Aanvullende informatie betreffende aanvraag IM09-004, t.b.v. Adviesvraag: veterinaire studie met R.equi vaccinstam (CGM/100414-02).

De COGEM heeft op 14 april 2010 om aanvullende informatie verzocht voor het ontwikkelen van een advies (CGM/100414-02) betreffende dossier IM09-004 met als titel ' Een niet-pathogene *Rhodococcus equi* stam als vaccin in paarden'. Met de onderstaande antwoorden proberen wij de COGEM van zoveel mogelijk aanvullende informatie te voorzien om de milieurisicoanalyse verder uit te kunnen voeren.

Wij zouden vooraf echter graag willen benadrukken dat het een aanvraag betreft om proeven te mogen doen met een bacterie waarin wij genen, die op het chromosoom liggen, hebben gedeleteerd, zonder dat er vreemd DNA is achtergebleven in de bacterie. Deze vaccinstam kan dus ook geen "vreemd" DNA overdragen aan andere micro-organismen.
De bacterie is door deze deletie verzwakt t.o.v. wildtype en kan geen ziekte meer veroorzaken in paarden.

In 2009 heeft de COGEM een advies uitgebracht over een soortgelijke studie van Intervet met dezelfde R. equi vaccinstam. In haar advies heeft de COGEM het belang aangegeven van shedding gegevens van veulens en merries voor milieurisico analyse van toekomstige experimenten en toepassingen. Deze gegevens worden in het onderhavige dossier slechts op uiterst summiere wijze samengevat en dienen veel uitvoeriger inzichtelijk gemaakt te worden, opdat de COGEM zich daarover een oordeel kan vormen.

In drie proeven is naar shedding gekeken. Die zijn hieronder samengevat. De resultaten zijn in de vorm van tabellen bijgevoegd.

Exp. 1 : De vaccinstam werd oraal toegediend aan 3-8 dagen oude veulens (dosis 2x10E10 CFU); twee weken later werd de vaccinstam nogmaals oraal toegediend (dosis 2x10E9 CFU). Twee veulens werden niet gevaccineerd en dienden als contact controles. Uitscheiding door de veulens werd getest mbv rectale swabs (Tabel 1) en nasale swabs (Tabel 2). Spreiding naar contact veulens of merries werd getest mbv rectale swabs (Tabel 3). Hieruit bleek dat de gevaccineerde veulens intermitterend uitscheiden en dat ook wildtype *Rhodococcus* aanwezig is in de veulens. Er werd geen spreiding naar contact veulens of merries waargenomen. Zij waren gedurende de hele proef negatief.

Exp 2 : De vaccinstam werd oraal of rectaal toegediend aan 5 maanden oude veulens (dosis 1x10E10 CFU); twee weken later werden de vaccinaties herhaald. In de periode 26 tot 32 dagen na eerste toediening werd uitscheiding getest m.b.v. rectale swabs (Tabel 4). *Rhodococcus equi* kon niet worden aangetoond.

Exp. 3 : De vaccinstam werd rectaal toegediend aan 8 maanden oude veulens (dosis variërend van 1x10E8 tot 1x10E10 CFU). In de periode 18 tot 28 dagen na toediening werd uitscheiding getest m.b.v. rectale swabs en selectieve agar (Tabel 5). Hieruit blijkt dat de veulens intermitterend uitscheiden.

De vaccinstam is geattenueerd in ziekteverwekkend vermogen, i.e. kan geen pneumonie meer veroorzaken omdat hij i.t.t. wildtype, niet meer in staat is te overleven in macrofagen. In de darmen van de dieren als ook in het milieu kan

hij naar verwachting evengoed overleven als de wildtype omdat macrofagen in die milieus geen rol spelen en/of niet voorkomen.

Een ander belangrijk aspect voor de milieurisicoanalyse vormt de stabiliteit van de ggo in het milieu. De aanvrager concludeert dat het ggo qua overleving in het milieu vergelijkbaar is met de uitgangsstam. Ook op dit punt worden zaken als proefopzet, uitkomsten en validatie van de gegevens uiterst summier beschreven. Om de conclusies te kunnen staven, dient de aanvrager een uitgebreide beschrijving van de uitgevoerde overlevingsstudies te overleggen.

Verdunningsreeksen van de vaccinstam en van de wildtype stam werden in drievoud geënt in buizen met kraanwater (Tabel 6-11), vijverwater (Tabel 12-17) en grond (Tabel 18-23). Deze werden vervolgens bij 4°C, KT en 37°C weggezet. Verder werden twee cultures (wildtype en vaccinstam) op een petrischaal ingedroogd en bij de drie temperaturen weggezet (Tabel 24-26). Van deze samples/cultures werden regelmatig swabs genomen en getest op *Rhodococcus equi* m.b.v. selectieve agar.
Uit deze overlevingsproeven blijkt dat de vaccinstam volgens verwachting onder de verschillende omstandigheden evengoed overleeft als het wildtype. Verder werd in enkele buizen die met de vaccinstam geënt waren ook wildtype genotype terug gevonden. Dit is waarschijnlijk het gevolg van het feit dat *Rhodococcus equi* algemeen overal voorkomt en/of is het gevolg van een werkbesmetting. Dit experiment loopt nog steeds.

Een gedetailleerdere beschrijving van de proeven in niet-doelwitorganismen (andere dieren dan paarden) die de vaccin stam via de oro-nasale route binnen zouden kunnen krijgen.

De experimenten in muizen, ratten en kippen zijn reeds als samenvatting gerapporteerd. We hebben nu de volledige rapporten bijgevoegd (zie bijlage 4-I, 4-II en 4-III). De vaccinstam is oraal toegediend aan groepen (n=5) muizen (6.6x10E8 CFU), ratten (1.3x10E9 CFU) en kippen (1.3x10E9 CFU). Vervolgens werden de dieren dagelijks klinisch geobserveerd en werden 2x per week gedurende 3 weken rectale swabs afgenomen en getest op de aanwezigheid van *Rhodococcus equi*. Na drie weken is een post-mortem onderzoek gedaan. Parallelle groepen (n=5) muizen, ratten en kippen zijn op dezelfde manier behandeld met de wildtype stam. Er zijn geen klinische effecten ten gevolge van de toediening waargenomen, en zowel wildtype als de vaccinstam zijn op geen enkel moment uit geen enkel dier teruggeïsoleerd uit rectale swabs. Ook tijdens het post-mortem onderzoek werden geen klinische effecten gevonden. Inmiddels is de veiligheid eveneens getest in kalveren en varkens. In deze dieren werd de vaccinstam zowel oraal als nasaal toegediend (totale dosis 1.1x10E9 CFU). Vervolgens werden de dieren dagelijks klinisch geobserveerd en werden 2x per week gedurende 3 weken rectale swabs en 1x per week neus swabs afgenomen en getest op de aanwezigheid van *Rhodococcus equi*. Na drie weken is een post-mortem onderzoek gedaan. Parallelle groepen (n=5) kalveren en varkens zijn op dezelfde manier behandeld met de wildtype stam.
Net als in muizen, ratten en kippen zijn in zowel varkens als kalveren geen klinische effecten ten gevolge van de toediening waargenomen en werd de vaccinstam uit geen enkel dier teruggevonden na toediening. Wel werd de wildtype stam uit 1 kalf van een neusswab (dag 7 na toediening) terug ge-

isoleerd. Tijdens het post-mortem onderzoek zijn eveneens geen Rhodococcus geassocieerde klinische effecten waargenomen.
Hiermee hebben wij in een 5 tal niet-doelwit organismen aangetoond dat er geen nadelige effecten zijn van de vaccinstam. Gezien de soortspecificiteit van *Rhodoccocus equi* (virulentieplasmide waarvan bekend is dat het soortspecifiek is), lijkt het onnodig om dit in alle mogelijke niet-doelwit organismen aan te tonen. Het principe is met een 5 tal diersoorten aangetoond.

Hierbij merkt de COGEM op dat de verminderde replicatie van de vaccinstam in paarden macrofagen geen maat is voor de vermeende verminderde virulentie in de mens. Aanvullende gegevens over de mogelijke effecten van het GGO in de mens zijn nodig.

Ten eerste willen wij graag opmerken dat er geen gebruik is gemaakt van paarden macrofagen maar van humane macrofagen cellijn, te weten U-937 (ATCC CRL-1593.2), en wij wel degelijk van mening zijn dat dit een maat is voor verminderde virulentie in de mens.
M.a.w. er is aangetoond dat de vaccinstam (i.t.t. de wildtype stam) niet meer kan overleven in humane macrofagen en hiermee dat deze dus niet meer in staat is om infectie te veroorzaken in de mens.

Verder willen wij hier nogmaals benadrukken dat *R. equi* nauwelijks virulent is voor de mens en zelden of nooit problemen veroorzaakt in immunocompetente personen. Het aantal infecties van gezonde personen door wildtype *R. equi* is zeer beperkt. Net als veel andere normaal gesproken apathogene bacteriën kan wildtype *Rhodococcus equi* infecties veroorzaken in immunodeficiënte personen (zie ook COGEM advies CGM/090416-01, *Humane infectie* en *Humane infectie door R. equi RG2837*).
De *R. equi* vaccinstam overleeft slecht in humane macrofagen, een niche waar een wild type *R. equi* goed overleefd (eigen macrofaag proeven), zonder dat dit blijkbaar ziekte tot gevolg heeft.

R. equi is sterk verwant aan *Mycobacterium tuberculosis* en de infectie bij veulens lijkt sterk op de *M. tuberculosis* infectie bij mensen. De bacteriële besmetting vindt meestal plaats via de longen alwaar ze worden gefagociteerd door de alveolaire macrofagen. Evenals *M. tuberculosis* is *R. equi* in staat het fuseren van het fagosoom met het lysosoom in de doel (mens of paard) macrofagen te voorkomen en zo een milieu te creëren waar de bacterie afgeschermd voor het immuun systeem kan repliceren.

Er zijn meer overeenkomsten. Uit onderzoek is gebleken dat de genen betrokken bij het cholesterol catabolisme ook een belangrijke rol spelen bij de pathogenese van deze beide bacteriën en andere pathogene Cornybacteriaceae (Mycobacterium, Corynebacterium, Gordonia, Norcardia, Tsukamurella en Rhodococcus). De genen van het cholesterol catabolisme zijn zeer geconserveerd binnen de Cornybacteriaceae (artikel Geize et al en unpublished results) en maken deel uit van een gencluster van meer dan 80 genen die in saprofytische Cornybacteriaceae zijn betrokken bij afbraak van plantaardige steroïden en andere aromatische (planten) verbindingen. In pathogene Cornybacteriaceae (waaronder *M. tuberculosis)* worden deze genen ook geïnduceerd tijdens groei in macrofagen en blijken een groot aantal genen (*kstD*, *hsaF*, *kshA*, *fadE30*, *ipdA*, *ipdB*) zelfs essentieel te zijn voor overleving in macrofagen en muizen (Sassetti et al, 2003). Ook voor *R. equi* hebben wij laten zien dat *ipdAB* essentieel is voor een groei in macrofagen, en dat de vaccinstam

geattenueerd is in veulens en niet meer in staat is pneumonie te veroorzaken. Intervet heeft ook de *ipdAB* deletie mutant gemaakt in *Nocardia serioliae*. Dit vissenpathogeen is gebruikt om White Tail te vaccineren en ook in deze proef bleek de Nocardia vaccinstam veilig en effectief, waarmee de centrale rol van *ipdAB* en het cholesterol catabolisme in de virulentie van pathogene Cornybacteriaceae wordt onderstreept. Dit laatste wordt nog eens benadrukt door het feit dat intact een *fadE30* gen ook essentieel voor groei in macrofagen in *R. equi* is.
Omdat *R. equi*, net als de meeste andere Corynebacteria gebruik kunnen maken van een groot aantal koolstofbronnen is het bijna onmogelijk om aan te tonen dat onze vaccinstam minder goed zou overleven in het milieu.

Bijlagen
Bijlage 4.1-I, II en III
Tabellen 1 – 26

Literatuur
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Bijlage 4-I

TITLE: SAFETY OF EQUILIS RHODE IN MICE AFTER ORAL ADMINISTRATION

Exp.no.: REV 09.13.015

Date: 14-01-2010

IMog-004 / 19 mei 2010

SUMMARY

The objective of this experiment was to investigate the safety of Equilis RhodE vaccine (vaccine strain RG2837) in mice after oral administration.
Two groups of five mice each were orally treated with $6.6x10^8$ CFU of either *Rhodococcus equi* strain RG2837 or with the wild parent type strain RE1. At T=0 and then twice weekly during three weeks rectal swabs were sampled and examined for re-isolation of *Rhodococcus equi* on selective agar. In addition the mice were daily observed for general health and/or clinical abnormalities. At T= 3 weeks after vaccination, the mice were killed and a full necropsy was performed.
One day after inoculation with strain RG2837, the mice in this group were less active. The next day these mice were normal again and further no abnormalities in either group were observed. During 3 weeks after inoculation with Equilis RhodE or with the wild type strain RE1, no abnormalities in the consistency of the faeces were observed and *Rhodococcus equi* was not re-isolated from rectal swabs.
During the necropsy one mouse inoculated with Equilis RhodE had an enlarged mesenterial lymph node and an enlarged spleen. Both tissues appeared sterile upon culture. Further no abnormalities of internal organs were detected in either group.
From the results it can be concluded that ingestion of *Rhodococcus equi* wild type strain RE1 or vaccine strain RG2837 by mice poses no risk to this species. It does not cause clinical signs, does not cause an infection and is not shed into the environment via mice after oral administration to mice.

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1 INTRODUCTION

The objective of this experiment was to investigate the safety of Equilis RhodE vaccine in mice after oral administration.

2 EXPERIMENTAL DESIGN

Two groups of five mice each were orally treated with $6.6x10^8$ CFU of either *Rhodococcus equi* vaccine strain RG2837 or with the wild type parent strain RE1. At T=0 and then twice weekly during three weeks rectal swabs were sampled and examined for re-isolation of *Rhodococcus equi* on selective agar. In addition the mice were daily observed for general health and/or clinical abnormalities. At T= 3 weeks after vaccination, the mice were killed and a full necropsy was performed. In case of any abnormalities, additional swab and/or tissue samples were taken for bacteriological and/or histopathological examination.

3 BIOSAFETY

Working with *Rhodococcus equi* was carried out using standard safe bacteriological techniques; the bacterium is a zoonotic (for immunocompromised persons) pathogen of EC class 2.
Handlings with the mutant strain at the laboratory were carried out under ML-II conditions. Animal experiments with the mutant strain were carried out under DM-II conditions (project no.: 99-123). All materials were transported from the ML-II to the DM-II units or vice versa in closed tubes or closed containers (decontaminated on the outside), double packed in appropriate DM-II approved containers. At the end of the experiment, the cages and isolation rooms were disinfected with formalin and the animals and waste were disposed of using VAT 4 WIVA (blue) containers.

4 MATERIALS AND METHODS

4.1 Test articles

4.1.1 Vaccine

Product name:	Equilis RhodE; *R. equi* strain RG2837.
Pharmaceutical form:	Lyophilisate
Batch number:	16A09
Storage conditions:	2-8 °C
Production date:	2 Feb 2009
Expiry date:	Feb 2012 (3 year stability to be confirmed)
Transport conditions:	Ambient temperature
Quality control:	The vaccine contained 3.7x10E9 CFU per vial. The vaccine was released according to standard procedures by the Microbiological R&D Department, Intervet International bv, Boxmeer, The Netherlands.

4.1.2 Solvent

Product name:	Nobivac Diluent (item nr. 012015)
Pharmaceutical form:	Solvent for parenteral/oral use
Batch number:	82123
Storage conditions:	15-25°C
Expiry:	Apr-2012
Quality control:	Quality Management Pharmaceuticals, Intervet International bv.

4.1.3 Preparation of vaccine

Each vial of Equilis RhodE contained $3.7x10^9$ CFU per 1 ml. For this experiment six vials were reconstituted in 1.4 ml each, pooled and then used to inoculate the mice orally with 0.25 ml (=$6.6x10^8$CFU).

Stability:	The vaccine was used immediately after preparation and is stable for at least 3 hours.
Infectivity titre:	After the vaccination, a live count was performed to confirm the infectivity titre. The titre found was $4.66x10^8$/dose

4.1.4 Wild type strain

Strain name:	*Rhodococcus equi* RE1
Pharmaceutical form:	Lyophilisate
Batch number:	100909
Storage conditions:	≤-15°C
Production date:	10 Sep 2009
Expiry date:	Not applicable
Transport conditions:	Ambient temperature
Quality control:	Viable count of $4.60x10^8$ CFU per vial.

