* KOPIE *

Intervet
Schering-Plough Animal Health

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INGEKOMEN 30 OKT. 2009

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Geachte mevrouw, heer,

Hierbij doet Intervet International bv een aanvraag van voorgenomen Introductie in het milieu van genetisch gemodificeerde organismen. Deze aanvraag is getiteld: Een niet pathogene *Rhodococcus equi* stam als vaccin in paarden.

Het betreft werkzaamheden met GGO zoals vermeld in beschikking IG99-123, lid 6. Deze aanvraag is een vervolg op deze vergunning voor ingeperkt gebruik (IG99-123), waarbij dierproeven met dit GGO zijn ingeschaald onder DM-I (lid 7). Een volgende stap om het GGO verder als vaccin te kunnen ontwikkelen is het vaccineren van grotere groepen dieren buiten inperking. Doel hiervan is het verzamelen van meer gegevens betreffende vaccinatieregime (m.b.t. leeftijd, ras, doses, vaccinvolume e.d.).

Met het aanvraagformulier sturen wij 8 bijlagen mee.

Met vriendelijke groet,

Dr Ir E. Joosten (MVF)
Intervet / Schering Plough Animal Health

Cc. J. van Raaij (directie)
P. Vermeij (VM-I)
R. Segers (VM-II)

Trade register 's-Hertogenbosch
16028015

VROM

10 October 2008

AANVRAAGFORMULIER INTRODUCTIE IN HET MILIEU:

VETERINAIRE TOEPASSINGEN VAN GENETISCH GEMODIFICEERDE ORGANISMEN

Indien u vragen heeft kunt u contact opnemen met Bureau GGO (email: bggo@rivm.nl, telefoon: 030-2742793).

[Alle informatie die in de aanvraag en de bijlagen wordt verstrekt, wordt voor zover deze niet als vertrouwelijk is aangemerkt, bij de ter inzage legging van de aanvraag en de (ontwerp) beschikking openbaar gemaakt. Van als vertrouwelijk aangemerkte onderdelen moet een openbare samenvatting worden verstrekt, waarin voldoende informatie staat voor een goed algemeen begrip van de aanvraag. Tevens moet schriftelijk gemotiveerd worden waarom bepaalde informatie als vertrouwelijk wordt aangemerkt, hierbij moet aannemelijk worden gemaakt dat het opheffen van de vertrouwelijkheid de concurrentiepositie van de aanvrager schaadt.

Getracht wordt de uiteindelijke beschikking zodanig op te stellen dat diverse veterinaire protocollen hieronder uitgevoerd kunnen worden. Hierbij wordt gebruik gemaakt van de in deze aanvraag beschreven informatie. Uiteraard moeten deze werkzaamheden passen onder de beschrijving van het experiment en de verstrekte risico-analyse.

Alvorens een dergelijke bredere vergunningaanvraag in te dienen is het aan te raden contact op te nemen met Bureau GGO voor informeel overleg over de mogelijkheden.

Onder de in het formulier genoemde 'proefdieren' wordt verstaan dieren die in het onderzoek opgenomen worden.]

INHOUDSOPGAVE

INTERNET www.bioveiligheid.nl

AFKORTINGEN

Regeling	Regeling genetisch gemodificeerde organismen
ggo	Genetisch Gemodificeerd Organisme

A. ALGEMENE GEGEVENS

A.1. Titel van de aanvraag:
Een niet pathogene *Rhododcoccus equi* stam als vaccin in paarden

A.2. Geef een korte inhoudelijke beschrijving van de aanvraag.
Rhodococcus equi is een bacterie die fatale pyogranulomateuze pneumonie kan veroorzaken bij jonge paarden. Vooral veulens (0-6 maanden) zijn zeer ontvankelijk voor een infectie met deze bacterie. Omdat de bacterie intracellulair overleeft in macrofagen, zijn langdurige antibiotica behandelingen noodzakelijk (Bijlage VII: Meijer and Prescot 2004 en Bijlage VIII: Vazques-Boland et al 2009). Er is geen vaccin beschikbaar. Intervet heeft besloten om een vaccin te ontwikkelen om veulens vanaf een jonge leeftijd te kunnen beschermen tegen een *R. equi* infectie. Passieve immunisatie (vaccinatie van de merrie en transfer van antilichamen naar het veulen via colostrum) bleek niet effectief, evenals actieve vaccinatie van het veulen met een geïnactiveerd vaccin (waarschijnlijk is cellulaire immuniteit nodig). Daarom blijft alleen een levend geattenueerd vaccin voor het veulen over om de juiste immuniteit op te wekken. Intervet heeft in samenwerking met de Universiteit Groningen een *R. equi* stam geselecteerd die niet meer pathogeen is voor paarden. In deze stam, *R. equi* RG2837, zijn een viertal genen verwijderd die een rol spelen bij het infecteren en groeien in macrofagen. Het vermogen om te overleven in macrofagen is een belangrijke virulentie karakteristiek voor *R. equi*. De genen zijn verwijderd met behulp van een homologe recombinatie techniek die recent is ontwikkeld en beschreven door onderzoekers van de Universiteit Groningen (Bijlage I: Van der Gijze et al., 2008 en Bijlage II: Van der Gijze 2008). Deze techniek maakt het mogelijk schone deleties te maken in *R. equi*. De ontstane stam bevat derhalve geen promotor, regulatoire of antibiotica resistenties coderende DNA sequenties die afkomstig zijn van vectoren gebruikt in de techniek. Omdat er geen "vreemd" DNA is achtergebleven in de vaccinstam kan er ook geen "vreemd" DNA overgedragen worden op andere micro-organismen.
De stam is volledig veilig gebleken in veulens. Verder is aangetoond dat de veulens tijdelijk lage hoeveelheden van de vaccinstam via de feces kunnen uitscheiden maar er is geen spreiding naar contact dieren gevonden. De vaccinstam blijft tijdelijk lokaal in de darm aanwezig maar dissemineert niet naar andere organen (Bijlage III, Bijlage IV en Bijlage V. Nadat de veiligheid in veulens was aangetoond is de stam ook getest op immuniserende eigenschappen. Uit deze proeven bleek dat orale toediening goede bescherming gaf tegen een challenge met wildtype bacteriën (Bijlage IV). Uit vervolg experimenten met een in de praktijk gangbare toedieningsvorm (kleiner volume, lagere dosis, jongere dieren etc. bleek dat er niet voldoende dieren beschermd waren (Bijlage VI) .
Om het vaccin nu verder door te kunnen ontwikkelen zullen er veel verschillende vaccinatie regimes, leeftijden, rassen toedienings volume etc. getest en vergeleken worden om het optimale schema te vinden. Dit is alleen goed mogelijk in testen buiten containment bv. Stoeterijen, gezien de samenstelling van de proefgroepen. Het houden van merries met veulen in containment komt niet ten goede aan het dieren welzijn. Daarbij is de capaciteit van de containment stallen te beperkt om goede statistiek te kunnen bedrijven.

A.3. **Geef een korte beschrijving van de voorgenomen werkzaamheden.**
Om deze vaccin stam verder te kunnen ontwikkelen tot een vermarktbaar product moet het vaccinatie schema verder geoptimaliseerd worden. Hiervoor zijn meer data uit grotere aantallen dieren nodig in normale houderij. De werkzaamheden zullen bestaan uit het vaccineren van dieren. Omdat er een correlatie is gevonden tussen serum titer en bescherming kan de werkzaamheid serologisch gevolgd worden. De dieren blijven gewoon op de stoeterij (stallen, weides) en worden alleen serologisch gevolgd, i.e. 3-5.x bloedafname. Nadat het optimale entschema bepaald is, zullen vergelijkbare proeven gedaan worden in het veld om de benodigde data voor product registratie te verzamelen. Na de proef kunnen de dieren op locatie blijven en/of verhandeld worden.

A.4. **Beoogde einddatum:**
We verwachten met deze proeven in 3-6 jaar voldoende data te hebben. Er zullen in deze fase 500-2000 veulens gevaccineerd worden.

DOEL VAN DE INTRODUCTIE IN HET MILIEU

A.5. **Algemeen doel van de werkzaamheden die worden aangevraagd:**
Het belangrijkste en finale doel is het ontwikkelen van een vaccin tegen *R. equi* pneumonie in veulens i.e. het verzamelen van voldoende data die nodig zijn voor product registratie.

A.6. **Specifiek doel van de werkzaamheden die worden aangevraagd:**
De specifieke doelen van de werkzaamheden die worden aangevraagd zijn het doen van proeven die noodzakelijk zijn voor de registratie van het nieuwe vaccin.
Om het vaccin verder door te kunnen ontwikkelen zullen er veel verschillende vaccinatie regimes, leeftijden, rassen, toedienings volumina etc. getest en vergeleken moeten worden om het optimale vaccinatie schema te vinden. Dit is alleen mogelijk als deze vaccinatie proeven buiten containment, in het veld, gedaan kunnen worden.
We zijn van mening dat er een aantal goede redenen zijn waarom de introductie in het milieu geen probleem is:
1) de vaccinstam is een deletie mutant die geen vreemd DNA bevat en daarom ook geen vreemd DNA in het milieu kan brengen. Hierdoor valt de risico analyse zeer gunstig uit: nihil.
2) attenuatie en veiligheid voor de gastheer zijn uitgebreid aangetoond
3) het is vrijwel onmogelijk de benodigde gegevens voor een product registratie te verkrijgen binnen de beperktere capaciteit van huisvesting in ingeperkte ruimtes.

VERGUNNINGAANVRAGER

A.8. **Rechtspersoon:**
Intervet International BV

A.9. **Afdeling/vakgroep:**
Directie

A.10. **Correspondentieadres:**
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A.11. **Postcode en plaatsnaam:**
5830AA Boxmeer

A.12. **Telefoonnummer:**
0485 587500

VERANTWOORDELIJK MEDEWERKERS (VM)

VERANTWOORDELIJK MEDEWERKER VOOR WERKZAAMHEDEN ANDERS DAN DE VETERINAIRE TOEPASSING VAN HET GGO (VM-I)

A.13. **Titel, voorletter, voorvoegsel, achternaam:**
Dr. P. Vermeij

A.14. **Instelling/bedrijf:**
Intervet International BV

A.15. **Afdeling/vakgroep:**
D&T

A.16. **Correspondentieadres:**
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A.17. **Postcode en plaatsnaam:**
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A.19. **E-mail adres:**
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VERANTWOORDELIJK MEDEWERKER VOOR DE VETERINAIRE TOEPASSING VAN HET GGO (VM-II)

A.20. **Titel, voorletter, voorvoegsel, achternaam:**
Dr. R. Segers

A.21. **Instelling/bedrijf:**
Intervet International BV

A.22. **Afdeling/vakgroep:**
Microbiological R&D

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MILIEUVEILIGHEIDSFUNCTIONARIS (MVF)

A.27. **Titel, voorletter, voorvoegsel, achternaam:**
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A.28. **Instelling/bedrijf:**
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A.30. **Correspondentieadres:**
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A.33. **E-mail adres:**
Ellen.joosten@sp.intervet.com

PLAATS VAN UITVOERING

A.34. Op welke locaties wordt de voorgenomen toepassing uitgevoerd:
De proeven zullen worden uitgevoerd op conventionele dierverblijven van Intervet (binnen of buiten Boxmeer) en nog te selecteren particuliere stoeterijen in Nederland. Na de proeven kunnen de dieren op locatie blijven en/of verhandeld worden.

ONDERTEKENING

Namens de Rechtspersoon
Naam: J. van Raay
datum 26/10/2009

MVF
Naam: Dr. E. Joosten
datum 26/10/2009

VM I (niet veterinaire toepassing)
Naam: Dr. P. vermeij
datum 22-10-2009

VM II (veterinaire toepassing)
Naam: Dr. R. Segers
datum 26-okt-2009

B. Beschrijving van het genetisch gemodificeerde organisme

B.1. Geef aan waaruit het genetisch gemodificeerde organisme dat aan de proefdier wordt toegediend bestaat.
Bacteriële stam (onderdeel B.3.), *R. equi* deletiemutant

B.3. BACTERIËLE STAMMEN

BACTERIESTAM WAARVAN HET GENETISCH GEMODIFICEERDE ORGANISME IS AFGELEID

B.3.1. Tot welke bacteriesoort behoort de stam die is gebruikt als uitgangsstam bij de constructie van het GGO.
Rhodococcus equi

B.3.2. Is de uitgangsstam een GGO.
Nee

B.3.3. Wat is de natuurlijke niche van de bacteriestam.
In principe is *R. equi* een bodem bacterie die overal in de natuur voorkomt. In het voorjaar en zomer tijdens droge periodes kunnen de bacteriën aan of met stof ingeademd worden door veulens en de longen bereiken. Daar infecteren zij de aanwezige macrofagen en vermenigvuldigen zich in deze cellen.

