citric acid cycle intermediates. We previously found that, although the gene order varied in the genomes of RHA1 *versus* H37Rv, these genes were all in the region upstream of the *mce4* loci of both genomes, with the *hsaADCB* and *hsaFGE* putative operons conserved in both organisms (20). For the current analysis, we examined the genomes of *M. avium* (104), *M. bovis* BCG, *M. bovis* (AF2122/97), *M. marinum* (M), *M. smegmatis* (MC2 155), *M. ulcerans* (Agy99), *M. paratuberculosis* (k-10), and *N. farcinica* (IFM 10152). Orthologs of all of the above steroid metabolism genes were found linked to (within 150 kbp of) the *mce4* locus in each genome. The encoded proteins had greater than 70% sequence identity to their RHA1 orthologs, with the sole exceptions of KshA_k-10 (63%) and HsaB_IFM 10152 (67%). Interestingly, the Mce proteins are much less conserved than these catabolic enzymes, with only 26–46% identity between orthologous Mce proteins of RHA1 and H37Rv. The *hsaADCB* and *hsaFGE* putative operons were conserved in all the genomes. Thus, in all

available genomes with *mce4* loci, those loci are genetically linked to steroid metabolism genes.

DISCUSSION

This study provides the first direct experimental evidence that an *mce* locus encodes an uptake system (Fig. 3), which substantiates previous suggestions that *mce* loci encode a novel type of ABC transporter (9, 15, 25). This study further demonstrates that the *mce4* locus of RHA1 encodes a system for steroid uptake. Like other ABC transporters (28), the Mce4 system of RHA1 is an active transporter that requires ATP. Minimally, this system transports cholesterol, 5-α-cholestanol, 5-α-cholestanone, and β-sitosterol (Fig. 4).

Several lines of evidence strongly indicate that all Mce4 systems function in steroid uptake. First, *mce4* loci are invariably associated with putative steroid metabolism genes in the currently available genome sequences. Second, of the species known to have *mce4* loci, four have been tested and consistently found to grow on cholesterol, *M. smegmatis* (29) *R. jostii* (20), *M. bovis* BCG (20), and *M. tuberculosis* (10). Third, the *mce4* genes and putative cholesterol metabolism genes are co-regulated in *M. tuberculosis* and *M. smegmatis* (25) as well as *R. jostii* (20). Fourth, additional *Mycobacterium* spp. can grow on steroids (29). Thus, steroid metabolism and the Mce4 uptake system may be nearly ubiquitous in the genus, *Mycobacterium*, and may be widespread in others such as *Rhodococcus* and *Nocardia*. Importantly, steroids are abundant in nature, being present in nearly all phyla, making steroids valuable growth substrates for saprophytic microorganisms, which include most known actinomycetes and the ancestors of contemporary pathogenic actinomycetes. It appears that the Mce4 system diverged from other Mce systems when it evolved the capacity to transport steroids and became associated with steroid metabolism genes. It further appears that the Mce4 system was subsequently recruited for a role in pathogenesis, accounting for its conservation in many pathogenic actinomycetes with reduced genomes.

In RHA1, the *sup*, *mce4*, and ro04706 genes appear to be co-transcribed and regulated as a very large 11-gene operon (Fig. 1), consistent with the prediction that the *mce1* operon of *M. tuberculosis* is transcribed as one large polycistronic message (30). Minimally, *supA* plus co-transcribed genes are induced during growth on cholesterol, which provides further evidence for the role of the *mce4* genes in steroid uptake. Analysis of *M. tuberculosis* and *M. smegmatis* (25) suggests that the *mce4* operon of RHA1 may be part of a much larger regulon that includes steroid metabolism genes and possibly additional genes and that is negatively regulated by KstR. Despite the inducibility of the *mce4* operon, there appears to be substantial constitutive expression in RHA1 because uptake activity was measurable in uninduced cells. This is consistent with the relatively modest, 4-fold, up-regulation of *sup4A* that we measured. Constitutive activity would presumably be adaptive for substrate uptake systems because cells with no uptake activity might be unable to detect the presence of available substrates in their environment.