4.1.5 Preparation of wild type strain

In total 33 vials of *R. equi* strain RE1 were reconstituted in 0.35 ml solvent each and subsequently pooled and then used to inoculate the mice orally with 0.5 ml (=$6.6x10^8$ CFU) of reconstituted material.

Stability:	The reconstituted material was used immediately after preparation and is stable for at least 3 hours.
Infectivity titre:	After the vaccination, a live count was performed to confirm the infectivity titre. The titre found was $7.38x10^8$/dose.

4.2 Test system

4.2.1 Animals

Ten 4 week-old outbred mice were used.

4.2.2 Acclimatisation, husbandry, housing, food and water

The two groups were housed in two different isolation rooms (i.e. 1.17 and 1.18) according to standard procedures. During the experiment the mice were fed according to standard procedures. The mice had access to fresh tap water ad lib.
The acclimatization was 1 week. During the acclimatization period the mice were daily observed.

4.3 Grouping and dosing

4.3.1 Grouping

The mice were assigned to two groups of five mice each. The groups were housed in two different isolation rooms.

4.3.2 Treatment

The mice were inoculated with either 0.5 ml of strain RE1 or 0.25 ml of strain RG2837. The inoculates were prepared as described under 4.1. The inoculations of the wildtype RE1 group were administered as three doses of 0.2 ml, 0.2 ml and 0.1 ml with one hour interval between the inoculations. The inoculations of the Equilis RhodE group were given as two doses of 0.15 ml and 0.1 ml with 1 hour interval between the inoculations. The oral inoculations were carried out by a biotechnician.

Treatment scheme

group	Treatment Oral dose	Observations day 0-21 Clin. Obs / rectal swabs	Necropsy
1 n=5	$6.6x10^8$ CFU strain RE1	Daily / twice a week	Day 21
2 n=5	$6.6x10^8$ CFU strain RG2837	Daily / twice a week	Day 21

4.4 Experimental procedures and parameters

4.4.1 Rectal swabs

Rectal swabs were sampled at T=0 and then twice a week during three weeks. The swab samples were resuspended and serially diluted in physiological salt solution, plated on selective agar and grown at 37°C during 24-72 hours. *R. equi* was initially identified by the typical colony morphology and further identified by API/Phoenix according to standard procedures. At least one isolate from each mouse (if present) was tested in a specific PCR (see below under 4.4.3) to confirm the identity of vaccine or wild type strain.

4.4.2 Clinical observations

During the study the mice were daily observed for any abnormalities of general health and/or behavior. The following scoring systems were used :
Systemic reaction scores : 0=normal, 1=less active, 2=slightly depressed, 3=depressed, 4=severely depressed.
Diarhea scores : 0=normal, 1=mild diarrhea, 2=moderate diarrhea, 3=severe diarrhea .

4.4.3 PCR

The identity of the vaccine strain Δ*ipdABipdAB2* genotype was confirmed by a quantitative PCR using a real time PCR detection system. PCR amplification was performed on colony material. The PCR mixture contained 20U/ml DNA polymerase (HT Biotechnology Ltd, Cambridge, UK), 1x Icycler buffer (100mM Tris-HCl and 500 mM KCl, pH 8.5), 0.2 mM dNTP's (HT Biotechnology, Ltd, Cambridge, UK), 4 mM $MgCl_2$, 200 nM primers and 100 nM probes.
Primers 5626-OLI-3159, -3158 and -3128 and probes 5626-OLI-3130 (TXR) and 5626-OLI-3160 (FAM) from the Intervet oligo collection were used for the *ipdAB* Q-PCR.
Primers 5626-OLI-3131, -3165 and -3166 and probes 5626-OLI-3134 (FAM) and 5626-OLI-3135 (TXR) from the Intervet oligo collection were used for the *ipdAB2* Q-PCR.
The PCR program is composed of a 5 minutes denaturation step at 95°C and 50 cycles of 10 seconds at 95°C, 15 seconds at 55°C and 30 seconds at 72°C. Data collection was performed at 72°C. A wild type strain (e.g. strain RE1) will be positive with the FAM probe 5626-OLI-3160 and TexasRed probes 5626-OLI-3130 and -3135). An *ipdABipdAB2* mutant will only be positive with the FAM probes 5626-OLI-3160 and -3134.

4.4.4 Post mortem examination and bacteriology

On day 21 post-challenge the mice were sedated by isofluraan, bled and subsequently killed by cervical dislocation. A complete post-mortem examination was performed on all animals with special attention to the lungs and gut with associated lymph nodes. Post-mortem findings were recorded on a dissection form. In case of abnormalities, samples for histology were taken as deemed necessary by the pathologist. Any samples for histological examination were recorded on the dissection form.
Swabs and/or tissue samples were sampled from all other abnormalities during necropsy and examined bacteriologically. These swabs were streaked on blood agar and/or on selective agar (in case of gut tissue) and incubated at 37°C for 16-72 hours. *Rhodococcus equi* colonies were initially identified by the typical non-hemolytic mucoid colony morphology, enumerated and expressed as CFU/ml. From each mouse the identity of at least one isolate (selected at random) if present was confirmed by: Gram stain, API/Phoenix and/or PCR.

5. EVALUATION OF RESULTS

All data on local and systemic reactions after the treatments and post-mortem findings were recorded for each mouse individually. This will give an impression of the number/percentage and severeness of reactions that might occur after oral ingestion of Equilis RhodE by mice in comparison with the wild type parent strain.

6. STORAGE OF DOCUMENTATION

All records concerning animal experimentation and analytical data will be kept at the Microbiological Research Department of Intervet International BV, Boxmeer.

7. RESULTS AND DISCUSSION

During inoculation of the first to be treated mouse (RE1 group) the mouse suffocated probably because of a too large inoculation volume of 0.5 ml (leaving 4 mice for the wild type group). Therefore, the inoculation volumes were reduced to maximally 0.2 ml and split into 2 or 3 inoculations with a 1 hour interval between the inoculations.
One day after inoculation with vaccine strain RG2837, mice in this group were less active (Table 1). The next day these mice were normal again and further no abnormalities in either group were observed.
During 3 weeks after inoculation with Equilis RhodE or with the wild type strain RE1, no abnormalities in the consistency of the faeces were observed (Table 2) and *Rhodococcus equi* was not re-isolated from rectal swabs (Table 3).
During the necropsy one mouse inoculated with the wild type strain RE1 had an enlarged mesenterial lymph node and an enlarged spleen. Both tissues appeared sterile upon culture and thus no relation with the inoculum could be demonstrated (Table 4). Further no abnormalities of internal organs were detected in either group.

8 CONCLUSION

From the results it can be concluded that ingestion of *Rhodococcus equi* wild type strain RE1 or vaccine strain RG2837 by mice poses no risk to this species. It does not cause clinical signs, does not cause an infection and is not shed into the environment after oral administration to mice.

AJ/IB/14-10-2010

Table 1 Systemic reactions after vaccination (day 0-21)

mouse no.	treatment group	local and/or systemic reactions at day																					
		0	0+4h	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
2	wild type strain RE1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
6	vaccine strain RG2837	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7		0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
8		0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
9		0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
10		0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Systemic reaction score: 0=normal, 1=less active, 2=slightly depressed, 3=depressed, 4=severely depressed.

Table 2 Diarrhea scores after vaccination (day 0-21)

mouse no.	treatment group	faeces score at day																					
		0	0+4h	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
2	wild type strain RE1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
6	vaccine strain RG2837	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
8		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
9		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
10		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Faecess score: 0=normal, 1=soft consistency of faeces, 2=mild diarrhea, 3=moderate diarrhea, 4=severe diarrhea

Table 3 Shedding of R. equi after vaccination

mouse no.	treatment group	isolation of R. equi (CFU/swab) from rectal swabs (days)						
		0	3	7	10	14	17	21
2	wild type strain RE1	0	0	0	0	0	0	0
3		0	0	0	0	0	0	0
4		0	0	0	0	0	0	0
5		0	0	0	0	0	0	0
6	vaccine strain RG2837	0	0	0	0	0	0	0
7		0	0	0	0	0	0	0
8		0	0	0	0	0	0	0
9		0	0	0	0	0	0	0
10		0	0	0	0	0	0	0

Table 4 Macroscopical findings at necropsy

treatment group	mouse no.	tissue/organ	macroscopical findings	re-isolation
wild type strain RE1	2	mesenterial lymph node spleen	enlarged enlarged	no growth no growth
	3	nad	-	-
	4	nad	-	-
	5	nad	-	-
vaccine strain RG2837	6	nad	-	-
	7	nad	-	-
	8	nad	-	-
	9	nad	-	-
	10	nad	-	-

nad= no abnormalities detected

BIJLAGE 4-II

TITLE: SAFETY OF EQUILIS RHODE IN CHICKENS AFTER ORAL ADMINISTRATION

Exp. No.: REV 09.13.017

Date: 14-01-2010

SUMMARY

The objective of this experiment was to investigate the safety of Equilis RhodE vaccine (vaccine strain RG2837) in chickens after oral administration.
Two groups of five chickens each were orally treated with $1.3x10^9$ CFU of either *Rhodococcus equi* strain RG2837 or the wild type parent strain RE1. At T=0 and then twice weekly during three weeks rectal swabs were sampled and examined for re-isolation of *Rhodococcus equi* on selective agar. In addition the chickens were daily observed for general health and/or clinical abnormalities. At T= 3 weeks after vaccination, the chickens were killed and a full necropsy was performed.
At day 14 post-inoculation one wild type treated bird had a small wound with crust on the left side of the head. This, most probably (obviously), is not related to the inoculation. At day 7 after inoculation one vaccine strain treated bird was less active. The next day this bird was normal again and further no clinical abnormalities were observed in either group, during the 3-week observation period. Likewise, during 3 weeks after inoculation with Equilis RhodE or with the wild type strain RE1, no abnormalities in the consistency of the faeces were observed and *Rhodococcus equi* was not re-isolated from rectal swabs. Moreover, during the necropsy no abnormalities of internal organs were observed.
From the results it can be concluded that ingestion of *Rhodococcus equi* wild type strain RE1 or vaccine strain RG2837 by chickens poses no risk to this species. It does not cause clinical signs, does not cause an infection and is not shed into the environment after oral administration to chickens.

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1 INTRODUCTION

The objective of this experiment was to investigate the safety of Equilis RhodE vaccine in chickens after oral administration.

2 EXPERIMENTAL DESIGN

Two groups of five chickens each were orally treated either with $1.3x10^9$ CFU of *Rhodococcus equi* vaccine strain RG2837 or with the wild type parent strain RE1. At T=0 and then twice weekly during three weeks rectal swabs were sampled and examined for re-isolation of *Rhodococcus equi* on selective agar. In addition the chickens were daily observed for general health and/or clinical abnormalities. At T= 3 weeks after vaccination, the chickens were killed and a full necropsy was performed. In case of any abnormalities, additional swab and/or tissue samples were taken for bacteriological and/or histopathological examination.

3 BIOSAFETY

Working with *Rhodococcus equi* was carried out using standard safe bacteriological techniques; the bacterium is a zoonotic (for immunocompromised persons) pathogen of EC class 2.
Handlings with the mutant strain at the laboratory were carried out under ML-II conditions. Animal experiments with the mutant strain were carried out under DM-II conditions (project no.: 99-123). All materials were transported from the ML-II to the DM-II units or vice versa in closed tubes or closed containers (decontaminated on the outside), double packed in appropriate DM-II approved containers. At the end of the experiment, the cages and isolation rooms were disinfected with formalin and the animals and waste were disposed of using VAT 4 WIVA (blue) containers.

4 MATERIALS AND METHODS

4.1 Test articles

4.1.1 Vaccine

Product name:	Equilis RhodE; *R. equi* strain RG2837.
Pharmaceutical form:	Lyophilisate
Batch number:	16A09
Storage conditions:	2-8 °C
Production date:	2 Feb 2009
Expiry date:	Feb 2012 (3 year stability to be confirmed)
Transport conditions:	Ambient temperature
Quality control:	The vaccine contained 3.7x10E9 CFU per vial. The vaccine was released according to standard procedures by the Microbiological R&D Department, Intervet International bv, Boxmeer, The Netherlands.

4.1.2 Solvent

Product name:	Nobivac Diluent (item nr. 012015)
Pharmaceutical form:	Solvent for parenteral/oral use
Batch number:	82123
Storage conditions:	15-25°C
Expiry:	Apr-2012
Quality control:	Quality Management Pharmaceuticals, Intervet International bv.

4.1.3 Preparation of vaccine

Each vial of Equilis RhodE contained $3.7x10^9$ CFU. For this experiment three vials were reconstituted in 1.4 ml each, pooled and then used to inoculate the chickens orally with 0.5 ml (=$1.32x10^9$ CFU).

Stability:	The vaccine was used immediately after preparation and is stable for at least 3 hours.
Infectivity titre:	After the vaccination, a live count was performed to confirm the infectivity titre. The titre found was $1.01x10^9$/dose

4.1.4 Wild type strain

Strain name:	*Rhodococcus equi* RE1
Pharmaceutical form:	Lyophilisate
Batch number:	100909
Storage conditions:	≤-15°C
Production date:	10 Sep 2009
Expiry date:	Not applicable
Transport conditions:	Ambient temperature
Quality control:	Viable count of $4.60x10^8$ CFU per vial.

4.1.5 Preparation of wild type strain

In total, 17 vials of *R. equi* strain RE1 were reconstituted in 0.35 ml solvent each and subsequently pooled and then used to inoculate the chickens orally with 1.0 ml (=$1.31x10^9$ CFU) of reconstituted material.

Stability:	The reconstituted material was used immediately after preparation and is stable for at least 3 hours.
Infectivity titre:	After the vaccination, a live count was performed to confirm the infectivity titre. The titre found was $1.26x10^9$/dose

4.2 Test system

4.2.1 Animals

Ten 4-week-old SPF (layer type) chickens were used.

4.2.2 Acclimatisation, husbandry, housing, food and water

The two groups were housed in two different isolators (i.e. B21 and B22) according to standard procedures. During the experiment the chickens were fed according to standard procedures. The chickens had access to fresh tap water ad lib.
The acclimatization was 1 week. During the acclimatization period the chickens were daily observed.

4.3 Grouping and dosing

4.3.1 Grouping

The chickens were assigned to two groups of five chickens each. The groups were housed in different isolators.

4.3.2 Treatment

The chickens were inoculated with either 1.0 ml of strain RE1 or 0.5 ml of strain RG2837. The inoculates were prepared as described under 4.1. The inoculations were carried out by a biotechnician.

Treatment scheme

group	Treatment Oral dose	Observations day 0-21 Clin. Obs / rectal swabs	Necropsy
1 n=5	$1.31x10^9$ CFU strain RE1	Daily / twice a week	Day 21
2 n=5	$1.32x10^9$ CFU strain RG2837	Daily / twice a week	Day 21

4.4 Experimental procedures and parameters

4.4.1 Rectal swabs

Rectal swabs were sampled at T=0 and then twice a week during three weeks. The swab samples were resuspended and serially diluted in physiological salt solution, plated on selective agar and grown at 37°C during 24-72 hours. *R. equi* was initially identified by the typical colony morphology and further identified by API/Phoenix according to standard procedures. At least one isolate from each chicken (if present) was tested in a specific PCR (see below under 4.4.3) to confirm the identity of vaccine or wild type strain.

4.4.2 Clinical observations

During the study the chickens were daily observed for any abnormalities of general health and/or behavior . The results were recorded. The following scoring systems were used :
Systemic reaction scores : 0=normal, 1=less active, 2=slightly depressed, 3=depressed, 4=severely depressed.
Diarhea scores : 0=normal, 1=mild diarrhea, 2=moderate diarrhea, 3=severe diarrhea .

