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A gene cluster encoding cholesterol catabolism in a soil actinomycete provides insight into *Mycobacterium tuberculosis* survival in macrophages

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*Rhodococcus* sp. strain RHA1, a soil bacterium related to *Mycobacterium tuberculosis*, degrades an exceptionally broad range of organic compounds. Transcriptomic analysis of cholesterol-grown RHA1 revealed a catabolic pathway predicted to proceed via 4-androstene-3,17-dione and 3,4-dihydroxy-9,10-secoandrost-1,3,5(10)-triene-9,17-dione (3,4-DHSA). Inactivation of each of the *hsaC*, *supAB*, and *meq* genes in RHA1 substantially altered their roles in cholesterol catabolism. Moreover, the *hsaC* mutant accumulated 3,4-DHSA, indicating that HsaC, formerly annotated as a biphynyl-degrading dioxygenase, catalyzes the oxygenolytic cleavage of steroid ring A. Bioinformatic analyses revealed that 51 rhodococcal genes specifically expressed during growth on cholesterol, including all predicted to specify the catabolism of rings A and B, are conserved within an 82-gene cluster in *M. tuberculosis* H37Rv and *Mycoberacterium bovis* bacillus Calmette–Guérin. *M. bovis* bacillus Calmette–Guérin grew on cholesterol, and *hsaC* and *kshA* were up-regulated under these conditions. Heterologously produced HsaC<sub>179Rv</sub> and HsaD<sub>179Rv</sub> transformed 3,4-DHSA and its ring-cleaved product, respectively, with apparent specificities ~40-fold higher than for the corresponding biphynyl metabolites. Overall, we annotated 28 RHA1 genes and proposed physiological roles for a similar number of mycobacterial genes. During survival of *M. tuberculosis* in the macrophage, these genes are specifically expressed, and many appear to be essential. We have delineated a complete suite of genes necessary for microbial steroid degradation, and pathogenic mycobacteria have been shown to catabolize cholesterol. The results suggest that cholesterol metabolism is central to *M. tuberculosis*’s unusual ability to survive in macrophages and provide insights into potential targets for novel therapeutics.

*catabolic pathway* | *oxygenase* | *Rhodococcus* | *steroid degradation*

Rhodococci are a genus of GC-rich, mycolic acid-producing bacteria within the order Actinomycetales that includes *Mycobacterium* (1). Rhodococci degrade a broad range of organic compounds, particularly hydrophobic ones, thereby playing a key role in the global carbon cycle. Analysis of the 9.7-Mb genome of RHA1 (www.rhodococcus.ca) reveals that this organism harbors a diverse armamentarium of enzymes (2), consistent with the catabolic versatility of the genus. These catabolic activities, together with robust and rapid rhodococcal growth, are of great interest to pharmaceutical, environmental, chemical, and energy industries (3).

The bacterial catabolism of steroids has attracted considerable attention (3) in part as a potential means of producing bioactive steroids from natural, low-cost sterols such as β-sitosterol and cholesterol. A pathway responsible for the aerobic degradation of the latter via 4-androstene-3,17-dione (AD) and 3-hydroxy-9,10-secoandrost-1,3,5(10)-triene-9,17-dione (3-HSA) may be pieced together from biochemical and genetic studies in diverse bacteria (Fig. 1). In some *Mycobacterium* (4) and *Rhodococcus* (5, 6) species, the aliphatic side chain at C17 is removed via a process similar to β-oxidation involving progressively shorter carboxylic acids. In these strains, 3-ketosteroid Δ1-dehydrogenase (KSTD) and 3-ketosteroid 9α-hydroxylase catalyze the opening of ring B and aromatization of ring A to yield 3-HSA (3, 7–9). The subsequent degradation of 3-HSA to 9,17-dioxo-1,2,3,4,10,19-hexanorandrostan-5-oi acid (DOHNA) via oxygenolytic cleavage of ring A is specified by the *tes* genes in the testosterone-degrading strain *Comamonas testosteroni* TA441 (10, 11). In *Rhodococcus equi*, the protopionate moiety of DOHNA is removed via β-oxidation (12). Many of the genes involved in steroid catabolism have yet to be identified, and many of the pathway enzymes are poorly characterized, particularly those involved in degrading the bicycloalkane originating from rings C and D. Detailed knowledge of steroid catabolism is essential to engineering strains for the biotransformation of sterols.