B.3.4. Geef relevante gegevens over pathogeniteit en eventuele attenuering en biologische inperking van de uitgangsstam.
R. equi is ingedeeld in pathogeniciteitsklasse 2. Een tot drie weken na de infectie worden de klinische verschijnselen, pneumonie, zichtbaar. De bacterie is alleen pathogeen voor paarden met een onvolwassen immuunsysteem. Oudere dieren zijn immuun tegen de infectie. Waarschijnlijk komt dit doordat *R. equi* altijd in het milieu (en in paarden) aanwezig is en de dieren langzaam een beschermende immuun respons opbouwen omdat ze regelmatig met het organisme in contact komen. Een wildtype *R. equi* kan ook humane macrofagen in vitro infecteren maar gezonde mensen worden nooit geïnfecteerd. Alleen in immuun gecompromeerde mensen (AIDS patiënten) kan de bacterie eveneens pneumonie veroorzaken (Bijlage VII: Meijer and Prescot 2004).

B.3.5. Geef informatie over voortplanting en overleving van de bacteriestam in natuurlijke gastheren.
In de gastheer infecteert *R. equi* de macrofagen in de longen van het veulen en vermenigvuldigd zich vervolgens in deze macrofagen. Ook is hij in staat om zich in de darm van paarden handhaven. In de literatuur is zeer weinig beschreven over de generatietijden in de gastheer. In onze proeven zien wij incubatie tijden (tussen besmetten en uitbreken klinische ziekte) die variëren van 1-3 weken na infectie. Zowel gedurende de infectie als buiten de gastheer vormt *R. equi* geen sporen of andere overlevings- of verspreidingsstructuren.
Behalve uit paarden wordt *R. equi* ook wel eens uit andere landbouwhuisdieren geïsoleerd maar veroorzaakt daar normaal gesproken geen ziekte. Deze stammen

blijken een afwijkend (gastheer specifiek) virulentie plasmide te bezitten en worden als intermediair pathogeen gekenmerkt.

B.3.6. Wat zijn de mogelijkheden voor overleving, vermenigvuldiging en verspreiding onder milieuomstandigheden anders dan in natuurlijke gastheren.
R. equi is een bodembacterie met eenvoudige eisen aan de omgeving waarin het groeit. Verder wordt de bacterie teruggevonden in de ingewanden van veel herbivoren en omnivoren en op hun leefgebieden. De bacterie kan lange tijd overleven in mest en in de bodem. De overleving en groei in het milieu zijn sterk afhankelijk van de omgevingstemperatuur, aanwezigheid van vluchtige vetzuren en de pH van de bodem. Onder de 10°C is *R. equi* inactief.

B.3.7. Kan de stam genetisch materiaal uitwisselen met andere organismen.
Alle *R. equi* stammen die geïsoleerd worden uit veulens met pneumonie bezitten een 80-90 kb virulentie plasmide. Dit plasmide is essentieel voor de virulentie van *R. equi* in paarden. Zodra virulente stammen het plasmide hebben verloren worden zij apathogeen. Het plasmide bevat, naast het gen voor de virulentie factor VapA, genen die mogelijk coderen voor eiwitten die een rol spelen bij conjugatie van plasmiden en replicatie. De homologie met deze eiwitten is echter zeer laag.
Conjugative transfer van het virulentie plasmide is geen probleem. Alle pathogene *R. equi* stammen hebben al een virulentie plasmide en kunnen vanwege incompatabiliteit geen twee plasmide opnemen. De vaccinstam heeft ook een virulentie plasmide zonder dat deze nog ziekte kan veroorzaken. Dit komt doordat de deleties die zorgen dat de vaccinstam verzwakt- en veilig is op het chromosoom liggen en niet op het virulentie plasmide.
De incompatibiliteitsklasse en het gastheer bereik lijkt beperkt tot *R. equi* aangezien het *vapA* gen dat op dit plasmide ligt nog nooit in een ander micro-organisme is terug gevonden.
Verder zijn er geen andere zelfoverdraagbare elementen, mobiliseerbare plasmiden of transposons beschreven voor *R. equi*.

DE GENETISCH GEMODIFICEERDE BACTERIE

B.3.8. Is bij de genetische modificatie gebruik gemaakt van een vector.
Ja, maar in de vaccinstam is geen DNA afkomstig van deze vector achtergebleven in het constructie proces.

B.3.9. Geef een beschrijving van de structurele genen en regulatoire sequenties die aanwezig zijn in de vector en in het in de vector geïnserteerde DNA.
Deze beschrijving is in dit geval niet relevant omdat er geen vector of onderdelen van de vector zijn achtergebleven in de vaccinstam. Echter voor de volledigheid bevatte de vector een OriT, apramycine resistentie gen, promotor regio van de kanamycin resistentie cassette met daarachter een *codA::upp* fusie gen dat codeert voor een cytosine deaminase en een uracil fosforibosyl transferase. Verder zijn er in de vector delen van de genen die in de constructie van de vaccinstam zijn verwijderd. Voor meer details zie Bijlage II: Van der Gijze 2008.

B.3.10. Vat de gegevens onder B.3.8 en B.3.9 samen in een schematische weergave ('kaart') van het genetisch gemodificeerde organisme.

Zie bijlage II: Van der Gijze 2008.

B.3.11. Welke fysiologische (daaronder mede begrepen ziekteverwekkende) effecten kan de genetisch gemodificeerde bacterie veroorzaken; welke behandelingen zijn beschikbaar.

Geen enkele.
De genen die verwijderd zijn uit de vaccinstam, coderen voor eiwitten die een rol spelen bij de virulentie en zijn essentieel voor het infecteren en overleven in macrofagen. Uit experimenten is gebleken dat de vaccinstam niet meer in staat is te overleven in macrofagen en niet in staat is om ziekte te veroorzaken. Hij is dan ook volledig veilig gebleken in veulens. In een proef met acht veulens werden vijf dieren intratracheaal besmet met de deletie mutant en werden drie andere dieren intratracheaal besmet met een gelijke dosis van de wildtype uitgangsstam.
Alle drie de veulens die besmet waren met de wildtype stam kregen pneumonie terwijl alle vijf de veulens die met de deletie mutant behandeld waren gezond bleven (zie bijlage III).

B.3.12. Geef aan in hoeverre het gastheerbereik van de genetisch gemodificeerde *bacterie gewijzigd is of kan zijn ten opzichte van de uitgangsstam.*

Het gastheer bereik is afgenomen.
Waar normaal *R. equi* kan overleven in macrofagen en lange tijd de longen en darmen van veulens kan koloniseren, is de vaccinstam al na korte tijd niet meer terug te vinden in de longen en andere organen van veulens die $4x10^6$ cellen van de vaccinstam intratracheaal toegediend hadden gekregen. Ook na orale toediening van $2x10^{10}$ CFU bleek de vaccin stam veilig. De bacterie blijft tijdelijk in de darm aanwezig (Bijlage III en bijlage V), maar veroorzaakt geen klinische verschijnselen.

B.3.13. Geef aan via welke routes de genetisch gemodificeerde bacterie zich kan verspreiden.

Het vaccin wordt oraal en/of rectaal toegediend. In de darmen van de veulens vind (waarschijnlijk) groei plaats en een interactie met het immuunsysteem. Als gevolg van deze interactie wordt er een immuniteit opgebouwd die de veulens beschermd tegen infectie in de longen. Het risico van orale toediening is dat de bacteriën via de faeces kunnen worden uitgescheiden. Dit vindt tijdelijk en in beperkte mate plaats (Bijlage V). Overlevings proeven met de mutant in leiding water, vijverwater en in aarde tonen aan dat de mutant bij verschillende temperaturen (range 4-37C°) maanden kan overleven. Dus hoewel hij in het dier geattenueerd is kan hij in het milieu nog goed overleven, vergelijkbaar met de wildtype *R. equi.*

C. MILIEUGERELATEERDE GEGEVENS AFKOMSTIG UIT EERDERE EXPERIMENTEN

C.1. Geef een beschrijving van de resultaten welke afkomstig zijn uit eerdere studies met het GGO, en die van belang zijn voor de milieurisicobeoordeling.

Onderzoek binnen ingeperkt gebruik:
De attenuering is duidelijk aangetoond in in vitro proeven (i.e. macrofaag overleving) en in veulens waarbij de vaccinstam i.t.t. de uitgangstam geen pneumonie veroorzaakt (Bijlage III). Daarna werd de veiligheid nog eens bevestigd in andere veulen proeven waarbij zeer hoge ($2x10^{10}$ CFU) en herhaalde doseringen oraal werden getest en veilig bleken voor 1-week oude veulens (Bijlage V). Verder werd de beschermende werkzaamheid van orale vaccinatie in veulens aangetoond door middel van vaccinatie-besmettings proeven (Bijlage IV en bijlage VI). Verder is aangetoond dat de veulens tijdelijk lage hoeveelheden van de vaccinstam via de feces kunnen uitscheiden maar er is geen spreiding naar contact dieren gevonden. De vaccinstam blijft tijdelijk lokaal in de darm aanwezig maar dissemineert niet naar andere organen (Bijlage III, Bijlage IV, en Bijlage V).

Introductie in het milieu onderzoek:
We hebben een selectieve agar ontwikkeld waarmee lage aantallen *R. equi* (i.e. 10 CFU/100 mg feces) kunnen worden gedetecteerd. Verder is een PCR beschikbaar die wildtype en vaccinstam kan onderscheiden met een vergelijkbare sensitiviteit.
De nu nog lopende overlevingsproeven in diverse omstandigheden (in aarde, kraanwater, vijverwater) tonen aan dat de vaccinstam tenminste maanden kan overleven in deze milieu's bij temperaturen variërend van 4°C tot 37°C. Hierbij is er (nog) geen verschil waargenomen tussen de vaccinstam en wildtype stam. Blijkbaar en volgens verwachting is de vaccinstam alleen maar geattenueerd in de gastheer.

D. PRODUCTIE VAN HET GGO OF NUCLEINEZUUR PREPARAAT

D.1. Geef aan onder welke verantwoordelijkheid productie van het GGO of nucleïnezuur preparaat wordt uitgevoerd.
Voor de productie wordt verwezen naar de reeds bestaande ingeperkt gebruik vergunning: IG99-123.

D.2. In welke stappen van de productie vindt kwaliteitscontrole plaats, welke testmethoden worden gebruikt en hoe worden de tests uitgevoerd.
De bacterie wordt gegroeid op vloeibaar medium. Naar gelang de schaal wordt de vloeibare cultuur doorgeënt. Na de groei wordt de cultuur gemengd met een vriesdroog stabilisator en wordt de bacterie gevriesdroogd. QC testen op het eindproduct bestaan uit: restvocht, reinheid, levendtelling, determinatie, een PCR ter bevestiging van de deletie, en tenslotte een PCR ter bevestiging van de aanwezigheid van het virulentie plasmide.

D.3. Welke criteria worden aan een batch van het GGO gesteld voordat deze wordt vrijgegeven voor de onderhavige toepassing.
Een aantal flacons met gevriesdroogd vaccin worden geresuspendeerd in water en een verdunningsreeks wordt uitgeplaat op bloed agar platen. Na 1-2 dagen worden de platen gecontroleerd op reinheid. Er mogen alleen *R. equi* kolonies op de platen zitten. Bovendien worden de kolonies geteld op de platen. Een batch wordt alleen vrijgeven als er meer dan 10^7 CFU per dosis vaccin in de flacon hebben gezeten. Verder worden een tiental kolonies van de plaat genomen en gebruikt in een *vapA* PCR en deletie PCR. Alle 10 kolonies moeten positief zijn in de *vapA* PCR en moeten beide gen deleties hebben. Zo niet dan wordt de batch niet vrijgeven Deaanwezigheid van het virulentie plasmid is essentieel anders kan hij geen immuniteit induceren).
Als laatste wordt ook nog een determinatie analyse ingezet om aan te tonen dat we te maken hebben met *R. equi*.

E. ASPECTEN BEHOREND BIJ DE VETERINAIRE TRIAL

E.1. Hoeveel proefdieren zullen opgenomen worden in het onderzoek.
Afhankelijk van het verloop van de proeven 500-2000.

E.2. Welke doses worden toegediend en op welke tijdstippen gedurende de studie vindt toediening plaats.
Doseringen variëren van 10^7 CFU tot $5x10^{10}$ CFU; waarbij een tot twee doseringen gegeven worden. In het geval van twee doseringen gebeurt dit met een interval van ± 2 weken. Veulens zullen gevaccineerd worden op een leeftijd van 3 dagen tot 6 maanden.

E.3. Op welke wijze wordt het GGO preparaat aan het proefdier toegediend.
Orale of rectale toediening;

E.4. Worden er monsters van het proefdier genomen die GGO's (kunnen) bevatten, en welke tests worden hiermee uitgevoerd.
Er worden geen monsters genomen die het GGO kunnen bevatten.
De enige monsters die worden genomen zijn bloed (serum) monsters voor antilichaam titraties. De kans dat daar GGO in zit is heel erg klein omdat aangetoond is dat de vaccin stam niet naar de bloedbaan of organen gaat en daar ook niet kan overleven (blijft alleen tijdelijk lokaal in de darm). Verder worden alleen monsters genomen indien daar aanleiding toe is.