4.4.3 PCR

The identity of the vaccine strain Δ*ipdABipdAB2* genotype was confirmed by a quantitative PCR using a real time PCR detection system. PCR amplification was performed on colony material. The PCR mixture contained 20U/ml DNA polymerase (HT Biotechnology Ltd, Cambridge, UK), 1x Icycler buffer (100mM Tris-HCl and 500 mM KCl, pH 8.5), 0.2 mM dNTP's (HT Biotechnology, Ltd, Cambridge, UK), 4 mM $MgCl_2$, 200 nM primers and 100 nM probes.
Primers 5626-OLI-3159, -3158 and -3128 and probes 5626-OLI-3130 (TXR) and 5626-OLI-3160 (FAM) from the Intervet oligo collection were used for the *ipdAB* Q-PCR.
Primers 5626-OLI-3131, -3165 and -3166 and probes 5626-OLI-3134 (FAM) and 5626-OLI-3135 (TXR) from the Intervet oligo collection were used for the *ipdAB2* Q-PCR.
The PCR program is composed of a 5 minutes denaturation step at 95°C and 50 cycles of 10 seconds at 95°C, 15 seconds at 55°C and 30 seconds at 72°C. Data collection was performed at 72°C. A wild type strain (e.g. strain RE1) will be positive with the FAM probe 5626-OLI-3160 and TexasRed probes 5626-OLI-3130 and -3135). An *ipdABipdAB2* mutant will only be positive with the FAM probes 5626-OLI-3160 and -3134.

4.4.4 Post mortem examination and bacteriology

On day 21 post-challenge the chickens were sedated by a CO_2/O_2,mixture, bled, and subsequently killed by cervical dislocation. A complete post-mortem examination was performed on all animals with special attention to the lungs and gut with associated lymph nodes. Post-mortem findings were recorded on a dissection form. In case of abnormalities, samples for histology were taken as deemed necessary by the pathologist. Any samples for histological examination were recorded on the dissection form.
Swabs and/or tissue samples were sampled from all other abnormalities during necropsy and examined bacteriologically. These swabs were streaked on blood agar and/or on selective agar (in case of gut tissue) and incubated at 37°C for 16-72 hours. *Rhodococcus equi* colonies were initially identified by the typical non-hemolytic mucoid colony morphology, enumerated and expressed as CFU/ml. From each chicken the identity of at least one isolate (selected at random) if present was confirmed by: Gram stain, API/Phoenix and/or PCR.

5. EVALUATION OF RESULTS

All data on local and systemic reactions after the treatments and post-mortem findings were recorded for each chicken individually. This will give an impression of the number/percentage and severeness of reactions that might occur after oral ingestion of Equilis RhodE by chickens in comparison with the wild type parent strain.

6. STORAGE OF DOCUMENTATION

All records concerning animal experimentation and analytical data will be kept at the Microbiological Research Department of Intervet International BV, Boxmeer.

7. RESULTS AND DISCUSSION

At day 14 post-inoculation one wild type treated bird had a small wound with crust on the left side of the head. This, most probably (obviously), is not related to the inoculation. At day 7 after inoculation one vaccine strain treated bird was less active (Table 1). The next day this bird was normal again and further no clinical abnormalities were observed in either group during the 3-week observation period. Likewise, during 3 weeks after inoculation with Equilis RhodE or with the wild type strain RE1, no abnormalities in the consistency of the faeces were observed (Table 2) and *Rhodococcus equi* was not re-isolated from rectal swabs (Table 3). Moreover, during the necropsy no abnormalities of internal organs were observed (Table 4).

8 CONCLUSION

From the results it can be concluded that ingestion of *Rhodococcus equi* wild type strain RE1 or vaccine strain RG2837 by chickens poses no risk to this species. It does not cause clinical signs, does not cause an infection in chickens and is not shed into the environment.

AJ/IB/14-10-2010

Table 1 Systemic reactions after vaccination (day 0-21)

chicken no.	treatment group	local and/or systemic reactions at day																					
		0	0+4h	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
586	wild type strain RE1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
587		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0[a]	0	0	0	0	0	0	0
588		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
589		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
590		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
726	vaccine strain RG2837	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
727		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
728		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
729		0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
730		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Systemic reaction score: 0=normal, 1=less active, 2=slightly depressed, 3=depressed, 4=severely depressed.

[a] small wound with crust on left side of head

Table 2 Diarrhea scores after vaccination (day 0-21)

chicken no.	treatment group	faeces score at day																					
		0	0+4h	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
586	wild type strain RE1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
587		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
588		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
589		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
590		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
726	vaccine strain RG2837	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
727		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
728		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
729		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
730		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Faecess score: 0=normal, 1=soft consistency of faeces, 2=mild diarrhea, 3=moderate diarrhea, 4=severe diarrhea

Table 3 Shedding of R. equi after vaccination

chicken no.	treatment group	isolation of R. equi (CFU/swab) from rectal swabs						
		0	3	7	10	14	17	21
586	wild type strain RE1	0	0	0	0	0	0	0
587		0	0	0	0	0	0	0
588		0	0	0	0	0	0	0
589		0	0	0	0	0	0	0
590		0	0	0	0	0	0	0
726	vaccine strain RG2837	0	0	0	0	0	0	0
727		0	0	0	0	0	0	0
728		0	0	0	0	0	0	0
729		0	0	0	0	0	0	0
730		0	0	0	0	0	0	0

Table 4 Macroscopical findings at necropsy

treatment group	chicken no.	tissue/organ	macroscopical findings	re-isolation
wild type strain RE1	586	nad	-	-
	587	nad	-	-
	588	nad	-	-
	589	nad	-	-
	590	nad	-	-
vaccine strain RG2837	726	nad	-	-
	727	nad	-	-
	728	nad	-	-
	729	nad	-	-
	730	nad	-	-

nad= no abnormalities detected

BIJLAGE 4-III

TITLE: SAFETY OF EQUILIS RHODE IN RATS AFTER ORAL ADMINISTRATION

Exp. No.: REV 09.13.016

Date: 14-1-2010

IM09-004/19mei2010

SUMMARY

The objective of this experiment was to investigate the safety of Equilis RhodE vaccine (vaccine strain RG2837) in rats after oral administration.
Two groups of five rats each were orally treated with $1.3x10^9$ CFU of either *Rhodococcus equi* strain RG2837 or with the wild type parent strain RE1. At T=0 and then twice weekly during three weeks rectal swabs were sampled and examined for re-isolation of *Rhodococcus equi* on selective agar. In addition the rats were daily observed for general health and/or clinical abnormalities. At T= 3 weeks after vaccination, the rats were killed and a full necropsy was performed.
During 3 weeks after inoculation no systemic reactions were observed in either group. Likewise, during 3 weeks after inoculation with Equilis RhodE or with the wildtype strain RE1, no abnormalities in the consistency of the faeces were observed and *Rhodococcus equi* was not re-isolated from rectal swabs. Moreover, during the necropsy no abnormalities of internal organs were observed.
From the results it can be concluded that ingestion of *Rhodococcus equi* wild type strain RE1 or vaccine strain RG2837 by rats poses no risk to this species. It does not cause clinical signs, does not cause an infection and is not shed into the environment after oral administration to rats.

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1 INTRODUCTION

The objective of this experiment was to investigate the safety of Equilis RhodE vaccine in rats after oral administration.

2 EXPERIMENTAL DESIGN

Two groups of five rats each were orally treated either with $1.3x10^9$ CFU of *Rhodococcus equi* vaccine strain RG2837 or with the wild type parent strain RE1. At T=0 and then twice weekly during three weeks rectal swabs were sampled and examined for re-isolation of *Rhodococcus equi* on selective agar. In addition the rats were daily observed for general health and/or clinical abnormalities. At T= 3 weeks after vaccination, the rats were killed and a full necropsy was performed. In case of any abnormalities, additional swab and/or tissue samples were taken for bacteriological and/or histopathological examination.

3 BIOSAFETY

Working with *Rhodococcus equi* was carried out using standard safe bacteriological techniques; the bacterium is a zoonotic (for immunocompromised persons) pathogen of EC class 2.
Handlings with the mutant strain at the laboratory were carried out under ML-II conditions. Animal experiments with the mutant strain were carried out under DM-II conditions (project no.: 99-123). All materials were transported from the ML-II to the DM-II units or vice versa in closed tubes or closed containers (decontaminated on the outside), double packed in appropriate DM-II approved containers. At the end of the experiment, the cages and isolation rooms were disinfected with formalin and the animals and waste were disposed of using VAT 4 WIVA (blue) containers.

4 MATERIALS AND METHODS

4.1 Test articles

4.1.1 Vaccine

Product name:	Equilis RhodE; *R. equi* strain RG2837.
Pharmaceutical form:	Lyophilisate
Batch number:	16A09
Storage conditions:	2-8 °C
Production date:	2 Feb 2009
Expiry date:	Feb 2012 (3 year stability to be confirmed)
Transport conditions:	Ambient temperature
Quality control:	The vaccine contained 3.7x10E9 CFU per vial. The vaccine was released according to standard procedures by the Microbiological R&D Department, Intervet International bv, Boxmeer, The Netherlands.

4.1.2 Solvent

Product name:	Nobivac Diluent (item nr. 012015)
Pharmaceutical form:	Solvent for parenteral/oral use
Batch number:	82123
Storage conditions:	15-25°C
Expiry:	Apr-2012
Quality control:	Quality Management Pharmaceuticals, Intervet International bv.

4.1.3 Preparation of vaccine

Each vial of Equilis RhodE contained $3.7x10^9$ CFU. For this experiment six vials were reconstituted in 1.4 ml each, pooled and then used to inoculate the rats orally with 0.5 ml (=$1.32x10^9$ CFU).

Stability:	The vaccine was used immediately after preparation and is stable for at least 3 hours.
Infectivity titre:	After the vaccination, a live count was performed to confirm the infectivity titre. The titre found was $0.93x10^9$/dose

4.1.4 Wild type strain

Strain name:	*Rhodococcus equi* RE1
Pharmaceutical form:	Lyophilisate
Batch number:	100909
Storage conditions:	≤-15°C
Production date:	10 Sep 2009
Expiry date:	Not applicable
Transport conditions:	Ambient temperature
Quality control:	Viable count of $4.60x10^8$ CFU per vial.

4.1.5 Preparation of wild type strain

In total, 33 vials of *R. equi* strain RE1 were reconstituted in 0.35 ml solvent each and subsequently pooled and then used to inoculate the rats orally with 1.0 ml (=$1.31x10^9$ CFU) of reconstituted material.

Stability:	The reconstituted material was used immediately after preparation and is stable for at least 3 hours.
Infectivity titre:	After the vaccination, a live count was performed to confirm the infectivity titre. The titre found was $1.48x10^9$/dose.

4.2 Test system

4.2.1 Animals

Ten 4-week-old outbred rats were used.

4.2.2 Acclimatisation, husbandry, housing, food and water

The two groups were housed in two different isolation rooms (i.e. 1.19 and 1.20) according to standard procedures. During the experiment the rats were fed according to standard procedures. The rats had access to fresh tap water ad lib.
The acclimatization was 1 week. During the acclimatization period the rats were daily observed.

4.3 Grouping and dosing

4.3.1 Grouping

The rats were assigned to two groups of five rats each. The groups were housed in two different isolation rooms.

4.3.2 Treatment

The rats were inoculated with either 1.0 ml of strain RE1 or 0.5 ml of strain RG2837. The inoculates were prepared as described under 4.1. The oral inoculations were carried out by a biotechnician.

Treatment scheme

group	Treatment Oral dose	Observations day 0-21 Clin. Obs / rectal swabs	Necropsy
1 n=5	1.31×10^9 CFU strain RE1	Daily / twice a week	Day 21
2 n=5	1.32×10^9 CFU strain RG2837	Daily / twice a week	Day 21

4.4 Experimental procedures and parameters

4.4.1 Rectal swabs

Rectal swabs were sampled at T=0 and then twice a week during three weeks. The swab samples were resuspended and serially diluted in physiological salt solution, plated on selective agar and grown at 37°C during 24-72 hours. *R. equi* was initially identified by the typical colony morphology and further identified by API/Phoenix according to standard procedures. At least one isolate from each rat (if present) was tested in a specific PCR (see below under 4.4.3) to confirm the identity of vaccine or wild type strain.

4.4.2 Clinical observations

During the study the rats were daily observed for any abnormalities of general health and/or behavior. The following scoring systems were used :
Systemic reaction scores : 0=normal, 1=less active, 2=slightly depressed, 3=depressed, 4=severely depressed.
Diarhea scores : 0=normal, 1=mild diarrhea, 2=moderate diarrhea, 3=severe diarrhea.

4.4.3 PCR

The identity of the vaccine strain Δ*ipdABipdAB2* genotype was confirmed by a quantitative PCR using a real time PCR detection system. PCR amplification was performed on colony material. The PCR mixture contained 20U/ml DNA polymerase (HT Biotechnology Ltd, Cambridge, UK), 1x Icycler buffer (100mM Tris-HCl and 500 mM KCl, pH 8.5), 0.2 mM dNTP's (HT Biotechnology, Ltd, Cambridge, UK), 4 mM $MgCl_2$, 200 nM primers and 100 nM probes.
Primers 5626-OLI-3159, -3158 and -3128 and probes 5626-OLI-3130 (TXR) and 5626-OLI-3160 (FAM) from the Intervet oligo collection were used for the *ipdAB* Q-PCR.
Primers 5626-OLI-3131, -3165 and -3166 and probes 5626-OLI-3134 (FAM) and 5626-OLI-3135 (TXR) from the Intervet oligo collection were used for the *ipdAB2* Q-PCR.
The PCR program is composed of a 5 minutes denaturation step at 95°C and 50 cycles of 10 seconds at 95°C, 15 seconds at 55°C and 30 seconds at 72°C. Data collection was performed at 72°C. A wild type strain (e.g. strain RE1) will be positive with the FAM probe 5626-OLI-3160 and TexasRed probes 5626-OLI-3130 and -3135). An *ipdABipdAB2* mutant will only be positive with the FAM probes 5626-OLI-3160 and -3134.

4.4.4 Post mortem examination and bacteriology

On day 21 post-challenge the rats were sedated by isofluraan, bled and subsequently killed by cervical dislocation. A complete post-mortem examination was performed on all animals with special attention to the lungs and gut with associated lymph nodes. Post-mortem findings were recorded on a dissection form. In case of abnormalities, samples for histology were taken as deemed necessary by the pathologist. Any samples for histological examination were recorded on the dissection form.
Swabs and/or tissue samples were sampled from all other abnormalities during necropsy and examined bacteriologically. These swabs were streaked on blood agar and/or on selective agar (in case of gut tissue) and incubated at 37°C for 16-72 hours. *Rhodococcus equi* colonies were initially identified by the typical non-hemolytic mucoid colony morphology, enumerated and expressed as CFU/ml. From each rat the identity of at least one isolate (selected at random) if present was confirmed by: Gram stain, API/Phoenix and/or PCR.

5. EVALUATION OF RESULTS

All data on local and systemic reactions after the treatments and post-mortem findings were recorded for each rat individually. This will give an impression of the number/percentage and severeness of reactions that might occur after oral ingestion of Equilis RhodE by rats in comparison with the wild type parent strain.

6. STORAGE OF DOCUMENTATION

All records concerning animal experimentation and analytical data will be kept at the Microbiological Research Department of Intervet International BV, Boxmeer.

7. RESULTS AND DISCUSSION

During 3 weeks after inoculation no systemic reactions were observed in either group (Table 1). Likewise, during 3 weeks after inoculation with Equilis RhodE or with the wild type strain RE1, no abnormalities in the consistency of the faeces were observed (Table 2) and *Rhodococcus equi* was not re-isolated from rectal swabs (Table 3). Moreover, during the necropsy no abnormalities of internal organs were observed in either group (Table 4).

8 CONCLUSION

From the results it can be concluded that ingestion of *Rhodococcus equi* wild type strain RE1 or vaccine strain RG2837 by rats poses no risk to this species. It does not cause clinical signs, does not cause an infection in rats and is not shed into the environment after oral administration to rats.

AJ/IB/14-10-2010

Table 1 Systemic reactions after vaccination (day 0-21)

rat no.	treatment group	local and/or systemic reactions at day																					
		0	0+4h	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1	wild type strain RE1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
6	vaccine strain RG2837	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
8		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
9		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
10		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Systemic reaction score: 0=normal, 1=less active, 2=slightly depressed, 3=depressed, 4=severely depressed.