Recent genomic analyses revealed that rhodococci may be useful models for many mycobacterial processes: ~60% of the 3,999 genes of *Mycobacterium tuberculosis* H37Rv are conserved in RHA1, including many of unknown function (2). *M. tuberculosis* is the leading cause of mortality from bacterial infection, killing 2 million to 3 million people worldwide each year, and extensive drug resistant strains such as XDR-TB are now emerging (ref. 13 and www.who.int/mediacentre/factsheets/fs104/en/index.html). One poorly characterized aspect of mycobacterial physiology that contributes to the prevalence of tuberculosis is the bacterium’s unusual ability to survive for long periods of time, and even to replicate, in the normally hostile environment of the macrophage (14, 15). The mechanisms enabling this persistence are poorly understood, but are logical targets for novel therapeutic strategies. Transposon site hybridization (TraSH), a genomewide microarray-based technique,
identified 126 genes that appear to be necessary for survival of H37Rv in macrophages under conditions that model the immune response (16) and many others that are critical for in vivo survival in mice (17). Further, transcriptomic studies have identified suites of genes that are specifically up-regulated during survival in the macrophage (18). Despite the importance of these genes, their physiological roles are largely unknown.

We investigated the cholesterol catabolic pathway in RHA1 by comparing the transcriptomes of cholesterol- and pyruvate-grown cells. Targeted gene deletion was used to substantiate key catabolic steps. Bioinformatic analyses enabled annotation of many of the cholesterol catabolic genes and also revealed their presence in M. tuberculosis (16, 32). The latter are important for H37Rv survival in the macrophage (16, 32) and predicted as essential for survival in the macrophage (16, 32) and in vivo in mice (17) are indicated by * and #, respectively.

**Results**

**The Cholesterol Transcriptome of RHA1.** In liquid medium containing 2 mM cholesterol as the sole organic substrate, RHA1 grew to a density of $2 \times 10^8$ cells per ml. Microarray analysis revealed 572 genes that were up-regulated at least 2-fold during growth on cholesterol compared with pyruvate. Many of the up-regulated genes are scattered throughout the 9.7-Mb genome (www.rhodococcus.ca) and likely reflect a general physiological adaptation of the bacterium to growth on a highly hydrophobic, polycyclic compound. However, six clusters of up-regulated genes were clearly discerned [supporting information (SI) Table 3]. The most striking of these was a cluster of 51 genes that occur within a 235-kb stretch of RHA1’s 7.9-Mb chromosome (ro04482–ro04705; Fig. 2A). As discussed below, these encode proteins with significant sequence identity with enzymes involved in the catabolism of steroid rings A and B by C. testosteroni TA441 (10, 11) and Rhodococcus erythropolis S01 (7, 8). A second cluster of chromosomal genes (ro06687–ro06698) also appear to be involved in...

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**Fig. 1.** The deduced cholesterol catabolic pathway of Rhodococcus sp. RHA1. M. tuberculosis H37Rv, and M. bovis bacillus Calmette-Guérin. The enzymatic steps of side-chain degradation and ring opening are depicted. The latter are important for H37Rv survival in the macrophage (Fig. 2). Dashed arrows indicate multiple enzymatic steps. The compound in brackets undergoes nonenzymatic hydrolysis. Genes responsible for the degradation of rings C and D in RHA1 are not conserved in H37Rv or bacillus Calmette-Guérin. ADD, 1,4-androstadiene-3,17-dione; 9OHADD, 9α-hydroxy-1,4-androstadiene-3,17-dione; KshAB, 3-ketosteroid 9α-hydroxylase.

**Fig. 2.** The cholesterol catabolic genes of Rhodococcus sp. RHA1 and M. tuberculosis H37Rv: comparison of their organization and their activities in different studies. (A) Genes in the physical map are color-coded according to assigned function: purple, uptake; red, side-chain degradation; blue, cleavage of rings A and B; orange, degradation of the DOHMAA propionate moiety; green, degradation of rings C and D. White arrows represent genes for which no reciprocal homologue is present. The nucleotide sequences of the M. tuberculosis H37Rv and M. bovis bacillus Calmette-Guérin clusters share 96% identity. (B) Heat map indicating correlation between gene expression (fold difference) during growth of RHA1 on cholesterol versus pyruvate (a), effect of gene disruption on H37Rv survival in IFN-γ-activated macrophages according to TraSH analysis (reciprocal of ratio) (16) (b), and gene expression in H37Rv after 48 h of growth in IFN-γ-activated macrophages (18) (c). M. tuberculosis genes predicted as essential for survival in the macrophage (16, 32) and in vivo in mice (17) are indicated by * and #, respectively.
cholesterol catabolism. The four other gene clusters (ro00440–ro00453, ro03461–ro03464, ro08053–ro08060, and ro10126–ro10162) do not appear to be directly involved in steroid catabolism, and some are described elsewhere (19).