E.5. Worden de proefdieren in isolatie gehouden of komen de proefdieren na behandeling met het GGO in contact met dieren die geen deel uitmaken van de onderhavige studie. Welke criteria worden gehanteerd voor het al dan niet implementeren van isolatie maatregelen. .
De dieren worden gehouden volgens de normale farm routines. De dieren worden echter altijd in stallen of afgerasterde weilanden gehouden en zijn als zodanig fysisch ingeperkt en komen gedurende de proef niet in aanraking met dieren die geen deel uitmaken van de studie.
Omdat 1) de vaccinstam sterk geattenueerd is in de gastheer en 2) het om een "schone" deletie mutant gaat, zijn de milieu risico's nihil. Speciale inperkings maarregelen zijn daarom niet nodig.

F. RISICO-ANALYSE

F.1. Geef aan volgens welk scenario het genetisch gemodificeerde organisme en/of een afgeleide van het nucleïnezuur preparaat zich vanuit het proefdier kan verspreiden in het milieu.

Via de feces kan de vaccinstam in het milieu geraken. Het gaat om een deletie mutant die in de gastheer veel moeilijker kan overleven. Dus verspreiding in het milieu is te allen tijde minder dan de verspreiding van de wild type (die alom aanwezig is) en indien dit zou gebeuren is het ongevaarlijk. Indien de deletie overgenomen wordt door soortgenoten zullen deze eveneens verminderd in staat zijn in macrofagen (dieren) te overleven. Alle *R. equi* stammen die geïsoleerd worden uit veulens met pneumonie bezitten een 80-90 kb virulentie plasmide. Dit plasmide is essentieel voor de virulentie van *R. equi* in paarden en is soort specifiek. *Rhodococci* die uit varkens geïsoleerd worden hebben een ander soortspecifiek virulentie plasmide. In het theoretische geval dat deze *Rhodococci* de deletie over zouden nemen zouden ze eveneens geattenueerd zijn.

F.2. Geef aan welke mogelijke nadelige effecten gepaard kunnen gaan met blootstelling van mens of milieu aan het GGO.

Omdat de vaccinstam een 4-tal genen mist die essentieel zijn om ziekte te veroorzaken is er geen kans dat deze ziekte veroorzaakt. Er is geen risico op nadelige effecten voor mens of milieu.

F.3. Geef een inschatting van de kans dat de in F.2. beschreven nadelige effecten ook daadwerkelijk kunnen optreden.

Een inschatting kan niet gemaakt worden omdat er geen nadelige effecten zijn. (zie F1 en F2)

F.4. Beschrijf de risico's die op kunnen treden ten gevolge van de toepassing van het GGO.

Het enige risico is dat de dieren de GGO uit zouden kunnen scheiden. Ook al zou dat gebeuren dan levert dat geen gevaar op omdat de GGO alleen maar stukken DNA met de bijbehorende eigenschappen mist, terwijl de WT bacterie overal in het milieu voor komt. Vaccinatie met deze stam is voor het dier aangetoond veilig.

G. RISICO MANAGEMENT

G.1. Welke criteria worden gehanteerd bij de selectie van proefdieren. En wat is het effect van deze criteria op de milieuveiligheid.
De dieren worden geselecteerd op algemene gezondheids toestand en leeftijd 3 dagen tot 6 maanden oud.

G.2. Welke beperking van de omvang van de studie, in relatie tot het aantal proefdieren en de toe te passen dosering wordt toegepast in het kader van risico management maatregelen.
Het aantal dieren is voorgeschreven door de overheid en/of is afhankelijk van de te verwachte spreiding in de resultaten i.v.m. statistisch verantwoorde conclusies. Doordat grotere aantallen dieren kunnen worden gevaccineerd, zullen de verschillen eerder significant zijn i.t.t. kleinere proefgroepen in containment.

G.3. Beschrijf welke maatregelen voorzien zijn ten aanzien van isolatie van het proefdier.
We hebben waargenomen dat de vaccin stam tijdelijk in lage hoeveelheden uitgescheiden wordt en dat hij zeer lang kan overleven in de mest en aarde (vergelijkbaar met de wildtype). Isolatie van het proefdier is echter niet nodig omdat het om een deletie mutant gaat waardoor de risico analyse zeer gunstig uit pakt. Desalniettemin zullen de dieren te allen tijde fysisch ingeperkt zijn omdat ze in stallen en/of afgerasterde weilanden gehouden worden (volgens de betreffende farm routines).

G.4. Beschrijf welke maatregelen worden getroffen om verspreiding van het GGO naar derden (waaronder bij de studie en de proefdieren betrokken personeel) te voorkomen.
Paarden worden verzorgd en behandeld volgens de normale farm routines waar wildtype *R. equi* altijd in grote hoeveelheden voor komt. Er worden voor deze vaccinatie proeven geen speciale maatregelen getroffen omdat de vaccin stam niet pathogeen is voor mensen of paarden en omdat het om een deletie mutant gaat. Normaal veilig werken en normale farm routines zijn afdoende.

G.5. Beschrijf welke maatregelen worden getroffen om verspreiding van het GGO naar derden (waaronder bij de studie en de proefdieren betrokken personeel) te voorkomen indien er sprake is van onverwachte gebeurtenissen zoals bijvoorbeeld ook de dood van een proefdier.
De veiligheid is zeer uitgebreid getest. De kans op een dood dier t.g.v. de vaccinatie is nihil. Mocht er toch onvoorzien een dier sterven tijdens de proef dan wordt er sectie verricht en worden er monsters genomen om de oorzaak te onderzoeken. Het kadaver kan vervolgens via de normale farm routines afgevoerd worden.

G.6. Beschrijf van welke monsters verwacht kan worden dat zij GGO's kunnen bevatten, en geef voor die monsters aan hoe bemonstering plaatsvindt, en hoe de monsters verder worden verwerkt.
De enige monsters die genomen worden is bloed (serum) voor antilichaam titratie. Het is zeer onwaarschijnlijk dat daar de vaccinstam in zit. Deze monster worden in een ELISA getest in het microbiologisch laboratorium van Intervet.

Andere monsters worden alleen genomen als daar aanleiding toe is.

H. MONITORING EN AFVALVERWERKING

H.1. Op welke wijze wordt het GGO preparaat gedetecteerd na de toediening.
Hoewel een selectieve agar en een specifieke PCR beschikbaar zijn achten wij detectie van de vaccin stam niet nodig omdat dit al zeer uitgebreid getest is. Het is bekend dat de paarden tijdelijk in geringe hoeveelheden vaccin stam kunnen uitscheiden en dat de vaccin stam zich geruime tijd kan handhaven in het milieu. Monstername en detectie zou slechts bevestigen wat we al weten.

H.2. Beschrijf hoe de monitoring wordt opgezet om eventuele verspreiding van het GGO waar te kunnen nemen.
Het is bekend dat paarden de vaccin stam tijdelijk in geringe hoeveelheden uit kunnen scheiden en dat de vaccin stam lang kan overleven in mest en aarde. Monitoring zou dit nogmaals kunnen bevestigen. Waar het om gaat is dat dit een deletie mutant is die geen vreemd DNA bevat waardoor de risico analyse voor effect van evt. verspreiding zeer gunstig uit valt.

H.3. Geef een overzicht van de aard en hoeveelheid van het geproduceerde afval en beschrijf hoe het afval wordt afgevoerd.
Afval dat de deletie mutant kan bevatten bestaat grotendeels uit paardenmest (in stal of weide). De mest in de stallen zal via de normale farm routines worden afgevoerd/verwerkt. Er zullen geen speciale maatregelen t.b.v. afvoer van afval genomen worden.

H.4. Beschrijf welke maatregelen gehanteerd worden om verspreiding van het genetisch gemodificeerd organisme te voorkomen.
Geen bijzondere maatregelen.
Het betreft een "schone" deletie mutant die geen vreemd DNA in het milieu kan introduceren. De attenuatie en veiligheid voor de gastheer zijn uitgebreid aangetoond. Bij de proeven op de stoeterijen worden de normale Farm routines gehanteerd. De dieren zijn te allen tijde fysisch ingeperkt omdat ze in stallen en/of afgerasterde weilanden gehouden worden.

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A novel method to generate unmarked gene deletions in the intracellular pathogen *Rhodococcus equi* using 5-fluorocytosine conditional lethality

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ABSTRACT

A novel method to efficiently generate unmarked in-frame gene deletions in *Rhodococcus equi* was developed, exploiting the cytotoxic effect of 5-fluorocytosine (5-FC) by the action of cytosine deaminase (CD) and uracil phosphoribosyltransferase (UPRT) enzymes. The opportunistic, intracellular pathogen *R. equi* is resistant to high concentrations of 5-FC. Introduction of *Escherichia coli* genes encoding CD and UPRT conferred conditional lethality to *R. equi* cells incubated with 5-FC. To exemplify the use of the *codA::upp* cassette as counter-selectable marker, an unmarked in-frame gene deletion mutant of *R. equi* was constructed. The *supA* and *supB* genes, part of a putative cholesterol catabolic gene cluster, were efficiently deleted from the *R. equi* wild-type genome. Phenotypic analysis of the generated Δ*supAB* mutant confirmed that *supAB* are essential for growth of *R. equi* on cholesterol. Macrophage survival assays revealed that the Δ*supAB* mutant is able to survive and proliferate in macrophages comparable to wild type. Thus, cholesterol metabolism does not appear to be essential for macrophage survival of *R. equi*. The CD-UPRT based 5-FC counter-selection may become a useful asset in the generation of unmarked in-frame gene deletions in other actinobacteria as well, as actinobacteria generally appear to be 5-FC resistant and 5-FU sensitive.

INTRODUCTION

An important molecular tool in functional genomics studies is the targeted inactivation of any gene of interest. Ideally, unmarked gene deletions are constructed using a positive selection step for the rare second recombination event. The *sacB* gene of *Bacillus subtilis* is one of the most widely used suicide genes, conferring sucrose sensitivity mostly in Gram-negative bacteria (1,2). Previously, we reported the use of *sacB* counter-selection to efficiently generate unmarked gene deletions in *Rhodococcus erythropolis* (3). This method subsequently has been applied in other *Rhodococcus* species (4–6). Sucrose sensitivity by *sacB* has also been reported for other mycolic acid containing actinobacteria, i.e. *Corynebacterium glutamicum* and *Mycobacterium* sp. (7). Despite considerable efforts, we have been unable to apply *sacB* as a counter-selectable marker in *Rhodococcus equi*, due to a lack of sucrose sensitivity. Similar observations have been reported for *Streptomyces lividans* (7). The *sacB* counter-selection system generally is not applicable to other Gram-positive bacteria, like *B. subtilis*. In *B. subtilis*, *mazF*, encoding a toxin, has been used as a suicide marker to generate unmarked gene deletion mutants (8). This method relies on the availability of a tightly regulated, inducible promoter, limiting applicability in bacteria with less developed molecular toolboxes. The use of alternative suicide genes have been reported as well, e.g. *glkA*, *pyrF*, *upp* and *rpsL* (9–12). A major drawback of these markers, however, is that they only function in *glkA*, *pyrF*, *upp* or *strA* null mutants, respectively, necessitating the construction, or availability, of a null mutant for every strain to be mutated. A PCR-targeted gene replacement strategy has

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been reported for streptomycetes that relies on the availability of ordered genomic libraries to efficiently generate mutants (13). This method, however, requires a laborious extra round of mutagenesis to remove the resistance marker integrated into the genome to generate unmarked mutants. Recently, a simplified method for marker removal in actinomycetes using Flp recombinase has been described (14).

Cytosine deaminase (CD, EC 3.5.4.5) and uracil phosphoribosyltransferase (UPRT, EC 2.4.2.9) are enzymes involved in the pyrimidine salvage pathway, converting cytosine via uracil into dUMP. CD activity has been found in certain prokaryotes and lower eukaryotes. The genes encoding these activities in *Escherichia coli*, *codA* and *upp*, respectively, have been cloned and characterized (15,16). Interestingly, microorganisms expressing CD convert the innocuous cytosine analog 5-fluorocytosine (5-FC) into 5-fluorouracil (5-FU), a highly toxic compound lethal to living cells. The cytotoxicity is largely exerted following UPRT mediated conversion of 5-FU into 5-fluoro-dUMP, which irreversibly inactivates thymidylate synthase inhibiting both RNA and DNA synthesis (17). Heterologous expression of *E. coli codA* was shown to confer 5-FC sensitivity to mammalian cells, ordinarily not producing CD (18). Concomitant expression of *E. coli* CD and UPRT as a fusion protein, encoded by *codA::upp*, was shown to further enhance the 5-FC cytotoxicity (19). A recent study on pyrimidine salvage in *Streptomyces* species indicated a lack of CD activity but sensitivity towards 5-FU (20). Some other actinobacteria, like *Rhodococcus* species, were also shown to be 5-FU sensitive (21,22). Most *Nocardia* species and certain *Mycobacterium* strains, however, were 5-FU resistant, while other mycobacteria strains were highly 5-FU sensitive (21).