Table 2 Diarrhea scores after vaccination (day 0-21)

rat no.	treatment group	faeces score at day																					
		0	0+4h	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1	wild type strain RE1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
6	vaccine strain RG2837	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
8		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
9		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
10		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Faecess score: 0=normal, 1=soft consistency of faeces, 2=mild diarrhea, 3=moderate diarrhea, 4=severe diarrhea

Table 3 Shedding of R. equi after vaccination

rat no.	treatment group	isolation of R. equi (CFU/swab) from rectal swabs (days)						
		0	3	7	10	14	17	21
1	wild type strain RE1	0	0	0	0	0	0	0
2		0	0	0	0	0	0	0
3		0	0	0	0	0	0	0
4		0	0	0	0	0	0	0
5		0	0	0	0	0	0	0
6	vaccine strain RG2837	0	0	0	0	0	0	0
7		0	0	0	0	0	0	0
8		0	0	0	0	0	0	0
9		0	0	0	0	0	0	0
10		0	0	0	0	0	0	0

Table 4 Macroscopical findings at necropsy

treatment group	rat no.	tissue/organ	macroscopical findings	re-isolation
wild type strain RE1	1	nad	-	-
	2	nad	-	-
	3	nad	-	-
	4	nad	-	-
	5	nad	-	-
vaccine strain RG2837	6	nad	-	-
	7	nad	-	-
	8	nad	-	-
	9	nad	-	-
	10	nad	-	-

nad= no abnormalities detected

Table 1 Shedding of R. equi by foals after vaccination as determined by rectal swabs

foal no	treatment group	isolation of R. equi (CFU/100 µl) from rectal swabs of foals at post-vaccination day																				
		0	1	2	3	6	8	10	13	14	15	16	17	20	22	24	27	28	29	30	31	34
4747	vaccinated	-	-	-	-	-	-	-	-	-	104[a]	3[a]	-	-	-	-	-	nd	nd	nd	nd	nd
4761	vaccinated	-	-	-	-	-	-	-	4[a]	2[a]	148[a]	2[a]	-	-	-	-	-	nd	nd	nd	nd	nd
0001	vaccinated	-	-	-	-	-	-	-	-	-	12[a]	-	-	-	-	-	nt	nd	nd	nd	nd	nd
0568	contact	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2692	vaccinated	-	-	-	-	-	-	2[a]	4[a]	5[a]	71[a]	-	-	-	-	-	-	nd	nd	nd	nd	nd
0002	vaccinated	-	-	-	-	-	-	-	-	-	-	1[a]	-	3[b]	1[a]	5[b]	4[b]	nd	nd	nd	nd	nd
8968	contact	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4208	vaccinated	-	>250[a]	±150[a]	125[a]	-	-	-	-	-	>250[a]	±100[a]	1[a]	-	-	-	-	nd	nd	nd	nd	nd
0360	vaccinated	-	-	-	-	-	-	-	2[a]	-	±125[a]	40[a]	5[a]	-	-	-	-	nd	nd	nd	nd	nd
6448	vaccinated	-	-	-	-	1[a]	-	-	-	-	2[a]	-	-	9[a]	-	1[a]	-	nd	nd	nd	nd	nd
6386	vaccinated	-	-	-	-	-	-	-	-	-	14[a]	15[a]	2[a]	-	-	-	-	nd	nd	nd	nd	nd
2426	vaccinated	-	-	-	-	-	-	-	-	-	25[a]	-	-	-	-	-	-	nd	nd	nd	nd	nd
0003	vaccinated	-	-	-	-	-	-	-	-	-	2[a]	20[a]	3[b]	2[b]	-	-	34[b]	nd	nd	nd	nd	nd

=contact animals nt=not tested; nd= not done

all re-isolations were confirmed as *Rhodococcus equi* [a]confirmed as vaccine strain by PCR [b]confirmed as wildtype strain by PCR

Table 2 Shedding of R. equi by foals after vaccination as determined by nasal swabs

foal no	treatment group	isolation of R. equi (CFU/100 µl) from nasal swabs of foals at post-vaccination day																				
		0	1	2	3	6	8	10	13	14	15	16	17	20	22	24	27	28	29	30	31	34
4747	vaccinated	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	nd	nd	nd	nd	nd
4761	vaccinated	-	-	1[a]	-	-	-	-	-	-	-	-	-	-	-	-	-	nd	nd	nd	nd	nd
0001	vaccinated	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	nt	nd	nd	nd	nd	nd
0568	contact	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2692	vaccinated	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	nd	nd	nd	nd	nd
0002	vaccinated	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	nd	nd	nd	nd	nd
8968	contact	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4208	vaccinated	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	nd	nd	nd	nd	nd
0360	vaccinated	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	nd	nd	nd	nd	nd
6448	vaccinated	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	nd	nd	nd	nd	nd
6386	vaccinated	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	nd	nd	nd	nd	nd
2426	vaccinated	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	nd	nd	nd	nd	nd
0003	vaccinated	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	nd	nd	nd	nd	nd

=contact animals nt=not tested; nd= not done

[a] re-isolated strain was confirmed as *Rhodococcus equi vaccine strain*

Table 3 Shedding of R. equi by mares after vaccination of foals as determined by rectal swabs

mare no	treatment group	isolation of R. equi from rectal swabs of mares (CFU/100 µl)) at post-vaccination day																	
		0	7	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35
4747	vaccinated	nt	-	-	-	-	-	-	-	-	-	-	-	nd	nd	nd	nd	nd	nd	nd
4761	vaccinated	nt	-	-	-	-	-	-	-	-	-	-	-	nd	nd	nd	nd	nd	nd	nd
0001	vaccinated	-	nt	-	-	-	-	-	-	-	-	-	-	nd	nd	nd	nd	nd	nd	nd
0568	contact	-	nt	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2692	vaccinated	-	nt	-	-	-	-	-	-	-	-	-	-	nd	nd	nd	nd	nd	nd	nd
0002	vaccinated	-	nt	-	-	-	-	-	-	-	-	-	-	nd	nd	nd	nd	nd	nd	nd
8968	contact	-	nt	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4208	vaccinated	-	nt	-	-	-	-	-	-	-	-	-	-	nd	nd	nd	nd	nd	nd	nd
0360	vaccinated	-	nt	-	-	-	-	-	-	-	-	-	-	nd	nd	nd	nd	nd	nd	nd
6448	vaccinated	-	nt	-	-	-	-	-	-	-	-	-	-	nd	nd	nd	nd	nd	nd	nd
6386	vaccinated	-	nt	-	-	-	-	-	-	-	-	-	-	nd	nd	nd	nd	nd	nd	nd
2426	vaccinated	-	nt	-	-	-	-	-	-	-	-	-	-	nd	nd	nd	nd	nd	nd	nd
0003	vaccinated	-	nt	-	-	-	-	-	-	-	-	-	-	nd	nd	nd	nd	nd	nd	nd

nt=not tested; nd= not done

- = no *Rhodococcus equi* isolated

Table 4 Shedding of R. equi by foals after vaccination as determined by rectal swabs

formulation / vaccination scheme	animal no.	Isolation of R. equi (CFU/100 µl) from rectal swabs at day ... post-vaccination						
		26	27	28	29	30	31	32
oral (Pronutrin)	71	-	-	-	-	-	-	-
at day 0 and 14	72	-	-	-	-	-	-	-
oral (alginate gel)	73	-	-	-	-	-	-	-
at day 0 and 14	74	-	-	-	-	-	-	-
10^{10} CFU/dose	75	-	-	-	-	-	-	-
Rectal vaccination	76	-	-	-	-	-	-	-
at day 0 and 1	77	-	-	-	-	-	-	-
and day 14 and 15	78	-	-	-	-	-	-	-
Rectal vaccination	79	-	-	-	-	-	-	-
at day 0 and 1	80	-	-	-	-	-	-	-

Table 5 Shedding of *R. equi* by foals after vaccination as determined by rectal swabs

formulation / vaccination scheme	animal no.	Isolation of R. equi (CFU/100 µl) from rectal swabs of foals at day ... post-vaccination										
		18	19	20	21	22	23	24	25	26	27	28
Rectal vaccination at day 0 and 1 10^{10} CFU/dose	50	-	-	-	-	1[a]	1[a]	1[a]	1[a]	-	-	-
	51	4[a]	-	12[a]	-	7[a]	8[a]	-	-	-	-	-
	52	-	-	-	-	-	-	-	-	2[a]	-	-
Rectal vaccination at day 0 10^{10} CFU/dose	47	-	-	-	-	-	-	-	-	-	-	-
	48	-	-	-	-	-	-	-	-	-	-	-
	49	-	-	-	-	-	-	-	-	-	-	-
Rectal vaccination at day 0 10^{9} CFU/dose	44	-	3[a]	-	-	-	-	-	-	2[a]	-	-
	45	-	-	-	-	-	1[a]	-	-	-	-	-
	46	-	-	-	-	-	-	-	-	-	-	-
Rectal vaccination at day 0 10^{8} CFU/dose	41	-	-	-	-	-	-	-	-	-	-	-
	42	-	-	-	-	-	-	-	-	-	-	-
	43	-	-	-	-	-	-	-	-	-	-	-

[a]confirmed as vaccine strain by PCR

Table 6: Course of inactivation *R.equi* strain RG2837 in Tap water (2-8°C)

Incubation time	Number of CFU *R.equi* detected in dilution								
	Undiluted	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
0 days	>250	>250	>250	>250	>250	47	11	3	0
1 days	>250	>250	>250	>250	24	7	2	1	0
3 days	>250	>250	>250	0	0	0	0	0	0
1 week	>250	>250	14	0					
2 weeks	>250	0	0	0					
3 weeks	>250	>250	0	0					
4 weeks	>250	>250	0	0					
5 weeks	>250	>250	0	0					
8 weeks	>250	>250[b]	0	0	0	0	0	0	0
13 weeks	>250	>250[b]	0	0	0	0	0	0	0
16 weeks	>250[b]	152	0	0	0	0	0	0	0
20 weeks	49	61[b]	0	0	0				
31 weeks	0	6[b]	0	0					
39 weeks	0	0	0						

Table 7: Course of inactivation *R.equi* strain RG2837 in Tap water (ambient temperature)

Incubation time	Number of CFU *R.equi* detected in dilution								
	Undiluted	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
0 days	>250	>250	>250	>250	82	17	2	5	2
1 days	>250	>250	>250	1	0	0	0	0	0
3 days	>250	>250	17	0	0	0	0	0	0
1 week	>250	>250	1	0					
2 weeks	>250	>250	0	0					
3 weeks	>250	>250	94	0					
4 weeks	>250	>250	>100	0					
5 weeks	>250	>250	>100	0					
8 weeks	>250	>250	>250[b]	0	0	0	0	0	0
13 weeks	>250	>250	>250[b]	0	0	0	0	0	0
16 weeks	>250	>250	>250[b]	0	0	0	0	0	0
20 weeks	>250	>250	>250[b]	0	0				
31 weeks	>250	>250	>250[b]	0					
39 weeks	+	173	143[b]	0					

Table 8: Course of inactivation *R.equi* strain RG2837 in Tap water (37°C)

Incubation time	Number of CFU *R.equi* detected in dilution								
	Undiluted	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
0 days	>250	>250	>250	>250	>250	36	9	4	2
1 days	>250	>250	123	0	0	0	0	0	0
3 days	>250	>250	>250	0	0	0	0	0	0
1 week	>250	>250	>250	7					
2 weeks	>250	>250	2	5					
3 weeks	>250	>250	>250	0					
4 weeks	>250	>250	>250	+					
5 weeks	>250	>250	116	2					
8 weeks	64	>100[b]	>250	0	0	0	0	0	0
13 weeks	+	>250	>250[b]	0	0	0	0	0	0
16 weeks	*	>250	133	0	0	0	0	0	0
20 weeks	>250	>250	55[b]	2	0				
31 weeks	>250	>250	>100	31[a]					
39 weeks	+	116	53[b]	8[a]					

* evaluation not possible; overgrown with aspecific bacteria

\+ overgrown but *R.equi* could be demonstrated (e.g. after cloning on blood agar plates)

[a] confirmed as wild type by genotype by PCR; [b] confirmed as double mutant by PCR

Table 9: Course of inactivation *R.equi* strain RE1 in Tap water (2-8°C)

Incubation time	Number of CFU *R.equi* detected in dilution								
	Undiluted	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
0 days	>250	>250	>250	>250	>250	41	16	4	2
1 days	>250	>250	>250	>250	37	6	1	0	0
3 days	>250	>250	>250	0	0	0	0	0	0
1 week	>250	>250	8	0					
2 weeks	>250	0	0	0					
3 weeks	>250	>100	0	0					
4 weeks	>250	>250	0	0					
5 weeks	>250	>250	0	0					
8 weeks	>250	>250[a]	0	0	0	0	0	0	0
13 weeks	>250	>250[a]	0	0	0	0	0	0	0
16 weeks	>250[a]	107	0	0	0	0	0	0	0
20 weeks	>250	35[a]	0	0	0				
31 weeks	10[a]	2	0	0					
39 weeks	0	1[a]	0						

Table 10: Course of inactivation *R.equi* strain RE1 in Tap water (ambient temperature)

Incubation time	Number of CFU *R.equi* detected in dilution								
	Undiluted	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
0 days	>250	>250	>250	>250	27	9	0	0	0
1 days	>250	>250	>250	0	0	0	0	0	0
3 days	>250	>250	19	0	0	0	0	0	0
1 week	>250	>250	2	0					
2 weeks	>250	>250	0	0					
3 weeks	>250	>250	116	0					
4 weeks	>250	>250	>250	0					
5 weeks	>250	>250	>250	0					
8 weeks	>250	>250	>250[a]	0	0	0	0	0	0
13 weeks	>250	>250	>250[a]	0	0	0	0	0	0
16 weeks	>250	>250	>250[a]	0	0	0	0	0	0
20 weeks	>250	>250	>250[a]	0	0				
31 weeks	50	80	>250[a]	0					
39 weeks	>250	33	165[a]	0					

Table 11: Course of inactivation *R.equi* strain RE1 in Tap water (37°C)

Incubation time	Number of CFU *R.equi* detected in dilution								
	Undiluted	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
0 days	>250	>250	>250	>250	>250	14	1	1	0
1 days	>250	>250	180	0	0	0	0	0	0
3 days	>250	>250	>250	0	0	0	0	0	0
1 week	>250	>250	>250	0					
2 weeks	>250	>250	0	0					
3 weeks	>250	>250	107	0					
4 weeks	>250	>250	55	0					
5 weeks	>250	>250	49	0					
8 weeks	*	>250[a]	51	0	0	0	0	0	0
13 weeks	+	>250	40[a]	0	0	0	0	0	0
16 weeks	>250	>250	62[a]	0	0	0	0	0	0
20 weeks	>250	32	20[a]	0	0				
31 weeks	>250	>250	105[a]	0					
39 weeks	+	101	70[a]	0					

* evaluation not possible; overgrown with aspecific bacteria

\+ overgrown but *R.equi* could be demonstrated (e.g. after cloning on blood agar plates)

[a] confirmed as wild type by genotype by PCR; [b] confirmed as double mutant by PCR

Table 12: Course of inactivation *R.equi* strain RG2837 in Pond water (2-8°C)

Incubation time	Number of CFU *R.equi* detected in dilution								
	Undiluted	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
0 days	>250	>250	>250	>250	>250	52	9	5	1
1 days	>250	>250	>250	>250	>250	64	6	3	2
3 days	>250	>250	>250	>250	28	6	2	1	0
1 week	>250	>250	>250	>250	12	3	2	1	0
2 weeks	>250	>250	>250	0	0	0	0	0	0
3 weeks	>250	>250	>250	43					
4 weeks	>250	>250	>250	20					
5 weeks	>250	>250	>100	10	0				
8 weeks	>250	>250	>250[b]	5	0	0	0	0	0
13 weeks	>250	>250	166	0	0	0	0	0	0
16 weeks	>250	>250[b]	84	0	0	0	0	0	0
20 weeks	>250	32[b]	20	0	0				
31 weeks	+[b]	10	4	0					
39 weeks	0	4[b]	0	0					

Table 13: Course of inactivation *R.equi* strain RG2837 in Pond water (ambient temperature)

Incubation time	Number of CFU *R.equi* detected in dilution								
	Undiluted	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
0 days	>250	>250	>250	>250	>250	27	3	8	0
1 days	>250	>250	>250	>250	115	11	5	2	0
3 days	>250	>250	>250	>250	18	1	0	0	0
1 week	>250	>250	>250	34	1	0	0	0	0
2 weeks	>250	>250	>250	0	0	0	0	0	0
3 weeks	>250	>250	>250	78					
4 weeks	>250	>250	>250	5					
5 weeks	>250	>250	>250	32	9				
8 weeks	>250	>250	>250[b]	34	0	0	0	0	0
13 weeks	>250	>250	>100[b]	8	0	0	0	0	0
16 weeks	+	>250	60[b]	30	0	2	0	0	0
20 weeks	20	>250	81[b]	1	3				
31 weeks	4	32	79[b]	4					
39 weeks	3[b]	12	85	2[a]					