Annotation of Cholesterol Catabolic Genes. Among the genes that were up-regulated during growth on cholesterol, the annotation of those predicted to specify cholesterol catabolism is summarized in SI Table 4. Most of these comprise the 51 genes of the ro04482–ro04705 cluster (Fig. 2A), and most of the encoded proteins have such sufficient sequence similarity to well-characterized enzymes that their function can be confidently predicted. Thus, sequences of KshA, KshB, and KSTD (Fig. 1) are 40–69% identical to those of orthologs in R. erythropolis SQ1 (SI Table 4) that act sequentially to transform AD to 3-HSA (3, 7, 8). Further degradation of 3-HSA was predicted to be specified by seven genes, annotated here as hsa, that are clustered with ksdT and kshA (Figs. 1 and 2A). The encoded proteins share significant amino acid sequence similarity (30–60%; SI Table 4) with the tet-encoded enzymes of C. testosteroni TA441 that transform 3-HSA during growth on testosterone (10, 11). HsaC and HsaD were respectively, because of the former’s ability to catalyze the extradiol cleavage of 2,3-dihydroxybiphenyl (DHB) and their sequence similarity to the corresponding biphenyl catabolic enzymes (20). However, HsaC shares greater sequence identity with TesB of C. testosteroni TA441 (11) than with extradiol dioxygenases that preferentially cleave DHB. Moreover, quantitative RT-PCR analyses confirmed that hsaC was up-regulated 15.4-fold during growth of RHA1 on cholesterol as compared with either biphenyl or pyruvate.

It was more difficult to assign specific roles to the numerous β-oxidation genes of the ro04482–ro04705 cluster. Most of these gene products share greatest sequence identity with homologs that occur in M. tuberculosis H37Rv (8). Phylogenetic analyses (SI Fig. 3) revealed comparable relationships (data not shown): for each type of enzyme, the known steroid-degrading homologs constitute a distinct subclass. None of the additional three sets of genes were up-regulated in RHA1 during growth on cholesterol and so appear to encode degradation of other steroids.

Mutational Analysis of Cholesterol Catabolic Genes. The critical role of Mce4A–Mce4F and SupAB proteins in cholesterol catabolism was confirmed by unmarked in-frame gene deletion of the entire mce4ABCDEF gene cluster and the supAB genes, respectively, in RHA1. Both the mce4 and sup mutants were severely impaired in the ability to grow on cholesterol in liquid mineral medium (Table 1). By contrast, growth on AD was not affected, supporting our hypothesis that Mce4 and SupAB are specifically involved in the uptake of cholesterol in RHA1. The doubling times of RHA1 and the mutants on AD (12 h) were approximately three times longer than on pyruvate or benzoate.

To substantiate the predicted role of HsaC in catalyzing the extradiol cleavage of 3,4-dihydroxy-9,10-secoandrost-1,3,5(10)-trien-9,17-dione (3,4-DHSA), a catechol, hsaC was deleted. In liquid media, the hsaC− mutant grew on cholesterol at a rate that was 60% that of the WT strain and developed a pink color. By contrast, growth on pyruvate was not affected. The slower growth on cholesterol may be caused by either degradation of the C17 side chain or complementary activity of one of the HSA homologs in RHA1 (SI Fig. 3B). The pink color is consistent with the accumu-
loration and nonenzymatic oxidation of a catechol. To identify the latter, metabolites were extracted from the supernatant of hsaC− cells incubated in the presence of cholesterol. HPLC analysis revealed a major metabolite, which, when derivatized with trimethylsilane (TMS), yielded a compound with a molecular ion m/z = 460 (SI Fig. 4). The molecular ion and its fragmentation pattern correspond to those predicted for TMS-derivatized 3,4-DHSA. Finally, transformation of the metabolite with HsaCH37Rv as described above yielded a product with a pH-dependent spectrum essentially identical to that reported for 4,5–9,10-diseco-3-hydroxy-5,9,17-trioxoandrost-1(10),2-diene-4-oic acid (4,9-DSHA) (9) (E92 = 7.64 mM−1 cm−1 at pH 8.0), confirming the metabolite’s identity as 3,4-DHSA (Fig. 1).