R. equi is a facultative intracellular pathogen that causes fatal pyogranulomatous bronchopneumonia in young foals. It is also an emerging opportunistic pathogen of immuno-compromised humans, particularly HIV infected patients (23,24). In addition to its pathogenic life-style, *R. equi* is a common soil-dwelling microorganism capable of rapid growth in soil and manure, using plant and animal sterols as sole carbon and energy sources (25,26). Knowledge on sterol metabolism in *R. equi* is extremely limited. So far, only the gene encoding the proposed first step in cholesterol degradation, cholesterol oxidase (*choE*), has been identified and inactivated in *R. equi* (27,28). A cholesterol catabolic gene cluster has been identified in the closely related *Rhodococcus jostii* RHA1 (6,29). This cluster also encodes a putative cholesterol uptake system, designated *mce4* operon. 'Mammalian cell entry' (*mce*) genes are critical virulence factors of the intracellular pathogen *Mycobacterium tuberculosis* (30) and, interestingly, *mce1*$^-$ and *mce4*$^-$ strains of *M. tuberculosis* H37Rv showed attenuated survival in mice (31). The *supAB* genes are part of the *mce4* operon and may encode the permease subunits of the cholesterol uptake system (6). The *supAB* and *mce4* genes were shown to be essential for growth of *R. jostii* RHA1 on cholesterol (6). Pandey and Sassetti (32) recently confirmed that cholesterol is used as a carbon and energy source by *M. tuberculosis* H37Rv, and that the *mce4* cluster in H37Rv is essential for growth on cholesterol.

The *R. equi* strain 103S genome sequence recently has become available (http://www.sanger.ac.uk/Projects/R_equi/). Proper genome annotation and identification of pathogenicity genes requires simple methods for gene deletion mutagenesis. A method for the isolation of gene deletion mutants of *R. equi* by the double homologous recombination strategy was first reported by Navas *et al.* (27). An improved method, using *lacZ* as counter-selectable marker, was subsequently reported by Jain *et al.* (33). These methods, however, often involve screening and handling of large numbers of colonies to select for the rare second recombination event (34,35). Here we show that introduction of the *codA::upp* cassette confers 5-FC sensitivity to *R. equi* allowing positive selection of the targeted gene deletion mutants. A simple and efficient procedure to generate unmarked in-frame gene deletions in *R. equi* is reported, exemplified by the construction and characterization of a Δ*supAB* mutant.

MATERIALS AND METHODS

Culture media and growth conditions for *R. equi* strain RE1

Virulent *R. equi* wild type strain RE1 was isolated from a foal with pyogranulomatous pneumonia in September 2007 in The Netherlands. *R. equi* strains were grown at 30°C (200 r.p.m.) in Luria-Bertani (LB) medium or mineral acetate medium (MM-Ac). MM-Ac contained K_2HPO_4 (4.65 g/l), $NaH_2PO_4 \cdot H_2O$ (1.5 g/l), sodium-acetate (2 g/l), NH_4Cl (3 g/l), $MgSO_4 \cdot 7H_2O$ (1 g/l), thiamine (40 mg/l, filter sterile) and Vishniac stock solution (1 ml/l). Vishniac stock solution was prepared as follows [modified from (36)]: EDTA (10 g/l) and $ZnSO_4 \cdot 7H_2O$ (4.4 g/l) were dissolved in distilled water (pH 8 using 2 M KOH). Then, $CaCl_2 \cdot 2H_2O$ (1.47 g/l), $MnCl_2 \cdot 7H_2O$ (1 g/l), $FeSO_4 \cdot 7H_2O$ (1 g/l), $(NH_4)_6 Mo_7O_{24} \cdot 4H_2O$ (0.22 g/l), $CuSO_4 \cdot 5H_2O$ (0.315 g/l) and $CoCl_2 \cdot 6H_2O$ (0.32 g/l) were added in that order at pH 6 and finally stored at pH 4. For growth on solid media Bacto-agar (15 g/l) was added. 5-Fluorocytosine (Sigma-Aldrich Chemie, Zwijndrecht, The Netherlands) and 5-fluorouracil (Sigma-Aldrich) stock solutions (10 mg/ml) were prepared in distilled water, dissolved by heating to 50°C, filter-sterilized and added to autoclaved media.

For growth on cholesterol, *R. equi* strains were inoculated in 25 ml MM-Ac liquid medium and grown for 24 h at 200 r.p.m. and 30°C. The pre-culture (0.5 ml) was used to inoculate 50 ml of MM medium containing 0.5 g/l cholesterol (Sigma-Aldrich) as sole carbon and energy source which had been finely dispersed by sonication. Regular turbidity (OD_{600nm}) measurements of cholesterol grown cultures were not possible due to high background of the cholesterol suspension. Protein content of the culture was used as a measure for biomass formation and was determined as follows. A sample (0.5 ml) of the culture was pelleted by centrifugation (5 min at 12 000*g*) and thoroughly resuspended in 0.1 ml Bacterial Protein Extraction Reagent (B-PER, Pierce, PerBio Science Nederland B.V., Etten-Leur, The Netherlands). Then, 0.4 ml distilled water

was added and the suspension was vortexed and incubated at room temperature for 5–10 min. An aliquot of 160 µl was mixed with 640 µl of distilled water and 200 µl of protein assay reagent (BioRad) was added. Protein content of the sample was determined using bovine serum albumin (BSA) as a standard as described by the manufacturer.

Cloning, PCR and chromosomal DNA isolation

E. coli DH5α was used as host for all cloning procedures. Restriction enzymes were obtained from Fermentas GmbH (St Leon-Rot, Germany). PCR was performed in a reaction mixture (25 µl) consisting of Tris–HCl (10 mM, pH 8), 10 × High-Fidelity polymerase buffer (Fermentas), dNTPs (0.2 mM), DMSO (2%), PCR primers (10 ng/µl each, Table 1) and High-Fidelity polymerase enzyme (1–2 U, Fermentas). For colony PCR, cell material was mixed with 100 µl of chloroform and 100 µl of 10 mM Tris–HCl pH 8, vortexed vigorously and centrifuged (2 min at 14 000*g*). A sample of the upper water phase (1 µl) was subsequently used as template for PCR. Chromosomal DNA of *R. equi* cell cultures was isolated using the GenElute Bacterial Genomics DNA Kit (Sigma-Aldrich) according to the instructions of the manufacturer.

Electrotransformation of *R. equi* strain RE1

R. equi strain RE1 cells were transformed by electroporation essentially as described (27). Briefly, cell cultures were grown in 50 ml LB at 30°C until OD_{600} reached 0.8–1.0. The cells were pelleted (20 min at 4500*g*) and washed twice with 10% ice-cold glycerol. Pelleted cells were re-suspended in 0.5–1 ml ice-cold 10% glycerol and 200 µl aliquots were put on ice. MilliQ-eluted plasmid DNA (5–10 µl; GenElute Plasmid Miniprep Kit, Sigma-Aldrich) was added to 200 µl cells in 2 mm gapped cuvettes. Electroporation was performed with a single pulse of 12.5 kV/cm, 1000 Ω and 25 µF. Electroporated cells were gently mixed with 1 ml LB medium and allowed to recover for 2 h at 37°C and 200 r.p.m. Aliquots (200 µl) were plated onto LB agar medium containing apramycin (50 µg/ml; Duchefa Biochemie, Haarlem, The Netherlands). Transformants appeared after 2–3 days of incubation at 30°C. The transformation efficiency for non-replicative plasmids integrating via homologous recombination was ~ 10 transformants/µg plasmid DNA.

5-Fluorocytosine positive selection in *R. equi*

R. equi transformants were inoculated in LB liquid medium (25 ml) and grown overnight (20–24 h) at 30°C and 200 r.p.m. 5-FC selection was performed by plating 100 µl aliquots of a dilution series (10^{-1} to 10^{-3} in MM-Ac medium) of the grown culture onto MM-Ac agar plates supplemented with 5-FC (100 µg/ml). Dilution of the culture prior to plating was crucial for effective 5-FC selection. 5-FC resistant colonies appeared after 2–3 days of incubation at 30°C.

Construction of plasmids pSET-Pkan-codA, pSET-Pkan-codAupp and pSelAct

The *aphII* promoter region was amplified from pRESQ (37) using PCR primers Pkan-F and Pkan-E5-R (Table 1). The obtained PCR product of 367 bp was blunt-ligated into *Eco*RV digested pBluescript(II)KS (Stratagene), resulting in pBs-Pkan. A *SalI*/*Not*I restriction released a 431 bp fragment comprising the *aphII* promoter which was then cloned into *SalI*/*Not*I digested pORF-codA::upp (InvivoGen, San Diego, USA), yielding plasmid pORF-Pkan-codAupp. The Pkan-codA::upp cassette was subsequently isolated from pORF-Pkan-codAupp as a 2.4 kb *Sma*I/*Nhe*I fragment and ligated into *Eco*RV/*Xba*I digested pSET152 resulting in plasmid pSET-Pkan-codAupp (Figure 2). The Pkan-codA cassette (1733 bp) was amplified from pSET-Pkan-codAupp using primers Pkan-F and codA-R2 (Table 1) and ligated into *Eco*RV digested pSET152, resulting in pSET-Pkan-codA (Figure 2). Suicide plasmid pSelAct (Figure 3) was constructed by ligating a 2.4 kb Klenow-treated *Eco*RI/*Nhe*I fragment of pORF-Pkan-codAupp into *Ssp*I digested pBs-Apra-ori dephosphoryated with alkaline phosphatase. Plasmid pBs-Apra-ori was constructed from pBluescript(II)KS in which the *bla* cassette was removed with *Bsp*HI, followed by Klenow treatment and replaced by an apramycin-*oriT* cassette obtained as a 1.3 kb *Xba*I fragment (Klenow treated) from pIJ773 (13).

Macrophage survival test

The human monocyte cell line U937 (38) was used to test for survival of *R. equi* strains. The monocytes were grown in RPMI 1640 (Invitrogen) + $NaHCO_3$ (1 g/l) + sodium pyruvate (0.11 g/l) + glucose medium (4.5 g/l) (RPMI 1640 medium), buffered with 10 mM HEPES (Hopax fine chemicals, Taiwan) and supplemented with penicillin (200 IU/ml), streptomycin (200 IU/ml) and 10% fetal bovine serum (FBS). The cells were grown in suspension at 37°C and 5% CO_2. For the macrophage survival assay, monocytes were grown for several days as described above. The culture medium was replaced with fresh culture medium and the cells were activated overnight with phorbol 12-myristate 13-acetate (60 ng/ml, PMA, Sigma-Aldrich) to induce their differentiation to macrophages. The differentiated cells were spun down (5 min at 200*g*) and the pellet was re-suspended in fresh, antibiotic free RPMI 1640 medium with 10% FBS. For each strain to be tested, a tube containing 10 ml of a cell suspension (~10^6 cells/ml) was inoculated with *R. equi*, pre-grown in nutrient broth (Difco, Detroit, MI., USA) at 37°C, at a multiplicity of infection (MOI) of approximately 10 bacteria per macrophage. The bacteria were incubated with the macrophages for 1 h at 37°C and 5% CO_2. The medium was replaced with 10 ml RPMI 1640 medium supplemented with 10% FBS and 100 µg/ml gentamycin and incubated again for 1 h to kill any extra-cellular bacteria. The macrophages (with internalized *R. equi*) were spun down (5 min at 200*g*) and the pellet was re-suspended in 40 ml RPMI 1640 medium, buffered with 10 mM HEPES and supplemented with 10% FBS and gentamycin (10 µg/ml). This suspension was divided over four culture

bottles (10 ml each) and incubated at 37°C and 5% CO_2. After 4, 28, 52 and 76 h the macrophages (one culture bottle per strain) were spun down (5 min at 200*g*) and the pellet washed twice in 1 ml antibiotic free RPMI 1640 medium. Finally the pellet was lysed with 1% Triton X-100 (Sigma-Aldrich) in 0.01 M phosphate buffered saline, followed by live count determination (plate counting).