The available evidence implicates at least 11 proteins in a functional Mce4 steroid uptake system. These include an

ATPase, two permease subunits (YrbE or Sup), and eight additional proteins of unknown function (Mce4ABCDEFHI). The absolute requirement for *supAB*, *mce4ABCDEF*, and *mce4HI* genes for uptake activity and the identical growth phenotypes of all three RHA1 deletion mutant strains (Figs. 2– 4) indicate that a partially active system does not exist in the absence of any of the corresponding groups of proteins. In particular, although some Mce systems appear to lack the MceHI (Mas) proteins, those proteins are essential for a functional Mce4 system. Our results do not prove that all 10 of the Sup and Mce4 proteins are essential for steroid uptake activity but are consistent with this hypothesis. The essentiality of all 10 Sup and Mce4 proteins is also strongly suggested by the conservation of all 10 genes in the *mce4* loci of diverse actinobacteria (15).

It is unclear why Mce systems require so many more proteins than do classical ABC transporters. It seems likely that the Mce proteins form a complex that fulfills the role of substrate-binding proteins in classical bacterial ABC uptake transporters. Additionally, such a complex may mediate movement of the substrate across thick cell walls. This function is consistent with the prediction that these proteins are extracytoplasmic, and in some cases, tethered to the membrane (15). This role might resemble that proposed for Isd proteins, relaying heme iron through Gram-positive bacterial cell walls to which Isd proteins are tethered (31). Notably, *mce* loci are almost exclusively found in mycolic acid bacteria, such as members of *Mycobacterium*, *Rhodococcus*, and *Nocardia*, which have thick, hydrophobic cell walls (32). The only exception is that *mce* loci are also present in members of *Streptomyces*. Interestingly, genes with similarity to *mce* genes are also present in some Gram-negative bacteria (15) and plants (33). Thus, this broader group of genes may encode a corresponding group of proteins functioning in transport across impermeable cell walls and outer membranes.

Understanding the biological function of the Mce4 system provides new insight to the apparent role of Mce4 proteins in pathogenesis of *M. tuberculosis*, suggesting many possible mechanisms. The published studies of mutations within the *M. tuberculosis mce4* locus report attenuation of virulence in mice (8, 9) or impairment of macrophage infection (11). A straightforward explanation for this effect is that steroid metabolism by *M. tuberculosis* is an important part of pathogenesis. One possibility is that host cell cholesterol, which is relatively abundant, is an important growth substrate for the bacterium at some time during infection, intracellular growth, or intracellular persistence. Another possibility is that metabolic transformation of a steroid might permit the pathogen to manipulate its host. Alternatively, uptake of cholesterol may be an important signal allowing the pathogen to recognize and respond to the host environment. It is also possible that the ability of Mce4 proteins to bind cholesterol might play an important role in pathogenesis by localizing the bacterium, modifying the host membrane or eliciting a signal response from the host. Consistent with these possibilities, it was proposed that a possible lipid-binding motif of Mce4 proteins may be involved in binding or other bacterial interactions with host cells (4). Finally, previous suggestions that Mce proteins elicit an important immune response from the host (14) or facilitate host cell entry (1, 2) may be correct, with induction of the Mce4 proteins by host cell cholesterol possibly stimulating the processes. All of the above possibilities are consistent with reports that the presence of cholesterol is essential for infection of host cells by various mycobacteria (34–36). Given the complex and variable virulence phenotypes of *M. tuberculosis mce* mutants, effects of Mce proteins are likely complex and, possibly, multiple. Knowing that Mce systems function in uptake and that the Mce4 system functions specifically in steroid uptake suggests numerous testable hypotheses that may ultimately lead to elucidation of roles for the Mce proteins in pathogenesis of diverse actinobacteria, foremost of which is *M. tuberculosis*.

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