**Conservation of the Cholesterol Catabolic Pathway in Mycobacteria.**

Further bioinformatic analyses revealed that 58 genes of the rot04482-rot04705 cluster in RHA1, including the 51 that were up-regulated during growth on cholesterol, are conserved together with much of their putative operonic structure within an 82-gene cluster in the genomes of M. tuberculosis H37Rv (Rv3492c–Rv3574c; Fig. 2A) and *M. bovis* bacillus Calmette–Guérin (Bcg3556c–Bcg3639; www.sanger.ac.uk/Projects/M.bovis) as well as within an 80-gene cluster in Mycobacterium avium (subsp. paratuberculosis) (Map0571–Map0491; ref. 29). As noted above, these genes appear to be sufficient to specify the uptake of cholesterol, the β-oxidation of the branched side chain at C17, and the catabolism of rings A and B to central metabolites via 3-HSA to yield DOHNAA (Fig. 1). The sequence identities of the RHA1, H37Rv, and bacillus Calmette–Guérin homologs are summarized in SI Table 4. Phylogenetic analyses revealed that among the four sets of steroid-degrading enzymes in RHA1 the mycobacterial enzymes are most similar to enzymes in RHA1 the mycobacterial enzymes are most similar to

**Table 1. Growth yields of RHA1 and mutants on different organic substrates**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Cholesterol, 1 mM</th>
<th>AD, 1 mM</th>
<th>Pyruvate, 20 mM</th>
<th>Benzoate, 20 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>73 (5)</td>
<td>77 (12)</td>
<td>170 (20)</td>
<td>470 (60)</td>
</tr>
<tr>
<td>ΔSupAB</td>
<td>1 (1)</td>
<td>75 (11)</td>
<td>170 (20)</td>
<td>520 (50)</td>
</tr>
<tr>
<td>Δmce4</td>
<td>3 (1)</td>
<td>78 (14)</td>
<td>150 (40)</td>
<td>440 (90)</td>
</tr>
</tbody>
</table>

Growth yields are expressed as micrograms of protein per milliliter of culture medium and are averages of triplicate cultures. Values in parentheses are standard errors.

**Discussion**

The current study identified clusters of genes that encode the catabolism of cholesterol in RHA1. These were initially identified through bioinformatic analyses of genes that were up-regulated during growth on cholesterol. Moreover, the involvement of Mce4 and SupAB proteins in cholesterol catabolism and the role of HsaC in RHA1, an extradiol dioxygenase, were substantiated by using gene deletion and characterization of the resultant mutants. Steroids such as cholesterol are ubiquitous in plants, animals, and some microbes and likely comprise an important energy source for saprophytic bacteria, particularly actinomycetes that efficiently use hydrophobic substrates. Although various aspects of steroid catabolism have been described in different bacteria (4–12, 22), in this study the genes of an entire catabolic pathway are delineated in a single organism. The identified genes include several involved in sterol uptake and side-chain degradation that are particularly good targets for cell and enzyme engineering studies. Thus, sterol uptake is believed to be rate-limiting, yet its mechanism is poorly understood. Similarly, efficient sterol side-chain degradation is critical for high yield production of steroid intermediates, particularly as most sterols used in microbial transformations consist of mixtures of compounds with slightly different side chains that are transformed with different efficiencies. Overall, this study facilitates the develop-

**Table 2. Steady-state kinetic parameters of HsaC_H37Rv and HsaD_H37Rv for steroid and biphenyl metabolites**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>K_m, μM</th>
<th>V_max, μMs⁻¹</th>
<th>V_max/K_m, s⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>HsaC_H37Rv</td>
<td>3,4-DHSA</td>
<td>0.9 (0.5)</td>
<td>12 (4)</td>
<td>790 (370)</td>
</tr>
<tr>
<td>HsaD_H37Rv</td>
<td>DHB</td>
<td>8.5 (0.8)</td>
<td>2.5 (0.4)</td>
<td>18 (3)</td>
</tr>
<tr>
<td>HsaD_H37Rv</td>
<td>4,9-DSHA</td>
<td>4 (1)</td>
<td>0.06 (0.02)</td>
<td>1.0 (0.2)</td>
</tr>
<tr>
<td>HsaD_H37Rv</td>
<td>HOPDA</td>
<td>19 (6)</td>
<td>0.009 (0.003)</td>
<td>0.028 (0.007)</td>
</tr>
</tbody>
</table>