Culture media and growth conditions for 5-FC selection in actinobacteria

Actinobacterial strains (Table 2) were grown until late exponential phase as shaken liquid cultures in complex medium at 30°C, except for *Amycolatopsis methanolica* and *Mycobacterium smegmatis* which were grown at 37°C. Tryptic Soy Broth (TSB) was used for *Corynebacterium glutamicum*, *Arthrobacter globiformis*, *Amycolatopsis orientalis*, *A. methanolica* and *M. smegmatis*. TSB supplemented with 2.5% NaCl was used for *Salinospora tropica*, and YEME:TSB (1:1) (39) for all *Streptomyces* strains and *Saccharopolyspora erythrea*. LB was used for *R. equi* and *Rhodococcus rhodochrous* and LBP (3) was used for *R. erythropolis* and *R. jostii*. Minimal regeneration medium (MRM) consisted of K_2SO_4 (0.25 g/l), $(NH_4)_2SO_4$ (2 g/l), $MgCl_2{\cdot}6H_20$ (0.6 g/l), 2-[(hydroxyl-1, 1-bis (hydroxymethyl)ethyl)amino] ethanesulfonic acid; TES (5.73 g/l) and trace elements (39). Sucrose (10.3% w/v) and L-proline (0.3% w/v) were added to MRM medium for *Streptomyces* strains, but omitted for all other strains. Glucose (20 mM) was used as carbon and energy source, except for all *Rhodococcus* strains which were grown in the presence of acetate (2 g/l). Autoclaved MRM was supplemented with KH_2PO_4 (0.1 g/l), $CaCl_2{\cdot}2H_20$ (3 g/l) and NaOH (0.28 g/l). For agar plates 2% granulated agar was added. Filter-sterilized thiamine (40 μg/ml) was added to autoclaved MRM medium for growth of *Rhodococcus* strains. 5-FC and 5-FU were freshly prepared as 10 mg/ml stocks in distilled water, dissolved by heating to 50°C and added to autoclaved medium.

RESULTS AND DISCUSSION

CD-UPRT mediated 5-fluorocytosine sensitivity of *R. equi* strain RE1

An obvious prerequisite for the applicability of a 5-FC based conditionally lethal positive selection system is natural resistance of *R. equi* strain RE1 towards 5-FC. To test this, *R. equi* RE1 was streaked onto acetate mineral (MM-Ac) agar plates supplemented with 5-FC or 5-FU and incubated for 3 days at 30°C. Examination of the plates revealed that *R. equi* was resistant to high concentrations of 5-FC (100 μg/ml), but highly sensitive to a lower concentration of 5-FU (50 μg/ml), indicating the feasibility of developing a 5-FC based positive selection system for *R. equi* (Figure 1). Next, we examined whether expression of the *E. coli* genes *codA* and *upp* in *R. equi* would confer sensitivity to 5-FC and thus could act as a suicide marker. The integrative *E. coli-Streptomyces* shuttle vector pSET152 provided a stable and convenient vehicle to introduce *codA* or a functional *codA::upp* fusion into

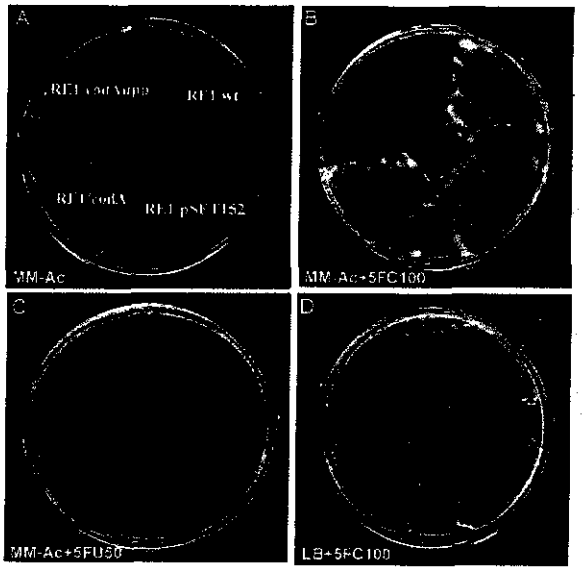

Figure 1. Introduction of *E. coli codA::upp* cassette, encoding CD and UPRT, confers 5-FC sensitivity to *R. equi* strain RE1. Panels show wild type strain RE1, recombinant strain RE1 containing pSET152, recombinant strain RE1 containing plasmid pSET-codA::upp streaked on mineral acetate agar (MM-Ac) containing (**A**) no addition, (**B**) 5-FC (100 μg/ml) or (**C**) 5-FU (50 μg/ml) and on (**D**) LB agar containing 5-FC (100 μg/ml).

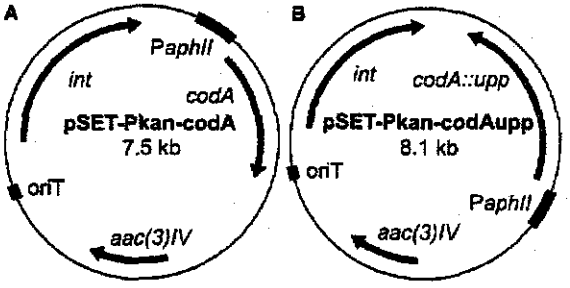

Figure 2. The pSET152 derived integrative plasmids used to introduce the (**A**) *codA* or (**B**) *codA::upp* cassette into *R. equi* RE1, expressed under control of the *aphII* kanamycin resistance cassette promoter (P_{aphII}). The apramycin resistance cassette (*aac(3)IV*), the *Streptomyces* PhiC31 integrase gene (*int*) and the RP4 origin of transfer (*oriT*) are also indicated.

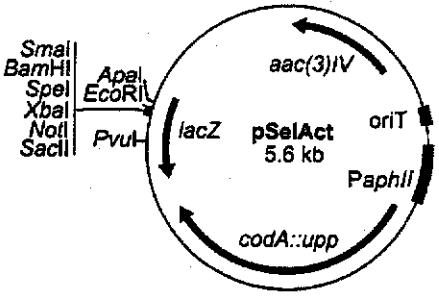

Figure 3. Schematic representation of the non-replicative plasmid pSelAct used to generate unmarked in-frame gene deletions in *R. equi* RE1. Restriction sites indicated are unique.

the *R. equi* genome (40,41). The *aphII* promoter region of the kanamycin resistance cassette (Pkan) was used to drive expression of *codA* or *codA*::*upp*, respectively. The two resulting integrative plasmids, pSET-Pkan-codA and pSET-Pkan-codAupp (Figure 2), were mobilized separately by electroporation to *R. equi* RE1. Transformants harboring either pSET-Pkan-codA or pSET-Pkan-codAupp were streaked onto MM-Ac agar plates (Figure 1A), MM-Ac agar plates supplemented with 100 µg/ml 5-FC (Figure 1B), or MM-Ac agar plates supplemented with 50 µg/ml 5-FU (Figure 1C) and incubated for 3 days at 30°C. The presence of the *codA*::*upp* cassette rendered *R. equi* sensitive to 5-FC, whereas expression of *codA* alone did not result in 5-FC sensitivity (Figure 1B). Growth of the *R. equi* strain carrying the *codA*::*upp* cassette under nonselective conditions was similar to that of wild type (Figure 1A), indicating that expression is conditionally lethal and not detrimental to cells when 5-FC is omitted from the medium. Importantly, no 5-FC selection was observed when complex agar media (LB) was used (Figure 1D). This may be due to repression of the pyrimidine salvage pathway affecting 5-FC uptake.

To enable the generation of unmarked in-frame gene deletions in *R. equi* using CD-UPRT based 5-FC conditional lethality, we next developed a non-replicative suicide vector designated pSelAct (Figure 3; see Materials and Methods section). Vector pSelAct is a small (5.6 kb) conjugative plasmid based on pBlueScript(II)KS, harboring the *aac(3)IV* cassette for apramycin resistance that can be used both in *E. coli* and *R. equi*. The vector contains a MCS with several unique restriction sites for cloning flanking regions of the gene of interest using *lacZ* dependent blue-white screening in *E. coli*.

Unmarked in-frame gene deletion of *supAB* in *R. equi*

A BLAST search with the *supA* (*ro04696*), *supB* (*ro04697*) and *mce4* (*ro04698-ro04703*) genes of *R. jostii* RHA1, predicted to encode the cholesterol uptake machinery (6), revealed that these genes and their apparent operonic organization were highly conserved in the *R. equi* 103S genome (Figure 4; http://www.sanger.ac.uk/Projects/R_equi/). Highest amino acid identities were observed for SupA (94%) and SupB (89%), whereas the *mce4* encoded proteins Mce4A-Mce4F showed lower identities (57–71%) (Figure 4).

The *supA* and *supB* genes are the first two genes of the *mce4* operon, and may encode the cholesterol permease subunits (Figure 4). To demonstrate a specific role of *supAB* in cholesterol catabolism, we aimed for their selective inactivation, without affecting expression of the downstream genes. This prompted us to develop the unmarked in-frame gene deletion system for *R. equi* based on counter-selection with 5-FC. The upstream and downstream flanking regions (~1.5 kb) of the *supAB* genes were amplified by PCR and ligated into pSelAct generating pSelAct-Δ*supAB* (Figure 4; Table 1 for primers). This construct was used to generate a Δ*supAB* mutant, by reducing the wild type *supA* (765 nt) and *supB* (852 nt) genes, encompassing 1637 bp, to a single in-frame open-reading-frame of 231 bp, encoding the first 20 amino acid of SupA and the last 54 amino acids of SupB, separated by a 6 bp *Sma*I restriction site. The first and last part of *supA* and *supB*, respectively, were left intact to ensure proper expression of downstream genes in the *mce4* operon. Introduction of plasmid pSelAct-Δ*supAB* into *R. equi* RE1, selecting for apramycin resistance, resulted in 29 transformants of which four were selected and grown non-selectively overnight for 20 h in LB medium (i.e. lacking apramycin and 5-FC). Aliquots (100 µl) of the overnight cultures were plated in 10^{-1} to 10^{-3} dilutions onto MM-Ac agar plates supplemented with 100 µg/ml 5-FC and incubated for 3 days at 30°C. Confluent growth on 5-FC selection plates was obtained for one of the transformants. Conceivably, the *codA*::*upp* suicide cassette had been inactivated in this transformant by spontaneous mutation or transposon insertion, as has been reported to occur during *sacB* counter-selection (3,42). Typically, $>10^2$ colonies grew on the 10^{-2} plate from which 50 colonies were replica picked onto LB agar with and without apramycin to select for 5-FC^R/Apra^S colonies. The frequency of 5-FC^R/Apra^S colonies amongst the other three transformants varied between 70–90%, indicating that the suicide cassette had been inactivated in 10–30% of the 5-FC^R/Apra^S colonies. For one transformant, eighteen 5-FC^R/Apra^S colonies were checked by colony PCR for the presence of the mutant Δ*supAB* genotype using PCR with oligonucleotides 6 and 7 amplifying the *supAB* genes (Table 1, Figure 4). Two of the eighteen FC^R/Apra^S colonies gave a PCR product of the expected size (231 bp) and were selected for further characterization. Genomic DNA was isolated from these two Δ*supAB* mutants and subjected to PCR analysis of the *supAB* locus and the up- and downstream flanking regions of *supAB* (Figure 5, see Table 1 for oligonucleotides used). This confirmed the presence of a genuine *supAB* gene deletion in both cases and revealed no aberrant genomic rearrangements at the *supAB* locus (Figure 5). One mutant strain was chosen, designated *R. equi* RE1Δ*supAB*, and was used for further characterization.

Phenotypical analysis and functional complementation of *R. equi* RE1ΔsupAB mutant

R. equi RE1 wild type and the RE1Δ*supAB* mutant strain were grown in MM-Ac liquid medium and used to inoculate MM-cholesterol liquid medium. The RE1Δ*supAB* mutant was completely blocked in growth on cholesterol as sole carbon and energy source (Figure 6). Growth on acetate or the steroid substrate 4-androstene-3,17-dione (AD) was unaffected and comparable to the wild type strain (data not shown). This indicated that *supAB* are essential for cholesterol catabolism, probably acting as the permease subunits of the cholesterol ABC transporter. These results are fully consistent with the phenotype of the *supAB* mutant of strain RHA1 (6).