Table14: Course of inactivation *R.equi* strain RG2837 in Pond water (37°C)

Incubation time	Number of CFU *R.equi* detected in dilution								
	Undiluted	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
0 days	>250	>250	>250	>250	>250	40	8	4	1
1 days	>250	>250	>250	138	1	1	0	0	0
3 days	>250	>250	>250	87	2	0	0	0	0
1 week	>250	>250	>250	10	0	0	0	0	0
2 weeks	>250	32	0	0	0	0	0	0	0
3 weeks	>250	>100	22	0					
4 weeks	>250	>100	32	14					
5 weeks	>250	134	25	48	0				
8 weeks	0	0	7	65[b]	1	0	0	0	0
13 weeks	>250	35[b]	5	3	0	0	0	0	0
16 weeks	+	9	2[b]	0	0	0	0	0	0
20 weeks	32[b]	2	0	0	0				
31 weeks	>250[b]	0	0	0					
39 weeks	10[b]	0							

* evaluation not possible; overgrown with aspecific bacteria

\+ overgrown but *R.equi* could be demonstrated (e.g. after cloning on blood agar plates)

[a] confirmed as wild type by genotype by PCR; [b] confirmed as double mutant by PCR

Table 15: Course of inactivation *R.equi* strain RE1 in Pond water (2-8°C)

Incubation time	Number of CFU *R.equi* detected in dilution								
	Undiluted	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
0 days	>250	>250	>250	>250	>250	81	12	3	6
1 days	>250	>250	>250	>250	>250	35	4	3	1
3 days	>250	>250	>250	>250	>250	18	2	2	1
1 week	>250	>250	>250	>250	15	2	1	1	0
2 weeks	>250	>250	>250	0	0	0	0	0	0
3 weeks	>250	>250	>250	49					
4 weeks	>250	>250	>250	14					
5 weeks	>250	>250	>100	9	0				
8 weeks	>250[a]	>250[a]	81	4	5	15	0	0	0
13 weeks	>250	>250	57[a]	0	0	0	0	0	0
16 weeks	>250	>250	27[a]	0	0	0	0	0	0
20 weeks	>250	130[a]	6	0	0				
31 weeks	75	35[a]	1	0					
39 weeks	4[a]	15[a]	1	0					

Table 16: Course of inactivation *R.equi* strain RE1 in Pond water (ambient temperature)

Incubation time	Number of CFU *R.equi* detected in dilution								
	Undiluted	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
0 days	>250	>250	>250	>250	>250	47	9	4	0
1 days	>250	>250	>250	>250	127	20	3	2	3
3 days	>250	>250	>250	>250	22	2	0	0	0
1 week	>250	>250	>250	99	13	1	0	0	0
2 weeks	>250	>250	>250	0	0	0	0	0	0
3 weeks	>250	>250	>250	19					
4 weeks	>250	>250	>250	167					
5 weeks	>250	>250	>250	>250	24				
8 weeks	>250	>250	>250	71	32[a]	1	0	0	0
13 weeks	>250	>250	>250	57[a]	2	0	0	0	0
16 weeks	*	>250	>250	49	20[a]	0	0	0	13
20 weeks	+	21[a]	>250	41	5				
31 weeks	0	8	110	29[a]					
39 weeks	0	12	123	19					

Table 17: Course of inactivation *R.equi* strain RE1 in Pond water (37°C)

Incubation time	Number of CFU *R.equi* detected in dilution								
	Undiluted	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
0 days	>250	>250	>250	>250	>250	55	6	1	1
1 days	>250	>250	>250	>250	0	9	26	9	8
3 days	>250	>250	>250	38	1	1	2	3	61
1 week	>250	>250	>250	17	0	1	1	2	10
2 weeks	>250	69	0	0	0	0	0	0	0
3 weeks	>250	>100	51	26					
4 weeks	>250	>250	26	41					
5 weeks	>250	44	43	15	1				
8 weeks	*	*	15[a]	22[a]	0	0	0	0	0
13 weeks	+	*	0	3[a]	0	1	0	0	0
16 weeks	+	0	30	21	0	0	0	0	0
20 weeks	>250[a]	0	0	4	0				
31 weeks	>250[a]	0	0	0					
39 weeks	>250[a]	0							

* evaluation not possible; overgrown with aspecific bacteria

\+ overgrown but *R.equi* could be demonstrated (e.g. after cloning on blood agar plates)

[a] confirmed as wild type by genotype by PCR; [b] confirmed as double mutant by PCR

Table 18: Course of inactivation *R.equi* strain RG2837 in Soil (2-8°C)

Incubation time	Number of CFU *R.equi* detected in dilution								
	Undiluted	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
0 days	>250	>250	>250	>250	>250	31	1	1	1
1 days	>250	>250	>250	>250	>50	6	2	0	0
3 days	>250	>250	>250	>250	19	6	3	1	0
1 week	>250	>250	>250	>250	20	8	0	0	1
2 weeks	>250	>250	0	0	0	0	0	0	0
3 weeks	>250	>250	>100	31					
4 weeks	>250	>250	>100	33					
5 weeks	>250	>250	>100	...	0				
8 weeks	>250	>250	100	13	2[b]	0	1	0	0
13 weeks	+	+	18[b]	0	0	0	0	0	0
16 weeks	+	+	+[b]	0	0	0	0	0	0
20 weeks	*	*	+[b]	+	*				
31 weeks	*	+[b]	*	*	*				
39 weeks	*	*	*	+					

Table 19: Course of inactivation *R.equi* strain RG2837 in Soil (ambient temperature)

Incubation time	Number of CFU *R.equi* detected in dilution								
	Undiluted	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
0 days	>250	>250	>250	>250	>250	28	3	2	1
1 days	>250	>250	>250	>50	26	7	2	0	0
3 days	>250	>250	>250	>50	>50	>50	5	1	0
1 week	>250	>250	>250	>50	17	2	3	0	0
2 weeks	>250	0	0	0	0	0	0	0	0
3 weeks	>250	+	+	+					
4 weeks	>250	>250	>100	+					
5 weeks	>250	>250	>100	...	0				
8 weeks	+	>100[b]	50	25	0	2	0	0	0
13 weeks	+	+	+[b]	>50	0	1	0	0	0
16 weeks	*	+	+	+[b]	0	0	0	0	0
20 weeks	*	+[b]	+	+	+				
31 weeks	+	+[b]	*	0	0				
39 weeks	+	*	-	-	*				

Table 20: Course of inactivation *R.equi* strain RG2837 in Soil (37°C)

Incubation time	Number of CFU *R.equi* detected in dilution								
	Undiluted	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
0 days	>250	>250	>250	>250	>250	33	4	1	2
1 days	>250	>250	>250	>250	>50	9	0	0	0
3 days	>250	>250	>250	>250	>50	11	9	1	1
1 week	>250	>250	>250	>250	45	8	7	10	4
2 weeks	0	0	0	0	0	0	0	0	0
3 weeks	*	1	0	0					
4 weeks	*	*	*	*					
5 weeks	*	*	*	*	...				
8 weeks	+	>250[b]	+	+	16	0	0	0	0
13 weeks	+	+	0	0	0	0	0	0	0
16 weeks	?	*	0	0	0	0	0	*	0
20 weeks	nd	*	*	*	0				
31 weeks	*	*	*	*	0				
39 weeks	*	*	*	*					

* evaluation not possible; overgrown with aspecific bacteria

\+ overgrown but *R.equi* could be demonstrated (e.g. after cloning on blood agar plates)

[a] confirmed as wild type by genotype by PCR; [b] confirmed as double mutant by PCR

Table 21: Course of inactivation *R.equi* strain RE1 in Soil (2-8°C)

Incubation time	Number of CFU *R.equi* detected in dilution								
	Undiluted	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
0 days	>250	>250	>250	>250	>250	16	5	1	1
1 days	>250	>250	>250	>250	>50	8	1	0	0
3 days	>250	>250	>250	>250	>50	0	3	1	0
1 week	>250	>250	>250	>50	32	8	1	3	0
2 weeks	>250	>250	0	0	0	0	0	0	0
3 weeks	>250	>250	>100	2					
4 weeks	>250	>250	>100	4					
5 weeks	>250	>250	>100	1	0				
8 weeks	*	>250	50[a]	+	+	1	0	0	0
13 weeks	+	+	27[a]	5	3	0	0	0	0
16 weeks	+	+	6[a]	0	0	0	0	0	0
20 weeks	>250	+[a]	+	0	*				
31 weeks	+[a]	+	+	+	+				
39 weeks	+[a]	-	*	+[a]	+[a]				

Table 22: Course of inactivation *R.equi* strain RE1 in Soil (ambient temperature)

Incubation time	Number of CFU *R.equi* detected in dilution								
	Undiluted	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
0 days	>250	>250	>250	>250	>250	19	11	1	0
1 days	>250	>250	>250	>250	>50	>20	1	0	0
3 days	>250	>250	>250	>250	>50	13	6	5	0
1 week	>250	>250	>250	>250	>50	16	1	2	0
2 weeks	>250	>250	0	0	0	0	0	0	0
3 weeks	>250	>250	>100	18					
4 weeks	>250	>250	>100	*					
5 weeks	>250	>250	>100	...	0				
8 weeks	>250	>250	50	20	1	>100	0	1[a]	0
13 weeks	+	+	+[a]	3	0	>20	0	0	0
16 weeks	*	+	+	+[a]	0	0	0	0	0
20 weeks	*	5[a]	2	0	0				
31 weeks	+[a]	*	+	*	0				
39 weeks	*[a]	+[a]	+[a]	*					

Table 23: Course of inactivation *R.equi* strain RE1 in Soil (37°C)

Incubation time	Number of CFU *R.equi* detected in dilution								
	Undiluted	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
0 days	>250	>250	>250	>250	>250	22	0	0	0
1 days	>250	>250	>250	>250	>50	19	7	3	0
3 days	>250	>250	>250	>250	35	3	2	1	0
1 week	>250	>250	>250	>250	54	7	2	5	2
2 weeks	0	27	0	0	0	0	0	0	0
3 weeks	>250	+	+	+					
4 weeks	>250	+	*	*					
5 weeks	>250	*	*	*	...				
8 weeks	>250	+	+[a]	+	20	2	+	1	0
13 weeks	-	-	0	3[a]	0	3	2	0	0
16 weeks	*	*	*	0	0	0	0	0	0
20 weeks	*	*	*	*	*				
31 weeks	+	+	0	0	0				
39 weeks	*	*	*	*					

* evaluation not possible; overgrown with aspecific bacteria

\+ overgrown but *R.equi* could be demonstrated (e.g. after cloning on blood agar plates)

[a] confirmed as wild type by genotype by PCR; [b] confirmed as double mutant by PCR

Table 24: Course of inactivation R.equi strains RG2837 and RE1 air dried on petridish (2-8°C)

Incubation time	Growth / No growth RG2837	RE1
0 day	+++	+++
1 day	+++	+++
3 days	+++	+++
1 week	+++	+++
2 weeks	+++	+++
3 weeks	+++	+++
4 weeks	++	++
5 weeks	++	++
8 weeks	+[b]	+[a]
13 weeks	+[b]	+[a]
16 weeks	-	-
20 weeks	-	-
31 weeks	-	-
39 weeks	-	-

Table 25: Course of inactivation R.equi strains RG2837 and RE1 air dried on petridish (ambient temperature)

Incubation time	Growth / No growth RG2837	RE1
0 day	+++	+++
1 day	+++	+++
3 days	+++	+++
1 week	+++	+++
2 weeks	-	-
3 weeks	++	++
4 weeks	+++	+++
5 weeks	+++	+++
8 weeks	+++[b]	+++[a]
13 weeks	+[b]	++[a]
16 weeks	-	-
20 weeks	+[b]	+[a]
31 weeks	+[b]	-
39 weeks	+[b]	+[a]

Table 26: Course of inactivation R.equi strains RG2837 and RE1 air dried on petridish (37°C)

Incubation time	Growth / No growth RG2837	RE1
0 day	+++	+++
1 day	+++	+++
3 days	-	+
1 week	-	-
2 weeks	-	-
3 weeks	-	-
4 weeks	ND	ND
5 weeks	ND	ND
8 weeks	-	-
13 weeks	-	-
16 weeks	-	-
20 weeks	-	-
31 weeks	-	-
39 weeks	-	-

[a] confirmed as wild type by PCR; [b] confirmed as double mutant by PCR

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

Robert Van der Geize*, Katherine Yam†, Thomas Heuser†, Maarten H. Wilbrink*, Hirofumi Hara†, Matthew C. Anderton‡, Edith Sim‡, Lubbert Dijkhuizen*, Julian E. Davies†, William W. Mohn†§, and Lindsay D. Eltis†§

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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-seconandrost-1,3,5(10)-triene-9,17-dione (3,4-DHSA). Inactivation of each of the *hsaC*, *supAB*, and *mce4* genes in RHA1 substantiated their roles in cholesterol catabolism. Moreover, the *hsaC*$^{-}$ mutant accumulated 3,4-DHSA, indicating that $HsaC_{RHA1}$, formerly annotated as a biphenyl-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 *Mycobacterium bovis* bacillus Calmette–Guérin. *M. bovis* bacillus Calmette–Guérin grew on cholesterol, and *hsaC* and *kshA* were up-regulated under these conditions. Heterologously produced $HsaC_{H37Rv}$ and $HsaD_{H37Rv}$ transformed 3,4-DHSA and its ring-cleaved product, respectively, with apparent specificities ~40-fold higher than for the corresponding biphenyl 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-seconandrost-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-oic acid (DOHNAA) 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 propionate moiety of DOHNAA 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 bicycloalkanone 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 agents. Transposon site hybridization (TraSH), a genomewide microarray-based technique,

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The authors declare no conflict of interest.

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Abbreviations: 3-HSA, 3-hydroxy-9,10-seconandrost-1,3,5(10)-triene-9,17-dione; 3,4-DHSA, 3,4-dihydroxy-9,10-seconandrost-1,3,5(10)-triene-9,17-dione; 4,9-DSHA, 4,5–9,10-diseco-3-hydroxy-5,9,17-trioxoandrosta-1(10),2-diene-4-oic acid; AD, 4-androstene-3,17-dione; DHB, 2,3-dihydroxybiphenyl; DOHNAA, 9,17-dioxo-1,2,3,4,10,19-hexanorandrostan-5-oic acid; KSTD, 3-ketosteroid Δ1-dehydrogenase; HOPDA, 2-hydroxy-6-oxo-6-phenylpentadienoate; TraSH, transposon site hybridization.

Data deposition: The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE6709).

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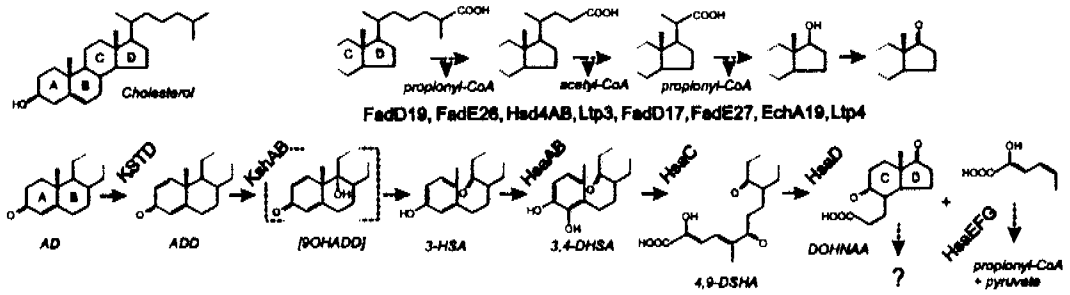

Fig. 1. The deduced cholesterol catabolic pathway of *Rhodococcus* sp. RHA1, *M. tuberculosis* H37Rv, and *M. bovis* bacillus Calmette–Guérin. The enzymatic steps of side-chain degradation and ring opening are depicted. The latter are important for H37Rv survival in the macrophage (Fig. 2). Dashed arrows 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.

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* and *Mycobacterium bovis*. Conditions to grow *M. bovis* on cholesterol were developed, and the expression of two pathway genes was shown by quantitative RT-PCR. Two of the *M. tuberculosis* pathway enzymes were heterologously produced and shown to efficiently catalyze the predicted transformations of steroid ring A. The results are discussed with respect to the survival of *M. tuberculosis* in the macrophage.