Parameters were normalized to the amount of cellular extract (milligrams of protein content) used in the assays. Values in parentheses represent standard errors.
A second important contribution of the current study is the discovery that the cholesterol catabolic pathway is conserved in related pathogenic actinomycetes, including *M. tuberculosis*, *M. bovis*, and *M. avium*. Thus, the latter appear to have retained the capacity for cholesterol metabolism and exploited it to survive in their hosts. Consistent with our bioinformatic predictions, *M. bovis* bacillus Calmette–Guerin used cholesterol as a carbon and energy source, and genes encoding the ring-degrading enzymes KshA and HsaC were up-regulated during this utilization. The substrate of the pathway in *M. tuberculosis* was verified by demonstrating the apparent specificity of HsaC, an extradiol dioxygenase, and HsaD were conserved in the pathogenic mycobacteria. These enzymes had been annotated as a probable DHB dioxygenase (NP_218085) and a HOPDA (CA07143), respectively. The current study further suggests that these enzymes do not play a direct role in mycobacterial cell wall synthesis as recently suggested (31). Of the pathway proteins conserved in RHA1 and *M. tuberculosis* those with the lowest amino acid sequence identities are the Mce4 proteins. It is possible that the latter have different functions in the two organisms. However, our findings in RHA1 are consistent with the recent proposal that the *supA* and *mce4* genes encode a lipid transport system (26). Moreover, some of the genes that were functionally linked to this system in that study include several cholesterol catabolic genes. Thus, the low sequence identities of the RHA1 and mycobacterial Mce4 homologs may instead reflect the different environments from which these two strains must scavenge cholesterol.

Several lines of evidence indicate that the identified steroid catabolic pathway is essential for the survival of *M. tuberculosis* in the macrophage. First, 41 of the pathway genes, including those specifying catabolism of rings A and B, are among those specifically up-regulated during survival in the macrophage (Fig. 2B and ref. 18). Second, TrSAH analyses predict that at least 11 of the pathway genes are essential for *M. tuberculosis* H37Rv to survive in the macrophage under conditions that model the immune response (Fig. 2B) (16). Most of the 11 encode enzymes such as KSTD, HsaA, and HsaD, which are involved in the degradation of steroid rings A and B (Fig. 1). Intriguingly, cholesterol catabolic genes that were not identified in TrSAH studies have functions that may be complemented by other similar genes in *M. tuberculosis* H37Rv. These include KshB and HsaB, the respective reductase components of the AD(D) and the 3-HSA hydroxylases. Some of the TrSH mutants, such as *mce4*, displayed a progressive increase in growth defect 2–4 weeks after infection in mice (17). Moreover, the essentiality of some of these genes has been substantiated. Thus, a *Δmce4* (i.e., *supA*) and *Δmce4* mutants show attenuated survival of *M. tuberculosis* H37Rv in mice (17, 26). Similarly, inactivation of *mce4* in *M. tuberculosis* CDC1551 (r3527 in H37Rv; Fig. 2B), a gene of unknown function adjacent to *kshA* and clustered with the *hsa* genes, had an impaired ability to arrest phagosome acidification and resulted in attenuated survival (32). Clearly, the essential nature of the cholesterol catabolic genes needs to be further substantiated. However, the available evidence suggests that cholesterol uptake and metabolism are important for *M. tuberculosis* to be able to persist in the macrophage for longer periods of time.

The deduced cholesterol catabolic pathway is consistent with at least two features of *M. tuberculosis* pathogenicity. First, cholesterol is essential for the phagocytosis of the bacterium by the macrophage and the inhibition of phagosome maturation (33–35). For example, depletion of cholesterol from macrophages abrogates the receptor-specific uptake of mycobacteria. Moreover, cholesterol depletion overcomes the block in phagosome maturation of *M. avium*-infected macrophages (35), further suggesting that cholesterol might play a similar role in other mycobacterial pathogens. Second, the large number of oxygenases in the pathway is consistent with the observation that tuberculosis infections are associated with the most O2-rich sites within the body (36). More specifically, the cholesterol catabolic genes encode six oxygenases, including two associated cytochromes P450 of unknown function. Reactivation of the disease occurs most frequently in the upper pulmonary lobes, likely the most oxygenated regions of the body (36).