To ensure that the growth deficiency of the RE1Δ*supAB* mutant was solely due to deletion of the *supAB* genes, a 3163-bp DNA fragment carrying the wild-type *supAB* genes was introduced by electroporation into the RE1Δ*supAB* mutant. The DNA fragment was obtained by PCR on strain RE1 wild type DNA

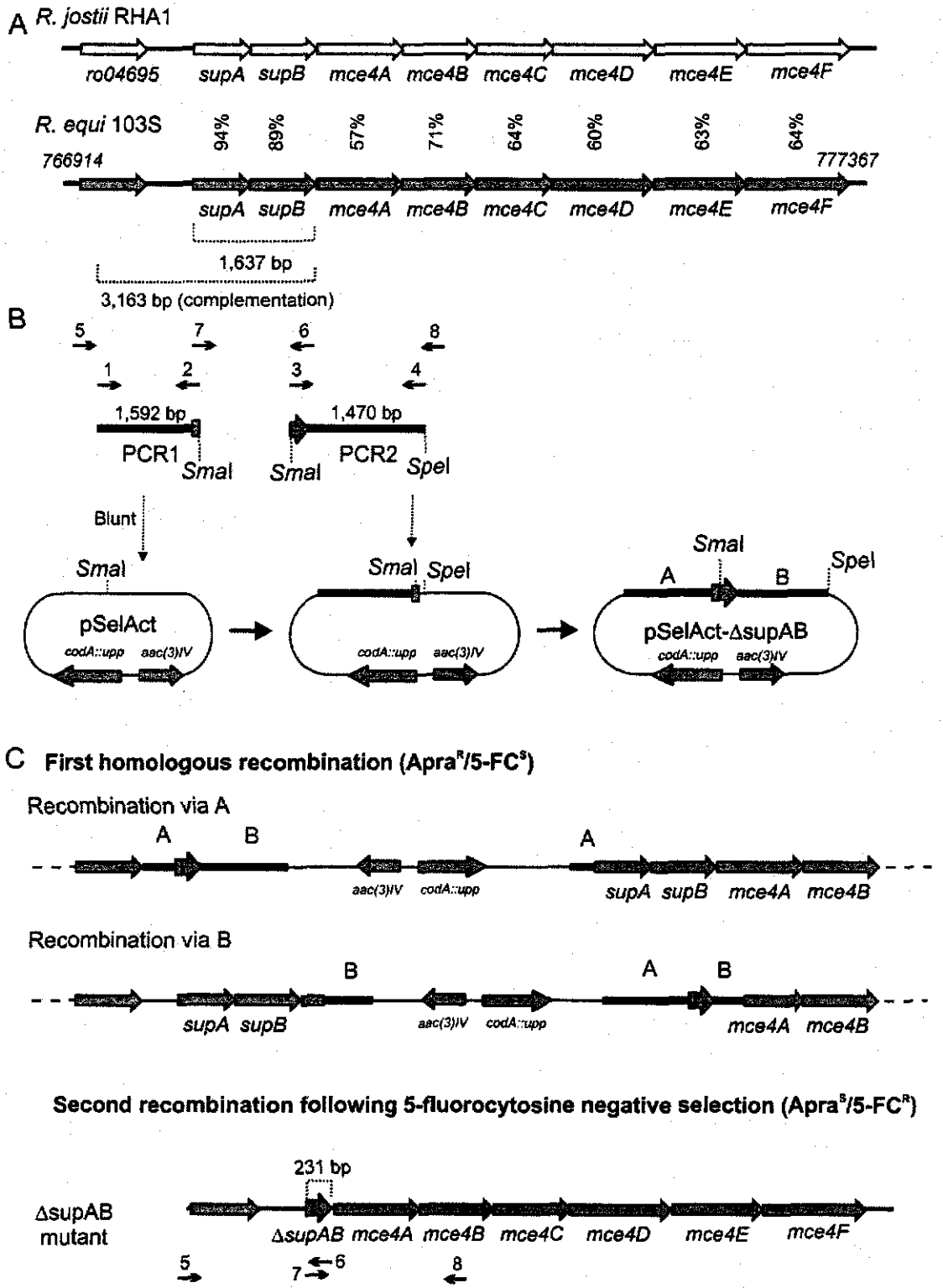

Figure 4. (A) Schematic overview of the molecular organization of the *mce4* gene clusters in *R. jostii* RHA1 and *R. equi* 103S. Percentages indicate amino acid sequence identities between the Mce4 proteins of *R. jostii* RHA1 and *R. equi* RE1. (B) Construction of mutagenic plasmid pSelAct-Δ*supAB* used to generate an unmarked *supAB* gene deletion mutant of *R. equi* RE1. Small black arrows with numbers indicate the PCR oligonucleotides used in this study (Table 1) and the site of their annealing. (C) Molecular organization of the *mce4* locus of *R. equi* RE1 following integration of pSelAct-ΔsupAB by single homologous recombination, and second homologous recombinant event after 5-FC counter-selection, resulting in *supAB* gene deletion.

Table 1. Oligonucleotides used in this study

No	PCR amplicon	Size (bp)	Oligonucleotide sequence (nr.)
1	Upstream region *supAB* (deletion construct)	1592	supABequiUP-F ATCGCGAGGTCAGCTTGGAG (1) supABequiUP-R CCCGGGCGCCGACATCGCGAAGAATC (2)
2	Downstream region *supAB* (deletion construct)	1470	supABequiDOWN-F CCCGGGCTCATCCACACCTACTACGG (3) supABequiDOWN-R ACTAGTGAGCTGCTGAATCTGAACTGG (4)
3	Upstream region *supAB* (confirmation deletion mutant)	1786 (wt: 3192)	supABequiContrUP-F CGGGAGTGCGTAGATGAGTGCA (5) supABequiContr-R TTATCCCGAAAGGTTGAAGTTG (6)
4	Downstream region *supAB* (confirmation deletion mutant)	1570 (wt: 2976)	supABequiContr-F GTGGTCGACCTCCTCGAGGTAC (7) supABequiContrDOWN-R GGACTTGAGCCCGGAGGCATCG (8)
5	*supAB* genes (confirmation deletion mutant)	231 (wt: 1637)	supABequiContr-F GTGGTCGACCTCCTCGAGGTAC (7) supABequiContr-R TTATCCCGAAAGGTTGAAGTTG (6)
6	*supAB* genes (complementation Δ*supAB* mutant)	3163	supABequiUP-F ATCGCGAGGTCAGCTTGGAG (1) supABequiContr-R TTATCCCGAAAGGTTGAAGTTG (6)
7	*aphII* promoter region	367	Pkan-F AGCTTCACGCTGCCGCAAGCACT Pkan-E5-R GATATCATGCGAAACGATCCTCATCCTG
8	*Pkan-codA* cassette	1733	Pkan-F AGCTTCACGCTGCCGCAAGCACT codA-R2 GTCAACGTTTGTAATCGATGGCTTCT
9	*vapA*	408	vapA-F GCAGCAGTGCGATTCTCAATAG (9) vapA-R TAACTCCACCGGACTGGATATG (10)

Primer numbering used in the text is shown between brackets following the nucleotide sequence.

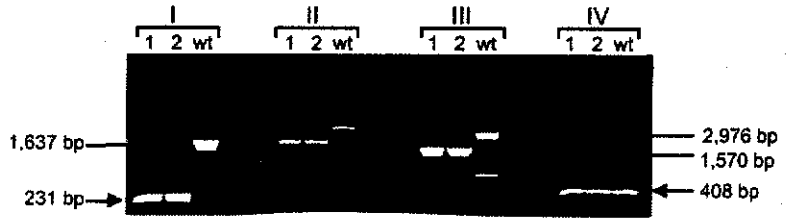

Figure 5. PCR analysis of wild type *R. equi* strain RE1 (wt) and two Δ*supAB* gene deletion mutants of *R. equi* strain RE1 (1 and 2). Panels show amplification of (I) the *supAB* locus [6 + 7], (II) the upstream region of *supAB* [5 + 6], (III) the downstream region of *supAB* [7 + 8], (IV) the *vapA* virulence gene [9 + 10]. The oligonucleotides used are mentioned between brackets and described in Table 1 and/or Figure 4.

using oligonucleotides 1 and 6 (Table 1, Figure 4) and cloned into the integrative plasmid SET152, resulting in pSET-supAB. The cholesterol growth negative phenotype of the RE1Δ*supAB* mutant harboring pSET-supAB was fully complemented, restoring growth on cholesterol to levels comparable to the wild type (Figure 6). We conclude that the *supAB* deletion did not exert any polar effects on the expression of other genes in the *mce4* operon.

These data show that we have developed a novel, simple and efficient method to generate unmarked in-frame gene deletions in *R. equi*, based on 5-FC lethality in the presence of the *E. coli codA*::*upp* fusion gene.

Deletion of *supAB* does not affect *R. equi* RE1 survival in macrophages

Intracellular survival and proliferation of the *R. equi* RE1Δ*supAB* mutant in the human monocyte cell line U937 was compared to those of wild type strain RE1 (Figure 7). The avirulent, plasmid free strain *R. equi* 103^- (43) was included as a negative control for macrophage survival (Figure 7). The results revealed that the RE1Δ*supAB* mutant is able to survive and proliferate in macrophages comparable to the wild-type parent strain RE1. By contrast, the avirulent strain 103^- failed to proliferate, resulting in reduced numbers of intracellular

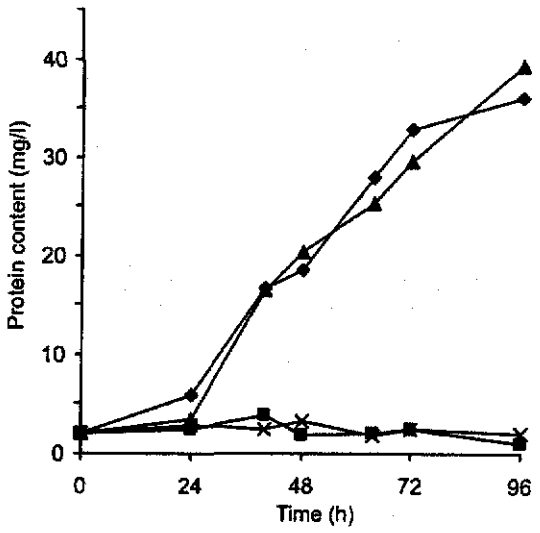

Figure 6. Growth curves in mineral medium supplemented with cholesterol (0.5 g/l) of *R. equi* RE1 wild-type (filled diamond), RE1 Δ*supAB* mutant strain (filled square), RE1 Δ*supAB* + pSET-supAB complemented strain (filled triangle), and RE1 Δ*supAB* + pSET152 control strain (x). Protein content (mg/l) of the culture was used as a measure for biomass formation. The data represent the averages for two independent experiments.

bacteria in time (Figure 7). These results indicate that cholesterol metabolism is not essential for macrophage survival of *R. equi* RE1 and suggest that cholesterol metabolism is not important for virulence of *R. equi* RE1 *in vivo*. These observations are consistent with the finding that ChoE is not important in the virulence of *R. equi* (28).

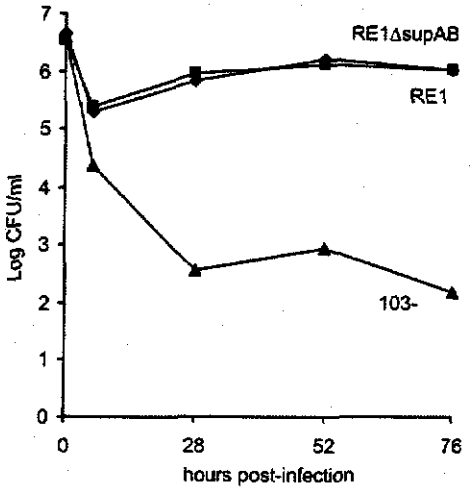

Figure 7. Survival and proliferation of *R. equi* strains in the human monocyte cell line U937. Macrophage cell suspensions were infected with wild type virulent strain *R. equi* RE1 (filled diamond), mutant strain *R. equi* RE1Δ*supAB* (filled square) and non-virulent (control) strain *R. equi* 103⁻ (filled triangle). Following a 1-h incubation to allow phagocytosis, cells were washed and treated with gentamycin to kill remaining extra-cellular bacteria. The numbers of intracellular bacteria were determined by plate counts following macrophage lysis. The data represent the averages for two independent experiments. Plate counts were carried out in duplicate.

Actinobacteria are generally 5-FC resistant

The CD-UPRT based 5-FC selection could also be a useful asset in the generation of unmarked in-frame gene deletions in other actinobacteria. To examine whether CD-UPRT selection potentially is an effective counter-selectable marker in this family of microorganisms, we tested the sensitivity of several actinobacteria towards 5-FC and 5-FU. A wide selection of actinobacterial strains were grown in complex medium until late exponential/early stationary phase and plated in 10-fold dilutions onto mineral selection media containing increasing concentrations of 5-FC or 5-FU (Table 2). The tested strains generally were resistant to high concentrations of 5-FC (100 μg/ml), but highly sensitive to lower concentrations of 5-FU (20–50 μg/ml), indicating the feasibility of developing a 5-FC based positive selection system for other actinobacteria.

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Conflict of interest statement. None declared.