Results

The Cholesterol Transcriptome of RHA1. In liquid medium containing 2 mM cholesterol as the sole organic substrate, RHA1 grew to a density of 2×10^8 cells per ml. Microarray analysis revealed 572 genes that were up-regulated at least 2-fold during growth on cholesterol compared with on 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. 2*A*). As discussed below, these genes 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* SQ1 (7, 8). A second cluster of chromosomal genes (*ro06687–ro06698*) also appear to be involved in

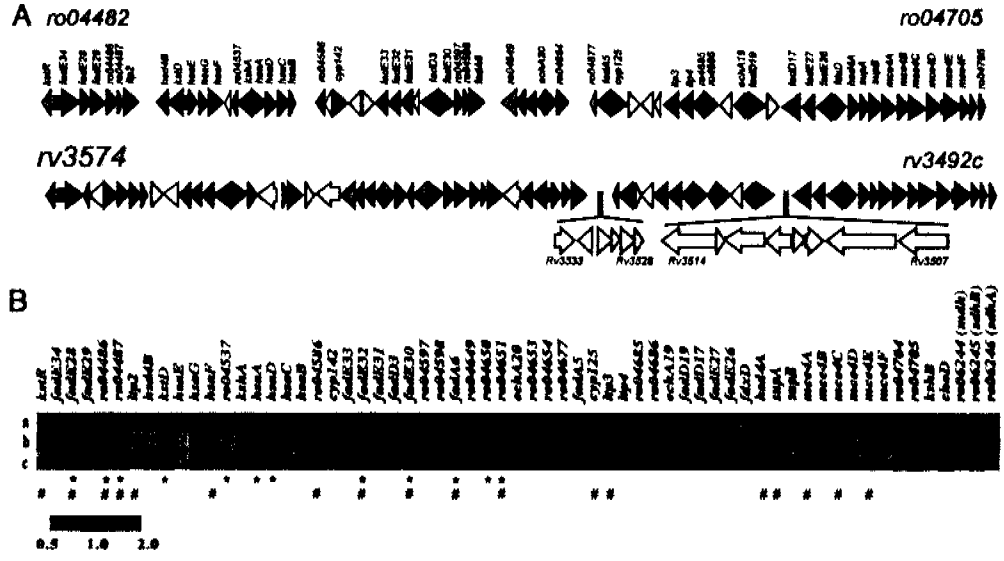

Fig. 2. The cholesterol catabolic genes of *Rhodococcus sp.* RHA1 and *M. tuberculosis* H37Rv: comparison of their organization and their activities in different studies. (*A*) Genes in the physical map are color-coded according to assigned function: purple, uptake; red, side-chain degradation; blue, cleavage of rings A and B; orange, degradation of the DOHNAA 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. 2*A*), 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 *kstD* and *kshA* (Figs. 1 and 2*A*). The encoded proteins share significant amino acid sequence similarity (30–60%; SI Table 4) with the *tes*-encoded enzymes of *C. testosteroni* TA441 that transform 3-HSA during growth on testosterone (10, 11). HsaC and HsaD were previously annotated as BphC5 and BphD2 in RHA1, 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 and were annotated accordingly (SI Table 4). One set of these genes (*hsd4A*, *hsd4B*, *fadD19*, *fadE26*, and *ltp3*) is highly up-regulated and encodes all of the enzymes necessary to perform one full cycle of β-oxidation. Hsd4A and Hsd4B share intriguing sequence similarity with the eukaryotic multifunctional 17β-hydroxysteroid dehydrogenase IV (17βHSD4) involved in peroxisome-related disorders (21). Hsd4A is homologous to the N-terminal domain of 17βHSD4, which acts as a 17β-hydroxysteroid dehydrogenase and, with branched fatty acids and bile acids, as a D-3-hydroxyacyl-CoA dehydrogenase. Hsd4B is homologous to the central domain of 17βHSD4, which is a 2-enoyl acyl-CoA hydratase proposed to be involved in cholesterol side-chain shortening. Accordingly, we predict that these RHA1 genes specify at least one cycle of β-oxidative transformation of the C17 side chain to propionyl-CoA and acetyl-CoA. A second near-complete set of β-oxidation genes (*echA19*, *fadD17*, *fadE27*, and *ltp4*) are up-regulated to a lesser extent, but are likely also involved in side-chain degradation. The bifunctional Hsd4A likely transforms the 17β-hydroxysteroid resulting from cleavage of the cholesterol side chain.

A third cluster of up-regulated genes related to β-oxidation, including *fadE28*, is similar to those involved in testosterone catabolism by *C. testosteroni* TA441 (10, 11). These genes may be involved in the degradation of the DOHNAA originating from steroid rings C and D (Fig. 1), as this part of the molecule is common to testosterone and cholesterol, whereas the C17 side chain is not. This set of genes is preceded by a gene encoding a TetR-type transcriptional regulator similar to the KstR (32% identity) repressor of *kstD* (7), suggesting that the RHA1 genes are also regulated by steroids. The propionate moiety of DOHNAA is likely degraded by β-oxidation encoded by the gene cluster that includes *echA20*. This cluster includes genes encoding a two-subunit, ATP-dependent CoA transferase of the type thought to initiate β-oxidation (22).

The *ro04482–ro04705* cluster also includes eight genes that appear to encode a multicomponent cholesterol uptake system: *supAB* and *mce4ABCDEF*. Conserved domain data revealed the presence of a domain related to an ABC-transport system involved in resistance to organic solvents in both SupA and SupB (23). The *mce* cluster is one of two such clusters in RHA1 that are highly similar to the four sets of "mammalian cell entry" (*mce*) genes of *M. tuberculosis* H37Rv (24). Mce proteins are critical virulence factors in *M. tuberculosis* (16), although the exact role of these genes is unknown. Heterologously expressed *mce1A* enhanced the entry of *Escherichia coli* into nonphagocytic HeLa cells (25), whereas *mce1*$^-$ and *mce4*$^-$ strains of *M. tuberculosis* H37Rv showed attenuated survival in mice (26). It has been proposed that Mce proteins are components of transport systems that translocate lipids between the bacterial cell and its host (26). Consistent with this proposed role, Mce1A is expressed at the cell surface of *M. tuberculosis* H37Rv (27). Indeed, signal sequences are predicted for all of the Mce4 proteins of RHA1 except Mce4C [SignalP (28)], indicating that these proteins are secreted or surface-exposed proteins. In summary, the 51 up-regulated genes of the *ro04482–ro04705* cluster appear to include all of those necessary to specify the catabolism of cholesterol to DOHNAA.

The separate *ro06687–ro06698* gene cluster, induced on cholesterol, includes genes typical of those encoding cycloalkanone catabolism (SI Table 4). These include *ro06698* and *ro06693*, which encode a probable monooxygenase and lactone hydrolase, respectively. We predict that these genes are involved in degrading the steroid ring D of DOHNAA.

Annotation of the cholesterol catabolic genes further revealed that these genes are but one of four sets in RHA1 that appear to specify the catabolism steroid-like compounds. Each of these sets encodes homologs of all ring-degrading enzymes: 3-ketosteroid 9α-hydroxylase, KstD, HsaAB, HsaC, HsaD, and at least one cyclohexanone monooxygenase. Sequence analyses revealed that all of the KshA homologs (ro02490, ro04538, ro05811, and ro09003) share at least 52% amino acid sequence identity with KshA of *R. erythropolis* SQ1 (8). Phylogenetic analyses (SI Fig. 3*A*) revealed that these enzymes define a subclass of Rieske nonheme oxygenases. Similarly, all of the HsaC homologs (ro02488, ro04541, ro05803, and ro09005) share at least 37% amino acid sequence identity and key active-site residues with TesB of *C. testosteroni* TA441. These enzymes constitute a subclass of type I extradiol dioxygenases (SI Fig. 3*B*) distinct from those involved in biphenyl and naphthalene catabolism. Similar analyses of HsaA and HsaD 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-seconandrost-1,3,5(10)-triene-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 HsaC homologs in RHA1 (SI Fig. 3*B*). The pink color is consistent with the accumu-

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

Protein	Cholesterol, 1 mM	AD, 1 mM	Pyruvate, 20 mM	Benzoate, 20 mM
WT	73 (5)	77 (12)	170 (20)	470 (60)
Δ*supAB*	1 (1)	75 (11)	170 (20)	520 (50)
Δ*mce4*	3 (1)	78 (14)	150 (40)	440 (90)

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.

Table 2. Steady-state kinetic parameters of $HsaC_{H37Rv}$ and $HsaD_{H37Rv}$ for steroid and biphenyl metabolites

Enzyme	Substrate	K_m, μM	V_{max}, μM·s^{-1}	V_{max}/K_m, s^{-1}
$HsaC_{H37Rv}$	3,4-DHSA	0.9 (0.5)	12 (4)	790 (370)
	DHB	8.5 (0.8)	2.5 (0.4)	18 (3)
$HsaD_{H37Rv}$	4,9-DSHA	4 (1)	0.06 (0.02)	1.0 (0.2)
	HOPDA	19 (6)	0.009 (0.003)	0.028 (0.007)

Parameters were normalized to the amount of cellular extract (milligrams of protein content) used in the assays. Values in parentheses represent standard errors.

lation 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 trimethyl-silane (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 $HsaC_{H37Rv}$ as described below yielded a product with a pH-dependent spectrum essentially identical to that reported for 4,5–9,10-diseco-3-hydroxy-5,9,17-trioxoandrosta-1(10),2-diene-4-oic acid (4,9-DSHA) (9) (ε_{392} = 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 *ro04482–ro04705* 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–Rv3574*; Fig. 2*A*) 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 those involved in cholesterol catabolism (SI Fig. 3).

Cholesterol Catabolism in *M. bovis* Bacillus Calmette–Guérin. Initial attempts to grow bacillus Calmette–Guérin on cholesterol as the sole energy source met with limited success, as for other pathogenic mycobacteria (30). However, with a liquid minimal medium containing asparagine, citrate, and Triton (18), the final growth yield of bacillus Calmette–Guérin was proportional to the initial concentration of cholesterol in the medium. Thus, in medium supplemented with 0, 0.25, and 0.5 mM cholesterol, respectively, the overall protein yields were 22 ± 7, 46 ± 9, and 70 ± 4 μg/ml. Further modification of the medium to reflect host factors or improve the availability of the cholesterol to the bacterium may improve growth.

To investigate whether the predicted cholesterol catabolic pathway is involved in this growth of bacillus Calmette–Guérin, quantitative RT-PCR analyses were performed on *kshA* and *hsaC* with *sigA* as a control. Normalized transcript levels were significantly higher in cultures growing on cholesterol (n = 4) than on glucose (n = 5) for both *kshA* ($P < 0.005$) and *hsaC* ($P < 0.05$), with relative fold differences of 3.7 and 2.4, respectively. Similar results were observed when comparing cholesterol- to pyruvate-grown cells. The relative fold differences for *kshA* and *hsaC* were very similar to the expression ratios determined for these genes (4.6 and 2.1, respectively) using the microarray to compare RHA1 growing on cholesterol versus on pyruvate (SI Table 3); although, a slightly higher fold difference was determined for *hsaC* in RHA1 with quantitative RT-PCR. The relative fold differences for *kshA* and *hsaC* were also very similar to those determined for these genes using a microarray to compare H37Rv growing in macrophages versus *in vitro* (18).

The Catalytic Activities of $HsaC_{H37Rv}$ and $HsaD_{H37Rv}$. To substantiate the predicted cholesterol catabolic pathway in *M. tuberculosis* H37Rv, the activities of two central enzymes, $HsaC_{H37Rv}$ and $HsaD_{H37Rv}$, were investigated. These enzymes were targeted in part because they were previously annotated as putative biphenyl-degrading enzymes (20). Accordingly, $HsaC_{H37Rv}$ and $HsaD_{H37Rv}$ were heterologously expressed in *E. coli*, and their steady-state kinetic parameters were evaluated with cell extracts. As summarized in Table 2, the enzymes preferentially transformed cholesterol metabolites as compared with biphenyl metabolites. Specifically, cell extracts containing $HsaC_{H37Rv}$ catalyzed the extradiol cleavage of 3,4-DHSA with an apparent specificity 44-fold higher than for DHB. Similarly, extracts containing $HsaD_{H37Rv}$ catalyzed the hydrolysis of 4,9-DSHA with an apparent specificity 34-fold higher than for 2-hydroxy-6-oxo-6-phenylpentadienoate (HOPDA). Equivalent extracts prepared from cells that contained the empty vector did not detectably transform either the steroid or biphenyl metabolites. These results strongly support the predicted roles of the mycobacterial enzymes in steroid metabolism and also indicate that the aliphatic side chain of cholesterol is removed before ring degradation.

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 $HsaC_{RHA1}$, 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 devel-

opment of whole-cell biotransformation processes for the synthesis of industrially relevant steroid compounds.

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–Guérin 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 $HsaC_{H37Rv}$, an extradiol dioxygenase, and $HsaD_{H37Rv}$, a C—C bond hydrolase, for the steroid metabolites. These enzymes had been annotated as a probable DHB dioxygenase (NP_218085) and a HOPDA (CAB07143), 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 *supAB* 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. 2*B* and ref. 18). Second, TraSH 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. 2*B*) (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 TraSH 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 TraSH mutants, such as *mce4*, displayed a progressive *in vivo* growth defect 2–4 weeks after infection in mice (17). Moreover, the essentiality of some of these genes has been substantiated. Thus, a Δ*yrbE4A* (i.e., *supA*) and Δ*mce4* mutants show attenuated survival of *M. tuberculosis* H37Rv in mice (17, 26). Similarly, inactivation *mt3626* of *M. tuberculosis* CDC1551 (*rv3527* in H37Rv; Fig. 2*B*), 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 O_2-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 metabolism of cholesterol rings C and D. First, the Baeyer-Villiger monooxygenase and hydrolase 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–Guérin 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 (OD_{600} of 1.0 for pyruvate and 2.0 for cholesterol). Bacillus Calmette–Guérin 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 KH_2PO_4, 2.5 g/ml Na_2PO_4, 10 mg/l $MgSO_4{\cdot}7H_2O$, 0.5 mg/liter $CaCl_2$, 0.1 mg/liter $ZnSO_4$, 50 mg/liter ferric ammonium citrate, and 0.5 ml/liter Triton wR1339 (Tyloxapol) (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–Guérin 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 σ^A were

used as internal standards in the multiplex reactions performed by using RHA1 and bacillus Calmette–Guérin cDNA, respectively (19). The C_t values were normalized (ΔC_t) by subtracting those of the internal standard. Significant differences in ΔC_t values were tested by using a two-sample t test assuming unequal variances. Relative fold differences were calculated as $2^{-\Delta\Delta Ct}$, where $\Delta\Delta C_t = \Delta C_{t\ \text{treatment}} - \Delta C_{t\ \text{control}}$.

Gene Replacement and Deletion. The *hsaC* gene was replaced in RHA1 with an apramycin resistance marker, *apra*^{R}, using a procedure in which the gene was first replaced in a fosmid by using λ-RED-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 *hsaADCB* 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 *supAB* 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 the six *mce4* genes were ro04698-F and ro04698-R(SpeI), and ro04703-F(SpeI) and ro04703-R(HindII), respectively. The upstream and downstream region of the *supAB* genes were amplified by using oligonucleotides SupA-F and SupA-R(SpeI), and Sup4B-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 *apra*^{R}.

Cloning and Expression of Mtb Genes. The $hsaC_{H37Rv}$ and $hsaD_{H37Rv}$ 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 Hcmt-F and Hcmt-R or Hdmt-F and Hdmt-R (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 to yield pT7HC1 and pT7HD1. $HsaC_{H37Rv}$ and $HsaD_{H37Rv}$ were produced by using *E. coli* GJ1158 transformed with pT7HC1 and pT7HD1, respectively, as described for DbfB (40).

Enzyme Assays. $HsaC_{H37Rv}$ and $HsaD_{H37Rv}$ 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 thermojacketed cuvette holder, essentially as described for biphenyl catabolic enzymes (40). Experiments were performed by using 20 mM 3-[4-(2-hydroxyethyl)-1-piperazinyl]propanesulfonic acid, 80 mM sodium chloride, pH 8.0 at 25.0 ± 0.1°C. Concentrations of 4,9-DSHA (ε_{392} = 7.64 $mM^{-1}\cdot cm^{-1}$) and HOPDA (ε_{434} = 32.5 $mM^{-1}\cdot cm^{-1}$) 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 at 280 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 Sylon BFT (Supelco, Bellefonte, 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 oxygraph assay (40).