At least two differences between the deduced cholesterol catabolic pathways in RHA1 and the pathogenic mycobacteria suggest distinct metabolic functions of cholesterol rings C and D. First, the Baeyer-Villiger monooxygenase and hydroxylase typically associated with the ring fission of cycloalkanones, and whose genes are up-regulated in the RHA1 cholesterol transcriptome, are not conserved in the mycobacteria. Second, the mycobacterial *hsa* operon includes an N-acetyl transferase gene (24, 31). Thus, it is possible that pathogenic mycobacteria transform this portion of the cholesterol molecule for an alternate function such as signaling or cell wall integrity. Moreover, the cholesterol metabolic enzymes reported herein may also transform other host steroids or their derivatives, such as vitamin D, recently shown to mediate an innate immune response to mycobacteria (37). Nevertheless, the identified mycobacterial pathway transforms most of the cholesterol molecule to central metabolites, consistent with growth of bacillus Calmette–Guerin on cholesterol in vitro and suggesting that the sterol is an important source of energy for *M. tuberculosis* during its survival in the macrophage. The essential nature of the cholesterol catabolic enzymes in vivo makes them promising targets for the development of therapeutic agents to combat XDR-TB and other strains, particularly as many of these enzymes have no human homolog.

**Materials and Methods**

**Bacterial Growth.** RHA1 was grown at 30°C on a shaker in one of two minimal media: W minimal salt medium (38) plus 20 mM pyruvate or 2 mM cholesterol or a similar medium supplemented with a different mineral solution (39) plus cholesterol, AD, pyruvate, or benzoate as indicated. RHA1 cells were harvested at midlog phase (OD600 of 1.0 for pyruvate and 2.0 for cholesterol). Bacillus Calmette–Guerin was grown at 37°C on a tube roller (10 rpm) in screw-capped 15-ml vials filled with 10 ml of liquid medium containing 0.5 g/ml asparagine, 1 g/ml KH2PO4, 2.5 g/ml Na2PO4, 10 mg/liter MgSO4·7H2O, 0.5 mg/liter CaCl2, 0.1 mg/liter ZnSO4, 50 mg/liter ferric ammonium citrate, and 0.5 ml/liter Triton WX1339 (Tyoconol) (18) plus the indicated amount of cholesterol, 10 mM pyruvate, or 10 mM glucose. Total protein content of cultures was determined in cells disrupted by sonication (10 cycles of 30 s at 6 μm) by using the Bradford protein assay (BioRad, Hercules, CA) and BSA as standard.

**RNA Extraction and Microarray.** RNA was isolated from RHA1 as described (19). RNA was similarly isolated from bacillus Calmette–Guerin except that both the RNeasy Plus and RNeasy Mini Kits (Qiagen, Valencia, CA) were used, and the sample was treated with 2 units of TURBO DNase (Ambion, Austin, TX). The RHA1 transcriptome was analyzed by using indirectly labeled cDNA and a microarray containing 70-mer probes for 8,313 genes as described (19). Data were analyzed by using GeneSpring (Agilent Technologies, Santa Clara, CA) and MeV 3.1 (The Institute for Genomic Research, Rockville, MD). For each condition, RNA was extracted from each of three independently grown cultures. Data were averaged and normalized by using Locally Weighted Linear Regression (Lowess). Details of the microarray design, transcriptomic experimental design and transcriptomic data have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo).

**Quantitative RT-PCR.** RT-PCR was performed as described (19) with TaqMan probes, and cDNA was synthesized by using the ThermoScript RT-PCR System (Invitrogen, Carlsbad, CA) and random hexamers. All oligonucleotide and probe sequences are provided in **SI Table 3**. The gene-encoding DNA polymerase IV and ρα were associated with the development of whole-cell biotransformation processes for the synthesis of industrially relevant steroid compounds.
used as internal standards in the multiplex reactions performed by using RHA1 and bacillus Calmette–Guérin cDNA, respectively (19). The Ct values were normalized (ΔCt) by subtracting those of the internal standard. Significant differences in ΔCt values were tested by using a two-sample t test assuming unequal variances. Relative fold differences were calculated as 2^ΔΔCt, where ΔΔCt = ΔCt treatment − ΔCt control.