Table 2. Growth of actinobacteria on MRM mineral agar media supplemented with different concentrations of 5-FC or 5-FU

Strain	5-FC (μg/ml)				5-FU (μg/ml)			Reference/origin
	0	20	50	100	20	50	100	
Amycolatopsis methanolica NCIB11946	++	+	–	–	–	–	–	(44)
Amycolatopsis orientalis ATCC 19795	++	++	+	+	–	–	–	ATCC
Arthrobacter globiformis DSM 20124	++	++	+	+	–	–	–	DSMZ
Corynebacterium glutamicum ATCC 13032	++	+	–	–	–	–	–	ATCC
Mycobacterium smegmatis mc^2155	++	+	–	–	–	–	–	(45)
Rhodococcus rhodochrous DSM 43269	++	++	++	++	+	–	–	DSMZ
Rhodococcus erythropolis SQ1	++	++	++	++	–	–	–	(46)
Rhodococcus jostii RHA1	++	++	++	++	+	–	–	M. Fukuda, Nagaoka (Japan)
Rhodococcus equi RE1	++	++	++	++	+	–	–	Schering-Plough, Netherlands
Salinospora tropica	++	++	++	++	–	–	–	DSMZ
Saccharopolyspora erythraea	++	++	++	++	–	–	–	DSMZ
Streptomyces albus J1074	++	++	++	++	–	–	–	(47)
Streptomyces avermitilis MA-4680	++	++	++	++	++	++	++	ATCC (ATCC 31267)
Streptomyces coelicolor M145	++	++	++	++	–	–	–	(39)
Streptomyces griseus DSM40236	++	++	++	++	–	–	–	DSMZ
Streptomyces lividans TK23	++	++	++	++	–	–	–	(39)
Streptomyces scabies ISP5078	++	++	++	+	–	–	–	Wellington, Warwick (UK)
Streptomyces tendae Tü 901/8c	++	++	++	+	+	–	–	(48)

Symbols indicate growth (++), moderate growth (+), slight/minor growth (–) or no growth (–).
ATCC, American Type Culture Collection; DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen.

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BIJLAGE II

Materials and methods

Culture media and growth conditions for R. equi *strain RE1*

R. equi strain RE1 was grown at 30^{0}C (200 rpm) in Luria-Bertani (LB) medium consisting of Bacto-Tryptone (BD), Yeast Extract (BD) and 1% NaCl (Merck), or mineral acetate medium (MM-Ac). MM-Ac contained K_2HPO_4 (4.65 g/l), $NaH_2PO_4 \cdot H_2O$ (1.5 g/l), Na-acetate (2 g/l), NH_4Cl (3 g/l), $MgSO_4 \cdot 7H_2O$ (1 g/l), thiamine (40 mg/l, filter sterile; Sigma), and Vishniac stock solution (1 ml/l). Vishniac stock solution was prepared as follows (modified from Vishniac and Santer, 1957): EDTA (10 g/l) and $ZnSO_4.7H_2O$ (4.4 g/l) were dissolved in distilled water (pH 8 using 2 M KOH). Then, $CaCl_2.2\ H_2O$ (1.47 g/l), $MnCl_2.7\ H_2O$ (1 g/l), $FeSO_4.7\ H_2O$ (1 g/l), $(NH_4)_6\ Mo_7O_{24}.4\ H_2O$ (0.22 g/l), $CuSO_4.5\ H_2O$ (0.315 g/l) and $CoCl_2.6\ H_2O$ (0.32 g/l) were added in that order at pH 6 and finally stored at pH 4. For growth on solid media Bacto-agar (15 g/l; BD) was added. 5-Fluorocytosine (Sigma-Aldrich Chemie, Zwijndrecht, The Netherlands) and 5-fluorouracil (Sigma-Aldrich) stock solutions (10 mg/ml) were prepared in distilled water, dissolved by heating to 50^{0}C, filter-sterilized and added to autoclaved media.

Cloning, PCR and genomic DNA isolation

Escherichia coli DH5α was used as host for all cloning procedures. Restriction enzymes were obtained from Fermentas GmbH (St.Leon-Rot, Germany). PCR was performed in a reaction mixture (25 µl) consisting of Tris-HCl (10 mM, pH 8), 10x High-Fidelity polymerase buffer (Fermentas), dNTPs (0.2 mM), DMSO (2%), PCR primers (10 ng/µl each) and High-Fidelity polymerase enzyme (1-2 U, Fermentas). For colony PCR, cell material was mixed with 100 µl of chloroform and 100 µl of 10 mM Tris-HCl pH 8, vortexed vigorously and centrifuged (2 min, 14,000 x g). A sample of the upper water phase (1 µl) was subsequently used as template for PCR. Chromosomal DNA of *R. equi* cell cultures was isolated using the GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich) according to the instructions of the manufacturer.

Electrotransformation of R. equi *strain RE1*

Cells of *R. equi* strains were transformed by electroporation essentially as described (Navas *et al.*, 2001). Briefly, cell cultures were grown in 50 ml LB at 30^0C until OD_{600} reached 0.8-1.0. The cells were pelleted (20 min at 4,500 x g) and washed twice with 10% ice-cold glycerol. Pelleted cells were re-suspended in 0.5-1 ml ice-cold 10% glycerol and 200 µl aliquots were put on ice. MilliQ-eluted plasmid DNA (5-10 µl; GenElute Plasmid Miniprep Kit, Sigma-Aldrich) was added to 200 µl cells in 2 mm gapped cuvettes. Electroporation was performed with a single pulse of 12.5 kV/cm, 1000Ω and 25 µF. Electroporated cells were gently mixed with 1 ml LB medium and allowed to recover for 2 h at 37^0C and 200 rpm. Aliquots (200 µl) were plated onto LB agar medium containing apramycin (50 µg/ml; Duchefa Biochemie, Haarlem, The Netherlands). Transformants appeared after 2-3 days of incubation at 30^0C.

Unmarked gene deletion in R. equi *strains using 5-fluorocytosine (5-FC) selection*

R. equi transformants obtained from electroporation of wild type or mutant cells were streaked onto LB agar medium supplemented with apramycin to confirm apramycin resistance ($Apra^R$). Four $Apra^R$ transformants per transformation were grown overnight (20-24 h) at 30^0C and 200 rpm in 25 ml LB medium, and plated in 10^{-1}-10^{-3} fold dilutions in MM-Ac medium onto MM-Ac agar plates supplemented with 5-FC (100 µg/ml) in 100 µl aliquots. 5-FC resistant colonies, appearing after 3 days of incubation at 30^0C, were replica streaked onto LB agar and LB agar supplemented with apramycin (50 µg/ml) to select for apramycin sensitive ($Apra^S$) and 5-FC resistant ($5\text{-}FC^R$) colonies. $Apra^S/5\text{-}FC^R$ colonies were checked for the presence of the desired gene deletion by colony PCR using primers amplifying the locus of the gene deletion. Genomic DNA was isolated from potential gene deletion mutants and used to confirm the gene deletion using primers amplifying the *geneAB* or *geneAB2* gene locus, as well as the upstream and downstream regions of these loci with the appropriate primers.

Construction of plasmids for geneAB and geneAB2 gene deletion

Earlier research had demonstrated that the operon that consists of *geneAB* is essential for development of disease in foals. For the generation of an unmarked gene deletion of the *geneAB* operon in *R. equi* RE1 plasmid pSelAct-gene1 (Fig. 1) was constructed as follows. The upstream (1,368 bp; primers geneABequiUP-F and geneABequiUP-

R) and downstream (1,398 bp; primers geneABequiDOWN-F and geneABequiDOWN-R) flanking regions of the *geneAB* genes were amplified by PCR. The obtained amplicons were ligated into *Eco*RV digested pBluescript(II)KS, rendering plasmids pEqui14 and pEqui16 for the upstream and downstream region, respectively. A 1.4 kb *SpeI*/*Eco*RV fragment of pEqui14 was ligated into *SpeI*/*Eco*RV digested pEqui16, generating pEqui18. A 2.9 kb *Eco*RI/*Hind*III fragment of pEqui18, harboring the *geneAB* gene deletion and its flanking regions, was treated with Klenow fragment and ligated into *Sma*I digested pSelAct suicide vector (Fig. 1). The resulting plasmid was designated pSelAct-gene1 for the construction of *geneAB* gene deletion mutant *R. equi* ΔgeneAB (RG1341).

Genome analysis revealed that *R. equi* RE1 harbors additional paralogous genes of *geneA* and *geneB*, designated *geneA2* and *geneB2*, respectively. Double gene deletion mutant *R. equi* ΔgeneABΔgeneAB2 (RG2837) was made by unmarked gene deletion of the *geneAB2* operon in *R. equi* ΔgeneAB mutant strain using plasmid pSelAct-ΔgeneAB2 (Fig. 1). Plasmid pSelAct-ΔgeneAb2 was constructed as follows. The upstream (1,444 bp; primers geneAB2equiUP-F and geneAB2equiUP-R) and downstream (1,387 bp; geneAB2equiDOWN-F, geneAB2equiDOWN-R) regions of *geneAB2* were amplified by PCR using genomic DNA as template.

The amplicons were ligated into *Sma*I digested pSelAct, resulting in plasmids pSelAct-geneAB2equiUP and pSelAct-geneAB2equiDOWN, respectively. Following digestion with *Bgl*II/*Spe*I of both plasmids, a 1,381 bp fragment of pSelAct-geneAB2equiDOWN was ligated into pSelAct-geneAB2equiUP, resulting in pSelAct-ΔgeneAB2 used for the construction of a Δ*geneAB2* gene deletion.

Construction of mutant strains R. equi ΔgeneAB (RG1341) and R. equi ΔgeneABΔgeneAB2 (RG2837)

R. equi unmarked gene deletion mutants of *geneAB* (RG1341) and *geneABgeneAB2* (RG2837) were constructed using a two-step homologous recombination strategy with 5-fluorocytosine counter-selection developed for *R. equi* (Van der Geize *et al.*, 2008).

For construction of the Δ*geneAB* mutant *R. equi* strain RG1341, the non-replicative plasmid pSelAct-gene1 (Fig. 1) was mobilized to *R. equi* strain RE1 by electrotransformation. Four Apra^R transformants, resulting from homologous recombination between plasmid pSelAct-gene1 and the RE1 genome, were

subsequently subjected to 5-FC selection in order to select for the occurrence of the second rare homologous recombination event resulting in gene deletion. Eighteen randomly picked $Apra^S/5FC^R$ colonies were subjected to colony PCR and three $FC^R/Apra^S$ colonies gave an amplicon of the expected size (296 bp). Genomic DNA was isolated from these three Δ*geneAB* mutants and subjected to PCR analysis of the *geneAB* locus and its up- and downstream flanking regions. This analysis confirmed the presence of a genuine *geneAB* gene deletion in two out of three cases and revealed no aberrant genomic rearrangements at the *geneAB* locus. The presence of *vapA* as a marker of the virulence plasmid was confirmed by PCR. One *geneAB* mutant strain was chosen, designated *R. equi* RG1341, and was used for further work.

Double gene deletion mutant strain RG2837 was constructed from strain RG1341 using plasmid pSelAct-ΔgeneAB2 (Fig. 1) essentially as described for the isolation of the Δ*geneAB* single mutant. Four $Apra^R$ transformants, obtained from electroporation of cells of strain RG1341 with pSelAct-ΔgeneAB2, were subjected to 5-FC selection to select for $Apra^S/5\text{-}FC^R$ colonies. Subsequent PCR analysis of eighteen $Apra^S/5\text{-}FC^R$ colonies confirmed that two colonies harbored a Δ*geneAB2* gene deletion, as evident from the obtained 123 bp amplicon using oligonucleotide developed to amplify the *geneAB2* operon. Further analysis of the upstream and downstream regions of the *geneAB2* locus by PCR confirmed the presence of an *geneAB2* gene deletion and revealed no aberrant genomic rearrangements. Also, the presence of the *vapA* virulence gene was confirmed by PCR. One Δ*geneAB*Δ*geneAB2* double gene deletion mutant strain RG2837 was chosen for further work.