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Genetic requirements for mycobacterial survival during infection

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Despite the importance of tuberculosis as a public health problem, we know relatively little about the molecular mechanisms used by the causative organism, *Mycobacterium tuberculosis*, to persist in the host. To define these mechanisms, we have mutated virtually every nonessential gene of *M. tuberculosis* and determined the effect disrupting each gene on the growth rate of this pathogen during infection. A total of 194 genes that are specifically required for mycobacterial growth *in vivo* were identified. The behavior of these mutants provides a detailed view of the changing environment that the bacterium encounters as infection proceeds. A surprisingly large fraction of these genes are unique to mycobacteria and closely related species, indicating that many of the strategies used by this unusual group of organisms are fundamentally different from other pathogens.

Tuberculosis has been a major killer throughout history, and is still the cause of millions of deaths every year. The prevalence of this infection is largely due to the ability of *Mycobacterium tuberculosis* to persist in the host for long periods of time and cause disease even in the face of a highly orchestrated host immune response (1). This unusual ability, as well as the phylogenetic distance between *M. tuberculosis* and other common bacterial pathogens, suggests that mycobacteria may use unique pathogenic mechanisms. Furthermore, the chronic subacute nature of this disease is likely to require the bacterium to adapt to a continually changing host environment, and recent evidence suggests that different virulence determinants may be used at distinct stages of the disease (2). To define the molecular mechanisms used by this pathogen at each stage of the infection, we used transposon site hybridization (TraSH) to identify *M. tuberculosis* genes that are specifically required for survival during infection in a mouse model of tuberculosis. TraSH is a microarray-based technique designed to analyze large pools of transposon mutants to comprehensively identify genes that are essential for growth under different conditions (3). The ability to simultaneously monitor the growth of thousands of mutant strains throughout the infection has allowed the identification of both specific stresses to which the bacterium is exposed as well as mechanisms used by the bacterium to resist these insults.

Methods

TraSH to Identify Genes That Are Required for Infection. A library of transposon mutants was made in *M. tuberculosis* strain H37Rv by using the MycoMarT7 phage, as described (3). C57BL/6J mice were infected intravenously with 10^6 colony-forming units of the mutant library. At the indicated times after infection, surviving bacteria (≈200,000 clones) were recovered from spleen homogenates by plating on 7H10 agar medium (*in vivo* pools). Four mice were killed at the 1 week time point, and five mice were used at each other time point. The *in vitro* pool was generated by replating the library. Genomic DNA was isolated from each pool, and TraSH probes were generated for each pool and hybridized to microarrays as described (4). Probes were generated from each pool twice and analyzed on duplicate microarrays. Microarray data were collected by using GENEPIX software (Axon, Union City, CA) and analyzed by using GENESPRING software (Silicon Genetics, Redwood City, CA). To define mutations that produce *in vivo* growth defects, the data for each time point (8–10 microarray hybridizations) was averaged and filtered to include only those features whose ratios were significantly different from 1 ($P < 0.05$ by t test). We then excluded mutants with subtle defects (ratios that differed from the median by <2.5-fold).

Determination of *In Vitro* Growth Rates Using TraSH. Relative *in vitro* growth rates were determined previously (4). Briefly, the *M. tuberculosis* transposon library was allowed to grow as separated colonies on 7H10 agar, collected, and replated. The resulting colonies were collected and TraSH probe was generated. This probe was mixed with differentially labeled chromosomal DNA and hybridized to the microarray. Ratios <0.2 (TraSH probe/chromosomal probe) indicated mutants with *in vitro* growth defects.

***In Vivo* and *In Vitro* Competition Experiments.** The Δ*bioF* and Δ*yrbE4A* mutants were generated by allelic exchange, using specialized transduction, as described (5). In the Δ*bioF* strain, nucleotides 1776702–1777776 (encoding *bioF*) in the genome of H37Rv (GenBank accession no. AL123456) were replaced with the hygromycin resistance gene of *Streptomyces hygroscopicus*. In the Δ*yrbE4A* strain, nucleotides 3,920,862–3,920,096 were replaced. The other strains represent transposon mutants that were isolated by sequencing the insertion sites in random strains. To determine the relative *in vivo* growth rates of individual mutants, each mutant strain was mixed with wild-type H37Rv (≈1:1 ratio), and 2×10^5 colony-forming units were inoculated into the tail vein of C57BL/6J mice. Groups of three mice were killed at the indicated times after infection and the ratio of wild-type to mutant was determined by plating lung or spleen homogenates on 7H10 agar with and without antibiotic. To determine relative *in vitro* growth rates, each mutant was mixed with wild-type H37Rv and inoculated into 7H9 broth containing OADC enrichment and 0.05% Tween 80. The ratio of mutant to wild-type bacteria was determined by plating either immediately after inoculation or after 10 days of growth at 37°C.

Results

The strategy used to identify *M. tuberculosis* genes that are required for bacterial survival during infection is depicted in Fig. 1. A library of 100,000 transposon mutants in *M. tuberculosis* strain H37Rv, in which virtually every nonessential gene was mutated (4), was used to infect mice intravenously. The surviving bacteria were recovered from the spleen after 1, 2, 4, and 8 weeks (*in vivo* pools), and mutants that were underrepresented relative to an *in vitro*-grown pool were identified by using TraSH. Because 97% of the predicted genes in the genome are repre-

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Abbreviations: TraSH, transposon site hybridization; RNI, reactive nitrogen intermediates; ROI, reactive oxygen intermediates; BER, base excision repair.

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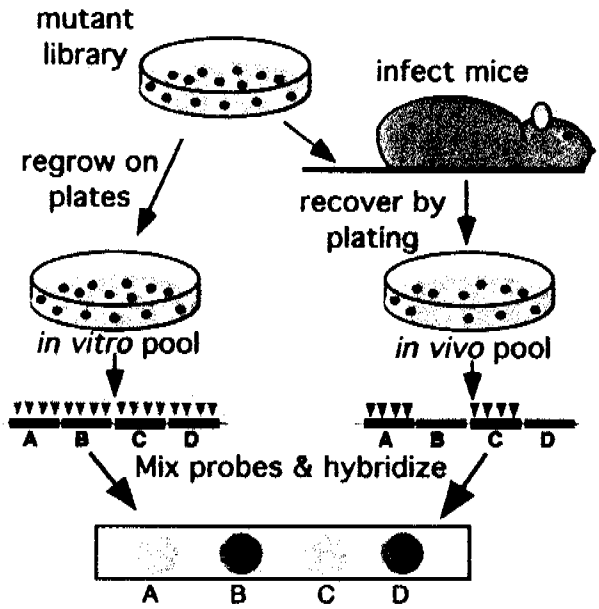

Fig. 1. TraSH to identify attenuated mutants. C57BL/6J mice were infected intravenously with 10^6 colony-forming units of the mutant library. At the indicated times after infection, surviving bacteria were recovered by plating on agar medium (*in vivo* pools). The *in vitro* pool was generated by replating the library. The *in vitro* pool contained mutants with insertions (represented by triangles) in each nonessential gene (black bars). Mutants harboring insertions in genes that are specifically required for survival in the mouse spleen (gray bars) were lost from the *in vivo* pool. Genomic DNA was isolated from each pool, and TraSH probe was generated that was complementary to the chromosomal sequence flanking each insertion in the pool (4). The probes from the two pools were labeled with different colored fluorophores and mixed. Probes were then hybridized to a microarray onto which DNA fragments (features) were immobilized that were complementary to each gene in the genome of *M. tuberculosis*. Features that hybridized to the probe from the *in vitro*, but not the *in vivo*, pool represented genes that were specifically required for growth in the mouse.

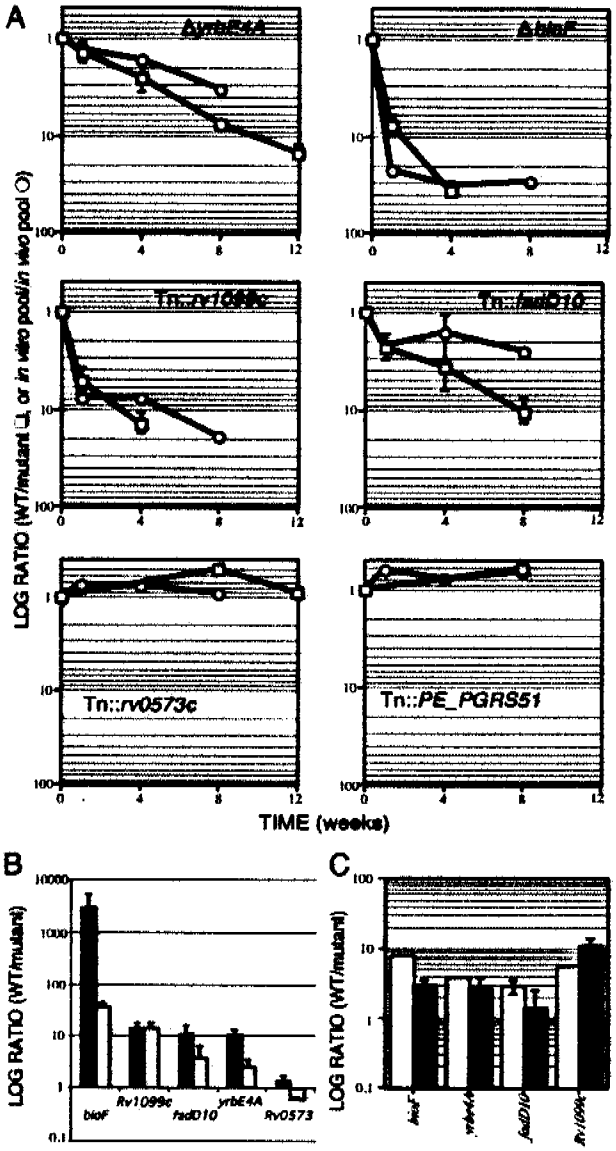

Fig. 2. TraSH accurately predicts the *in vivo* growth characteristics of individual mutants. (*A*) Average TraSH ratios for the indicated genes (*in vitro* pool/*in vivo* pool) are plotted on a log scale as a function of time (circles). Individual strains carrying mutations in these genes were mixed with wild-type bacteria and inoculated into mice. The ratio (wild type/mutant) at each time point after infection is plotted (squares) on the same scale as the TraSH data. Data were normalized so the ratio of the inoculum equaled 1. Error bars represent standard deviations. (*B*) Growth of individual mutants in lung (filled bars) and spleen (open bars). Data were collected as in *A* from organs harvested after 4 weeks of infection except for Tn::*Rv0573* (8 weeks). (*C*) Attenuated strains do not have *in vitro* growth defects. Each mutant strain was mixed with wild-type H37Rv and grown in triplicate broth cultures. The ratio of wild type to mutant was determined after inoculation (open bars) and after 10 days of growth (filled bars). Only the ratio for the Δ*bioF* mutant changed significantly ($P = 0.0005$; mutant grew faster than wild type).

sented on the microarray, we were able to monitor the relative *in vivo* growth rate of nearly every viable mutant.

For each time point, the data from 8–10 independent TraSH experiments were averaged (see Table 2, which is published as supporting information on the PNAS web site, www.pnas.org, for the complete data set). Mutations that resulted in substantial *in vivo* growth attenuation were defined as those producing significantly reduced ratios of *in vivo*/*in vitro*-grown probes (defined in *Methods*). By applying these criteria to the data from each time point, 194 growth-attenuating mutations were defined (Table 3, which is published as supporting information on the PNAS web site). This represents ≈5% of the genome, which is similar to the number of attenuating mutations found in several large signature-tagged mutagenesis screens of other bacterial pathogens (6).

To assess the predictive value of the TraSH data, we isolated several mutant strains that we predicted to have varying *in vivo*-growth phenotypes. Mutants were isolated either by allelic exchange (Δ*bioF* and Δ*yrbE4A*) or by identifying strains with transposon insertions in appropriate genes (Tn::*fadD10* and Tn::*Rv1099c*). The *in vivo* growth rate of each strain was then determined relative to wild-type bacteria in mixed infections (Fig. 2 *A* and *B*). Each of the strains that was predicted to be attenuated for growth did indeed show a significant defect compared with control strains (Tn::*Rv0573c* and Tn::*PE_PGRS51*). In addition, the TraSH data accurately predicted the time at which attenuation can first be detected (Fig. 2*A*) and, therefore, these data can be used to distinguish between mutants with different *in vivo* growth kinetics. Although the TraSH ratio was correlated with the degree of attenuation, in some cases it underestimated the ratio of WT to mutant bacteria. It appears that the maximal attainable microarray ratio varies for each gene and, thus, although changes in the

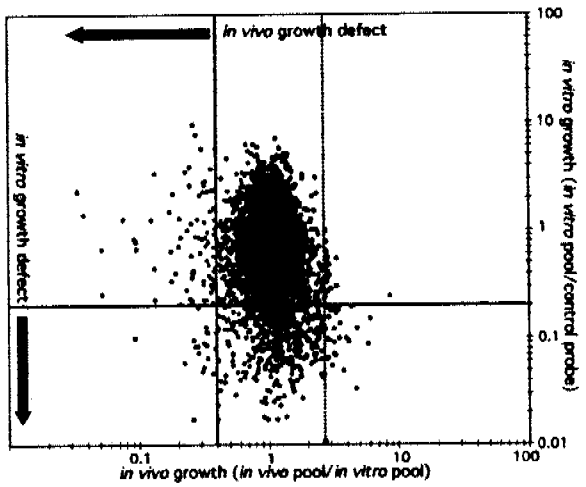

Fig. 3. Comparison of *in vivo* and *in vitro* growth rates. Predicted *in vivo* growth rates are represented as TraSH ratios (8-week *in vivo* pool/*in vitro* pool) for each gene on the *x* axis. Solid vertical line represents cutoff value of 0.4×, which defines attenuating mutations. Dotted line indicates cutoff for the definition of mutants with increased *in vivo* growth rates. Relative *in vitro* growth rates were determined previously (4) and are represented on the *y* axis as the ratio of TraSH probe from the *in vitro* pool divided by a control probe of labeled chromosomal DNA. Horizontal line represents 0.2× cutoff value, identifying mutants with *in vitro* growth defects.

ratio for a particular gene over time are very informative, caution must be used when comparing the absolute ratios for different genes.

In humans, where *M. tuberculosis* is generally transmitted via aerosol, the lung is the primary focus of infection. However, pathogenic mycobacteria can disseminate and grow in virtually any organ in the body; in the mouse model, both lung and spleen are infected. In this study, we analyzed the ability of mutants to survive in mouse spleens, because this allowed us to colonize a single organ with the entire library of 10^5 mutants. This model appears to be generally applicable to other sites of infection, because the four attenuated mutant strains that we have isolated based on TraSH data from spleen were also defective for growth in the lung (Fig. 2*B*).

It is possible this analysis might identify mutants with generalized growth defects, because the *in vivo* pool has undergone several more rounds of division than the *in vitro* pool. To minimize this effect, we compared predicted *in vivo* growth rates of each mutant with previous data (4) on *in vitro* growth rates (Fig. 3). Only 22 of the 194 mutants that were predicted to have *in vivo* growth defects also grow poorly *in vitro* (annotated in Table 3). Furthermore, none of the four attenuated mutants that we isolated grew significantly slower than wild type in broth culture (Fig. 2*C*). Thus, the vast majority of the genes that we have identified are selectively required for growth in the mouse.

Genes of Known Function. Forty-five percent of the genes that we identified can be classified based on predicted function (Table 1). The distribution of functions for the genes that are necessary for *in vivo* growth differs greatly from those that were defined previously as important for growth on agar medium. Lipid metabolic genes and those involved in the transport or metabolism of inorganic ions and carbohydrates are prominently represented in the genes required *in vivo*. Genes with essential *in vitro* functions, such as those involved in translation and nucleotide and amino acid metabolism, are also likely to be important *in vivo* but, because strains with these mutations do not survive, they are not represented in the pools used for infection. Although functional predictions are lacking for only 29% of the genes that are required for growth on agar medium, the majority of those that are important for survival during infection have no annotated function. This observation highlights our relative lack of knowledge regarding bacterial adaptation to the environment in the host.