Gene Replacement and Deletion. The hsaC gene was replaced in RHA1 with an apramycin resistance marker, apraR, by using a procedure in which the gene was first replaced in a fosmid by using λRED-based methodology and then in RHA1 by using the modified fosmid and allelic exchange (39). The parent fosmid, RF00128O15, contained 38.3 kb of RHA1 DNA including the hsaACDB cluster. The oligonucleotides used to generate the resistance cassette used to replace hsaC were hsaC-for1 and hsaC-rev1 (SI Table 5). The six mce4 genes and the superAB genes were deleted separately in RHA1 by using the sacB counterselection system essentially as described (7). Oligonucleotides used to amplify the upstream and downstream region of mce4 genes were cloned into similarly digested RF00128O15, containing 38.3 kb of RHA1 DNA including the hsaACDB cluster. The oligonucleotides used to generate the resistance cassette used to replace mce4 were rof04698-F and rof04698-R(SpeI), and rof04703-R(HindII), respectively. The upstream and downstream region of superAB genes were amplified by using oligonucleotides SupA-F and SupA-R(SpeI), and SupB-F(SpeI) and SupB-R (SI Table 5). Gene replacements and deletions were verified by using a series of PCRs using: (i) primers matching sequences within the target gene(s), (ii) primers matching sequences flanking the target gene, and, when appropriate, (iii) primers matching a region within apraR.

Cloning and Expression of Mtb Genes. The hsaC3HTR and hsaD3HTR genes were amplified by PCR using Expand High Fidelity DNA polymerase (Roche Diagnostics, Indianapolis, IN) and cloned essentially as described for dbfB (40). The genes were amplified by using M. tuberculosis H37Rv genomic DNA and either HcmF and HcmR or HdmF and HdmR (SI Table 5). The amplicons were digested with NdeI and BamHI and cloned into similarly digested pT7–7, and their respective nucleotide sequences were confirmed by using pT7HC1 and pT7HD1. HsaC3HTR and HsaD3HTR were produced by using E. coli GJ1158 transformed with pT7HC1 and pT7HD1, respectively, as described for DbfB (40).

Enzyme Assays. HsaC3HTR and HsaD3HTR activities in cellular extracts were measured by following the formation (HsaC) or consumption (HsaD) of the ring-cleaved product on a Cary 5000 spectrophotometer (Varian, Walnut Creek, CA) equipped with a thermostatted cuvette holder, essentially as described for biphenyl catalytic enzymes (40). Experiments were performed by using 20 mM 3-[4-(2-hydroxyethyl)-1-piperazinyl] propanesulfonic acid, 80 mM potassium chloride, pH 8.0 at 25.0 ± 0.1°C. Concentrations of 4,9-DSHA (E_a2 = 7.64 mM−1 cm−1) and HOPDA (E_a2 = 32.5 mM−1 cm−1) were monitored at 392 and 434 nm, respectively. Initial velocities were determined from a least-squares analysis of the linear portion of the progress curves by using the kinetics module of Cary software. Steady-state rate equations were fit to data as described (40).

Metabolite Preparation and Characterization. Culture supernatant was acidified by using 0.5% orthophosphoric acid then extracted twice with 0.5 volume of ethyl acetate. The ethyl acetate extracts were pooled, dried with anhydrous magnesium sulfate, and evaporated to dryness with a rotary evaporator. The residue was dissolved in a 7:3 mixture of methanol/water containing 0.5% phosphoric acid and purified by HPLC with a 2695 separation module (Waters, Milford, MA) and a Prodigy 10-µm ODS-Prep column (21.2 × 250 mm; Phenomenex, Torrance, CA). Metabolites were eluted by using the same methanol/water solvent at a flow rate of 5 ml/min. The eluate was monitored by using a photodiode array detector at 434 nm. The retention time of the major metabolite was ~21 min. The fractions containing this metabolite were pooled, added to 10 volumes of water, and extracted as described above. The metabolite was derivatized by using N-acetyl-BT (capsule, Biotrend, PA) and analyzed by using a 6890 gas chromatograph (Agilent Technologies) and 5973N mass-selective detector (Agilent Technologies) in electron ionization mode. The extinction coefficient of 4,9-DHSA was determined with an oxygen assay (40).

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