The functions that are predicted to be important during growth in the host suggest specific stresses to which the bacterium is exposed at different points during infection. In the mouse model, the first week after infection is a period of unrestrained growth during which the bacteria multiply at approximately the same rate as in broth culture. Despite this rapid growth, it

Table 1. Predicted functional classifications of genes in this study

	In vivo		*In vitro*	
Functional classification	No. of genes	Percent of category	No. of genes	Percent of category
Lipid metabolism	15	7.5	30	14.9
Carbohydrate transport and metabolism	9	8.4	24	22.4
Inorganic ion transport and metabolism	8	8.0	8	8.0
Cell envelope biogenesis, outer membrane	8	7.3	32	29.4
Amino acid transport and metabolism	8	4.3	80	43.0
Transcription	7	5.4	15	11.6
Coenzyme metabolism	7	6.0	38	32.8
DNA replication, recombination and repair	5	4.6	19	17.4
Translation, ribosomal structure	5	3.9	76	59.4
Signal transduction mechanisms	4	5.2	12	15.6
Secretion	3	13.6	8	36.4
Energy production and conversion	3	1.6	31	16.3
Cell division and chromosome partitioning	2	8.7	8	34.8
Posttranslational modification, chaperones	2	2.5	27	34.2
Nucleotide transport and metabolism	1	1.5	25	38.5
Unknown	107	4.7	181	8.0
Total	194	5.0	614	15.7

In vivo and *in vitro* refer to genes that are required for optimal growth under either condition. *In vitro* genes were identified previously (4). "Percent of category" refers to the fraction of genes of particular functional class that are important for growth under each condition.

appears that the *in vivo* environment is still quite challenging, because we identified 80 mutants that were already underrepresented at the 1-week time point. Clearly, the nutrients available to the bacteria are limiting during infection, as several auxotrophic mutants are known to be unable to survive in the mouse even at very early time points (7–10). Similarly, the set of genes that we identify at this time contains several that are involved in nutrient acquisition and cofactor biosynthesis. For example, mutations in each of the genes in the biotin biosynthetic pathway (except *bioD*, for which no data were available) result in some of the most dramatic *in vivo* growth defects in our study (Fig. 2*A* and Table 3). In addition, four genes (*sugA-C* and *lpqY*) that are highly homologous to four-subunit disaccharide importers were predicted to be required at 1 week after infection. At later time points after infection, mycobacteria are thought to mainly use a C2 carbon source, such as fatty acid (11). Our data suggest that, at early time points, the nutrients available to the bacterium are quite different, and the inability to metabolize carbohydrates results in a severe growth defect. Other genes required at one week include several predicted to be involved in lipid metabolism or cell wall synthesis (Table 3). Mutations affecting the lipid-rich cell envelope of *M. tuberculosis* are known to result in reduced bacterial survival several weeks after infection (12–14). The TraSH data indicate that the integrity of this structure may also be critical at early time points, perhaps to resist elements of the innate immune system such as complement or antimicrobial peptides.

At ~10 days after infection, the host environment changes dramatically, as do the genetic requirements for bacterial survival. The adaptive immune response is responsible for arresting the growth of *M. tuberculosis* at this time, and bacterial numbers in the spleen decrease by 10-fold between 2 and 4 weeks. This control of bacterial growth is largely dependent on increased production of reactive nitrogen intermediates (RNI) by activated macrophages (15). A potential mechanism to minimize the damage caused by these oxidizing agents is encoded by the *kefB* gene (*Rv3236c*). The *Escherichia coli* KefB protein is a K^+ channel that protects the cell from the toxic effects of electrophilic species by reducing intracellular pH (16). Because the mutation of this gene in *M. tuberculosis* results in a significant growth defect at 4 weeks after infection, it is possible that the mycobacterial KefB is acting in an analogous fashion to limit the damage caused by host-derived radicals. Despite this system, RNI are still toxic to mycobacteria. These species have the ability to cause several different types of DNA damage, including deamination of cytosine (17), and the base excision repair system (BER) is important for mycobacterial resistance to RNI *in vitro* (18). We found that members of the BER system, uracil glycosylase and exonuclease III (encoded by *ung* and *xthA*, respectively), are required for *in vivo* growth at the 2-week time point. Uracil glycosylase specifically removes deaminated cytosine (uracil) residues from DNA. Thus, although RNI have the capacity to damage many different cellular constituents, a significant effect of RNI exposure *in vivo* appears be this specific DNA lesion. Interestingly, another BER enzyme, endonuclease IV (encoded by *end*, the mycobacterial ortholog of *nfo*), is required earlier, at 1 week after infection. In *E. coli*, exonuclease III and endonuclease IV have differing levels of activity toward different DNA lesions (19). Therefore, our data suggest that mycobacteria are exposed to different DNA-damaging agents as infection proceeds. As reactive oxygen intermediates are responsible for much of the DNA damage sustained by *Salmonella typhimurium* during the first week of infection (20), it is likely that these compounds are at least partly responsible for mycobacterial DNA damage at this time.

Genes of Unknown Function. No functional predictions were available for 55% of the genes that we identified as important for *in vivo* growth, and even more strikingly, 25% of these genes have no obvious homologs outside of mycobacteria and closely related species (Table 3). In part, this is a result of the unusual biochemistry and cellular architecture of these organisms, but it also undoubtedly reflects the presence of virulence mechanisms that are unique to mycobacteria. Many of these genes belong to three relatively large gene families, discussed below, that appear to have arisen by duplication and the TraSH data suggest that several members of these families have acquired specialized functions.

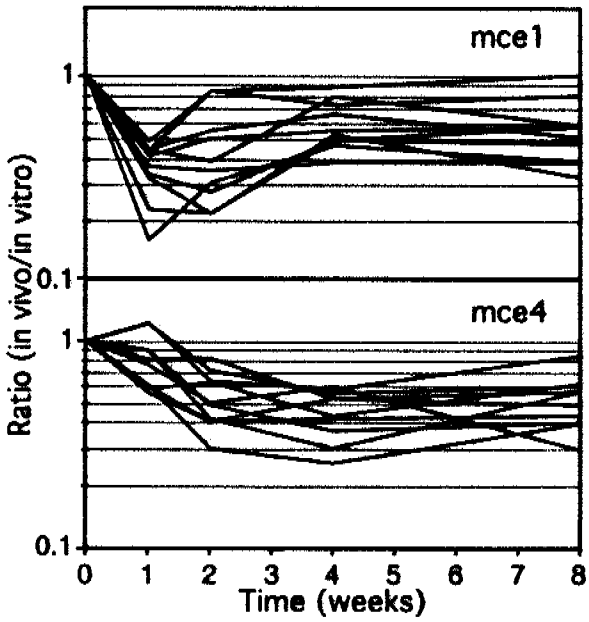

Fig. 4. *In vivo* behavior of *mce* mutants. TraSH ratios for each gene in the *mce1* (*Rv0169-Rv0178*) or *mce4* (*Rv3492c-Rv3501c*) loci are plotted as a function of time (*yrbE1A* and *yrbE1B* of *mce1* locus were omitted because of lack of data). TraSH data compare mutants grown *in vitro* with those isolated from mouse spleens.

The first member of one such family (*mce*) was identified as a factor that increased the uptake of bacteria into nonphagocytic cells (21). This gene (*mce1A*) is located in a 12 gene operon (22) that has been duplicated four times around the chromosome (*mce1-mce4*). We find that two of these gene clusters are required for infection and, surprisingly, show kinetically distinguishable phenotypes. Mutations in the *mce1* locus produce a growth defect one and two weeks after infection, and a partial recovery thereafter (Fig. 4). In contrast, *mce4* mutants show a progressive growth defect that is not detectable until 2–4 weeks after infection. The microarray data are corroborated by the behavior of the Δ*yrbE4A* mutant (Fig. 2*A*), in which the first gene of the *mce4* operon was deleted. Thus, these structurally similar gene clusters are specialized to perform distinct functions and appear to be required at different times during infection. Notably, several of the genes in the *mce1* and *mce4* loci do not meet our strict criteria for attenuation. However, the similar phenotypes of the individual mutants (Fig. 4) strongly suggests that they are involved in a coordinate pathway and indicates that more extensive analyses that incorporate operon structure can reveal these subtle phenotypes.

During the serial *in vitro* passage used to create the attenuated vaccine strain, *M. bovis* bacillus Calmette–Guérin, several large deletions occurred. One of these, termed RD1, removed the locus surrounding the secreted antigen ESAT-6 (23), and this event appears to be responsible for much of the attenuation seen

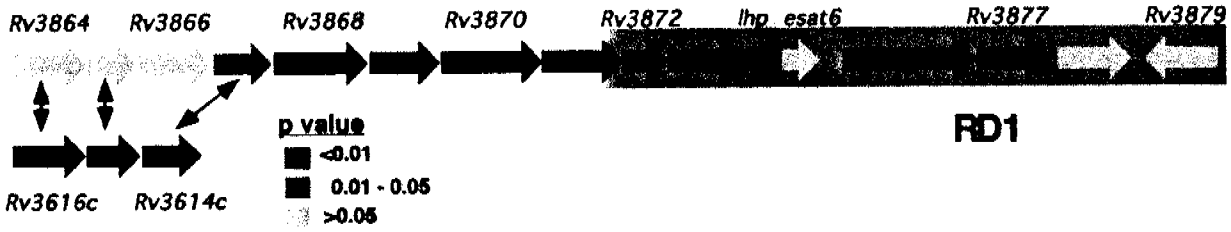

Fig. 5. Genes surrounding *esat6* are required for *in vivo* survival. The *esat-6* locus is depicted and shaded according to the significance level (*P* value) that the ratio for each gene differs from 1 (at the 4-week time point). Homology between *Rv3614c-Rv3616c* and the *esat-6* locus is indicated by arrows. "RD1" refers to the region deleted in *M. bovis* bacillus Calmette–Guérin.

in this strain (24, 25). Because this deletion removed all or part of nine genes, it remained uncertain which of these was responsible for the attenuated phenotype. We find that nearly all of the genes that surround *esat-6* are required for infection (Fig. 5). In part, this could be caused by polar effects of transposon insertions on the transcription of downstream genes. However, the presence of a promoter in the transposon mitigates the attenuation of downstream transcription, and polar effects have not been observed in TraSH data (4). Furthermore, the transcription of downstream genes is unaffected in individual strains harbouring transposon insertions in two of the genes in this locus, *Rv3870* and *Rv3876* (D. Sherman, personal communication). Once again, the organization of these genes into an apparent transcriptional unit and the similarity of their mutant phenotypes suggest that the genes of this locus are performing a coordinated function, perhaps as steps in a biochemical pathway or subunits in a larger structure. This model is supported by recent work demonstrating that the secretion of the Esat-6 protein depends on at least two other genes in this locus (26).

The *esat-6* locus is a member of another gene family that appears to be specific to mycobacteria and close relatives (27). The genome of *M. tuberculosis* contains five imperfect copies of this gene cluster and one of these, *Rv0282-Rv0291*, was predicted to be essential for optimal growth on laboratory medium (4). Thus, like the *mce* genes, the different loci belonging to this family appear to have been retained because they have become specialized to function under different conditions. Although we cannot predict the importance of the *Rv0282-Rv0291* locus *in vivo*, we suspect that it may play a role because only this cluster and the *esat-6* locus are conserved in *Mycobacterium leprae*, an obligate pathogen. In addition to these large duplications, at least 10 smaller loci in the *M. tuberculosis* genome share some homology with these genes. One such cluster, *Rv3614c-Rv3616c*, is also predicted by our data to be essential for *in vivo* growth. Mutation of any of the genes in this locus results in severe attenuation at all time points (Fig. 5).

The largest and most distinctive class of mycobacteria-specific genes encode a group of 167 proteins of repetitive sequence belonging to the PE and PPE families, which have been implicated in the pathogenesis of several mycobacterial species (28–30). In our experiments, only three of these genes met the criteria for defining growth-attenuating mutations, *Rv1807*, *Rv3872*, and *Rv3873*, two of which are encoded in the RD1 region. Although mutations in several other PE and PPE genes appeared to have subtle defects, the fact that such a small fraction are detected in our system suggests either that most of these genes are able to functionally complement each other, or that they are required under conditions that we are unable to test.

Discussion

The set of attenuating mutations described here includes several genes that were previously found to be necessary for bacterial survival in the spleen. These include the alternative *secA* gene, *secA2* (31); *pirG*, encoding a secreted protein of unknown function (32); and the RD1 locus (24). In addition, we identified the locus responsible for the synthesis and export of a cell wall-associated lipid, PDIM, which was identified in two different signature-tagged mutagenesis screens for mutants with growth defects in the lung (13, 29). Several of the genes in this locus were identified, such as *drrA-C* and *mmpl7* (Table 3). Other attenuated mutants may not have been identified for several reasons. Mutations in some genes, such as *icl*, the gene that encodes the glyoxylate shunt enzyme isocitrate lyase (11), were not detected because they cause *in vitro* growth defects in the *M. tuberculosis* strain that we studied (data not shown). Other genes, such as *relA*, which is required during stationary phase growth (33), did not produce sufficient signal in all experiments to meet our criteria for attenuation. A closer analysis of gene clusters, such as the *mce* loci, suggests that the use of these strict criteria excluded several mutants that are truly attenuated, and likely resulted in an underestimation of the actual number of attenuating mutations. The specific animal model that was used for these experiments also influenced which genes were identified. For example, genes that are necessary for the initial colonization of the lung during an infection with aerosolized bacteria were likely missed in our study because of the route of infection that was used. Disease in mice differs markedly from human illness (34). Additionally, the phenotypes of individual mutants could have been influenced by the presence of the fully virulent bacteria that predominate the pool. This could have lead to an accentuation of mutant phenotypes caused by competition, or alternatively, specific mutants could have been complemented in trans by neighboring bacteria.

In many other bacterial pathogens, a surprisingly large number of mutations cause increased virulence in animal models of infection and the corresponding genes have been described as "anti-virulence" genes (35). It is thought that these genes are maintained either because they favor host survival and thereby increase transmission or alternatively, they may promote bacterial survival in a nonhost environment. When we used similar criteria as those that were used to identify attenuated mutants, we found 19 mutants that were overrepresented in the *in vivo* pool at the 8-week time point (Fig. 3; genes are annotated in Table 2). Interestingly, most of these mutants were predicted to grow poorly *in vitro*, suggesting that the increase in bacterial growth *in vivo* is balanced by a decrease in growth rate under other conditions. Although growth on laboratory medium is clearly not directly relevant to the evolution of *M. tuberculosis*, it is likely that the decrease in growth rate under this condition is indicative of a general deficit in the fitness of these mutants.

Several parallels can be drawn between the set of genes that we have defined as critical for mycobacterial infection and those that have been identified in other pathogens. Mutations that affect nutrient uptake systems or result in auxotrophy are attenuating in *M. tuberculosis*, as well as a variety of other intracellular and extracellular bacteria (9, 36, 37). This suggests

that one of the common barriers to *in vivo* growth is the acquisition of essential nutrients. Similarly, as the production of reactive oxygen intermediates and RNI is a common host response to bacterial infection, most pathogens are able to resist the damage done by these toxic species. DNA repair mechanisms appear to be critical for surviving these insults in diverse species of bacteria, but the primary mechanisms that are used differ. Recombinational repair is of primary importance in Gram-negative organisms (20, 38). In contrast, the BER system is required for resistance to RNI in high G+C Gram-positive organisms, such as mycobacteria (18). We found that genes of the BER system, but not the *recA* gene were essential for mycobacterial growth *in vivo*, implying that this system, and not recombinational repair, is predominantly used by *M. tuberculosis* to resist oxidative damage during the first weeks of infection. At very late time points, an additional mechanism involving an error-prone polymerase is important for mycobacterial survival (39). We have also identified the KefB potassium channel as a potential mechanism of resistance to oxidative stress. Interestingly, expression of the *kefB* gene of *Salmonella typhimurium* is induced during intracellular growth in macrophages (40), indicating that the activity of this gene may represent a common virulence mechanism.

A surprisingly large fraction of the genes that we identified are unique to mycobacteria and this has profound implications for understanding the evolution of *M. tuberculosis*. For many bacteria, the acquisition of a pathogenic phenotype is associated with the horizontal transfer of DNA from another organism and this has resulted in many pathogens using conserved virulence mechanisms (41). However, recent acquisition of foreign DNA is not apparent in the genome of *M. tuberculosis*, and most of these common virulence systems are not used by this organism. These observations suggest a model in which mycobacterial evolution from saprophyte to pathogen has occurred largely through the adaptation of ancestral genes to function in the host environment. This model provides a rationale for the large fraction of the genome that appears to have arisen through duplication. Members of the resulting gene families have evolved different functions, and this specialization is critical to bacterial survival in the continually changing environment of the host.

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