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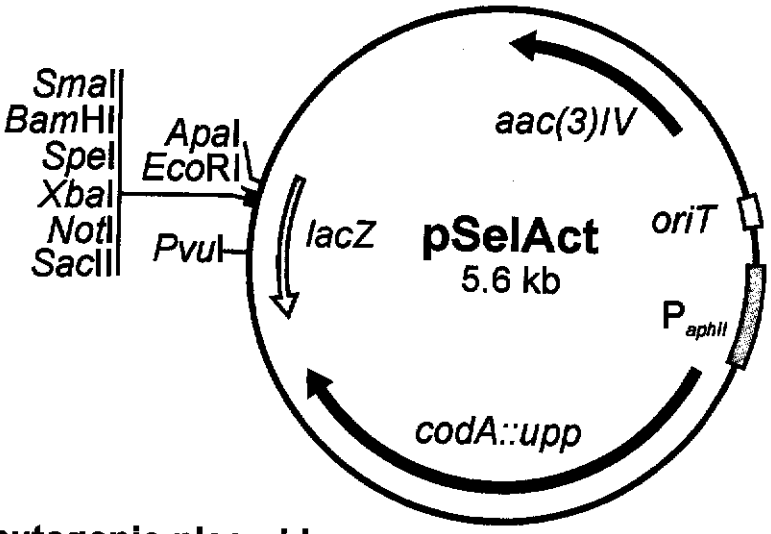
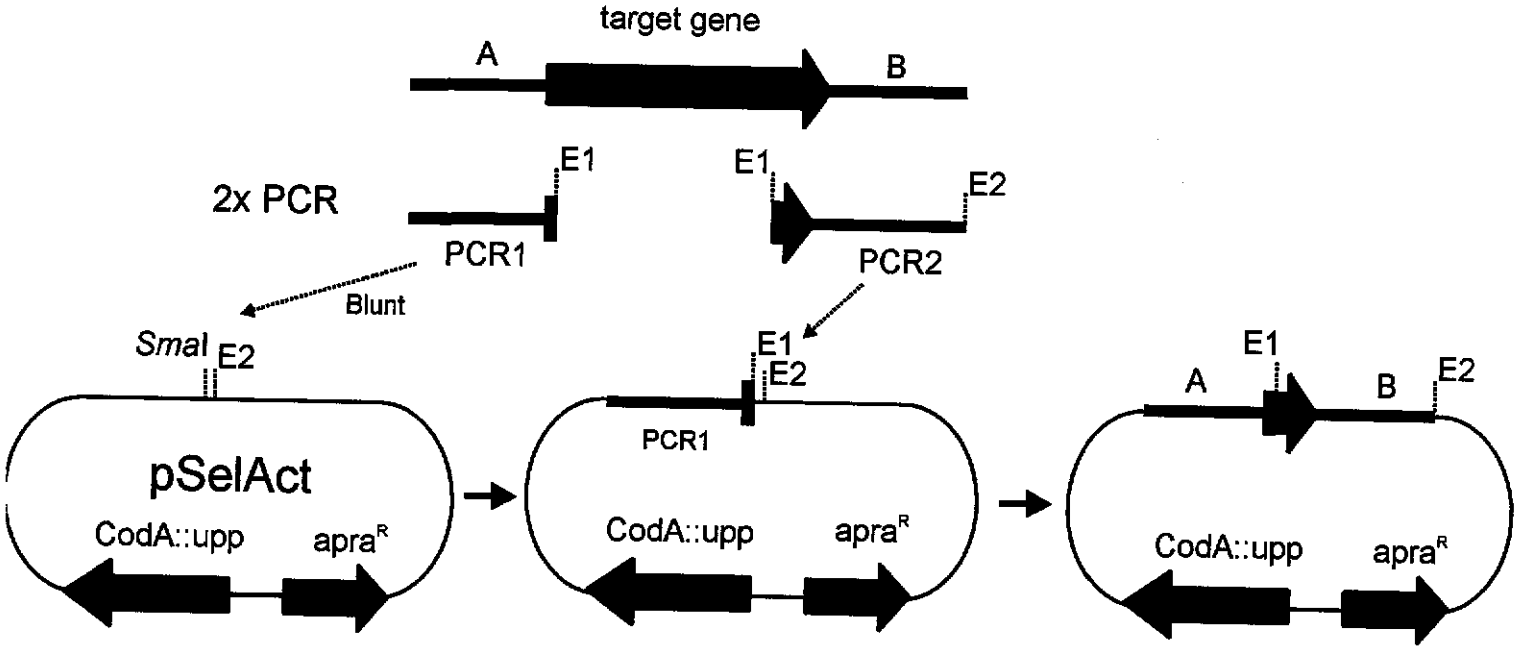
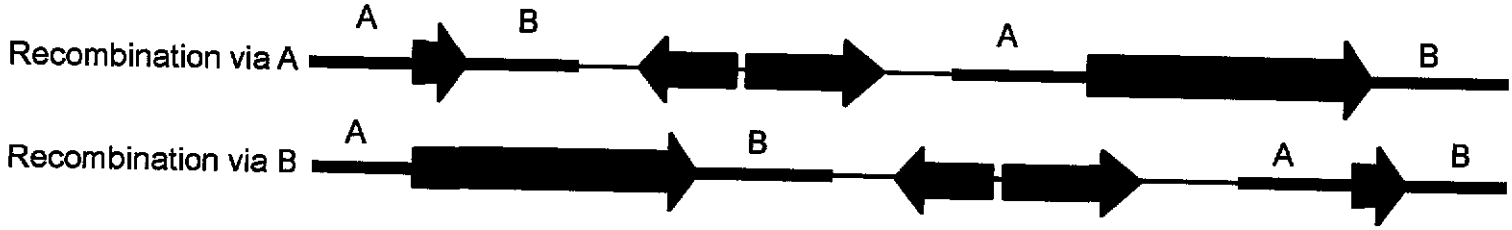


I Construction of mutagenic plasmid

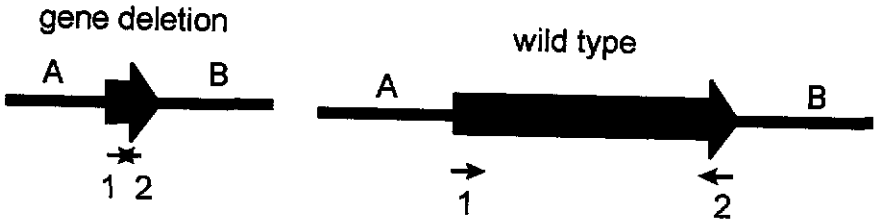


II Transformation: First homologous recombination (Apra^R/5-FC^S)



III Non-selective growth: Second homologous recombination

IV 5-Fluorocytosine negative selection (Apra^S/5-FC^R)



V Check Apra^S/5-FC^R colonies by PCR

BIJLAGE III

INTERVET - BOXMEER
R & D Laboratories

SUBJECT REPORT

Release no. : 08R/0234
Name of project : *Rhodococcus equi vaccine* **(REV)**
Date : 25-09-2008
Pages : 1 - 35

TITLE: IN VITRO AND IN VIVO ATTENUATION OF RHODOCOCCUS EQUI STRAIN RE1Δ*AB* IN COMPARISON WITH THE WILDTYPE PARENT STRAIN RE1

SUMMARY

The objective of this experiment was to test for in vitro and in vivo attenuation of two *Rhodococcus equi AB* deletion mutant(s). The (mutant) strains were tested in the macrophage survival test and one mutant was tested in foals by intratracheal challenge.
In the macrophage survival test the following strains were tested: RE1 wildtype parent strain (positive control), strain 103- which does not contain the 80- to 90 kb virulence plasmid and known to be non-virulent (negative control), strain RE1Δ*AB* (an unmarked in-frame gene deletion mutant of the *A* and *B* genes on the chromosome and strain RE1Δ*ABAB2* (unmarked in-frame gene deletion mutant of the *A, A2, B and B2* genes present on the chromosome. The second copy of the *A* and *B* genes, show ± 50% homology with the *A* and *B* genes.
Survival in macrophages of strain RE1Δ*AB*, RE1Δ*ABAB2* and strain 103-, was clearly reduced (more than 2 logs) if compared to the wildtype parent strain RE1. These results indicate that an intact *AB* gene is essential for survival in macrophages. The results also indicate that the *AB* single or double mutants (in parallel with strain 103- which is apathogenic in foals) might be attenuated in foals as well.
Therefore, strain RE1Δ*AB* was administered intratracheally to foals (using the normal challenge procedure) and compared with the wild type parent strain RE1.
Eight 3 to 7-week-old foals were used for the challenge study. The foals were divided in two groups of 3 and 5 foals that were challenged intratracheally with strain RE1 or RE1Δ*AB*, respectively. During a period of 3 weeks after challenge the horses were clinically evaluated and at 3 weeks after exposure the foals were euthanized and necropsied with special attention to the respiratory tract and associated lymph nodes. Although the dose of the wild type strain RE1 was lower compared to that of the mutant strain, the foals in the RE1 challenge group developed clear signs of pneumonia which was confirmed at post-mortem where severe extensive pyogranulomatous pneumonia was found from which pure cultures of the wild type strain (identity confirmed by PCR) were isolated. In contrast, foals challenged with RE1Δ*AB* had virtually no signs of disease, had no pneumonia at post-mortem and *R. equi* was not isolated except from a slightly enlarged bronchial lymph node of 1 foal and from a healthy piece of lung tissue of another foal where low numbers were isolated. Apparently the foals had not yet completely cleared the mutant.
From the results it can be concluded that strain RE1Δ*AB* is clearly impaired in macrophage survival and is attenuated in foals. Knocking out the second copy of the *AB* gene (resulting in RE1Δ*ABAB2)* did not seem to have an additional effect in the macrophage survival test and probably also not in foals.
The combined in vivo and in vitro results with strain 103- (Takai et al. 2000) and RE1Δ*AB* indicate a good correlation between macrophage survival and horse pathogenicity.
The above results warrant testing of RE1Δ*AB* and/or RE1Δ*ABAB2* as vaccine candidate.

BIJLAGE IV

INTERVET - BOXMEER
R & D Laboratories

SUBJECT REPORT

Release no. : 08R/0247
Name of project : *Rhodococcus equi vaccine* **(REV)**
Date : 07-10-2008
Pages : 1 - 41

TITLE: SAFETY AND EFFICACY IN FOALS OF LIVE ATTENUATED RHODOCOCCUS EQUI STRAIN RE1Δ*AB*, ADMINISTERED VIA THE ORAL ROUTE.

SUMMARY

The objective of this experiment was to test the dose response in foals (with regard to safety and efficacy) of a live attenuated *Rhodococcus equi* strain RE1Δ*AB*, administered by the oral route, against intratracheal challenge with virulent *Rhodococcus equi*. Sixteen 2 to 4-week-old foals were used for the study. The foals were divided in four groups of 4 foals and vaccinated orally with an 1 ml dose containing different amounts of strain RE1Δ*AB*. Group 1 was vaccinated with $5x10^9$ CFU/animal, group 2 was vaccinated with $5x10^8$ CFU/animal; group 3 was vaccinated with $5x10^7$ CFU/animal and group 4 was left as unvaccinated controls. Vaccinations were given at T=0 and at T=2 weeks. At T=4 weeks all foals were challenged intratracheally with virulent *Rhodococcus equi* strain 85F. During a period of 3 weeks after challenge the horses were clinically evaluated. In addition, other parameters were determined as well such as weight gain, blood cell analysis and fibrinogen content. At 3 weeks after challenge (or earlier in case of severe clinical signs) the foals were euthanized and a complete post-mortem examination was performed with special attention to the lungs and respiratory lymph nodes as well as the gut and associated lymph nodes. Tissue samples from all lung lobes were sampled for bacteriological examination and counting. Additional samples for bacteriology and histology were collected from all abnormalities encountered during necropsy.

The post-vaccination results indicate that all three doses of vaccine strain tested are safe in young foals. No indications for adverse effects were found. In addition, after vaccination *R. equi* was not re-isolated from rectal swabs, indicating that the vaccine strain does not result in massive colonization of the alimentary tract (N.B. a selective agar was not available at that time).

After challenge all 4 controls developed severe signs of pyogranulomatous pneumonia caused by *R. equi* as the sole pathogen. Two vaccinates had signs of *Rhodococcus* pneumonia comparable to the controls. All other vaccinates had much milder signs or virtually no signs. Among those less affected foals was one foal which appeared to have pneumonia caused by aspecific bacteria and in fact should be regarded as completely protected, since *Rhodococcus* was not isolated. In addition, from three vaccinated foals with pneumonia, mixed infections were isolated, which negatively influenced the protection level.

Although the protective effect of the vaccine was evident, no dose response effect was observed. Therefore, all vaccinates were combined and compared to the controls for the main parameters using the two-sided Mann-Whitney U test. Despite the small group size of the controls, significant differences were found in daily weight gain, % lung weight per total weight, pneumonia lung score and quantitative re-isolation from the lungs. The clinical scores, evaluated as the highest daily total score occurring between day 7 and 20, was not statistically different between the vaccine and control group. However, more advanced statistical methods such as Generalized Estimating Equations (GEE) for the daily total clinical score over time and heart beat as ordinal score over time, or ANOVA for repeated measurements for rectal temperature, showed a significant effect of the combined vaccine groups on the daily total clinical score, rectal temperature and heart beat, and, even between each separate vaccine group and the control group, except for the mid dose group 2.

In conclusion, all three oral doses ($5x10^7$ CFU, $5x10^8$ CFU and $5x10^9$ CFU) of strain RE1Δ*AB* appeared to be safe for young foals and induced partial protection against a severe intratracheal challenge. No dose-response effect of the tested doses was observed.

BIJLAGE V

INTERVET - BOXMEER
R & D Laboratories

SUBJECT REPORT

Releasenummer : 09R/0227-01
Name of project : Rhodococcus Equi Vaccine **(REV)**
Date : 30-09-2009
Pages : 1 – 29

TITLE: SAFETY, SHEDDING, SPREADING AND DISSEMINATION STUDY OF EQUILIS RHODE ADMINISTERED ORALLY TO FOALS: OVERDOSE FOLLOWED BY A SINGLE DOSE.

SUMMARY

The objective of this experiment was to investigate the safety of Equilis RhodE vaccine strain RG2837 in young susceptible foals after an overdose followed by a single dose. A second objective was to test for shedding, spreading and dissemination of the vaccine strain.
Thirteen foals (age range 2-10 days) were used for the experiment. Eleven foals were vaccinated orally with $2x10^{10}$ CFU of vaccine strain RG2837. Two weeks later the foals were vaccinated with $2x10^{9}$ CFU of vaccine. Age range of treated foals was 2-8 days.Two sentinel foals were placed in contact with two vaccinated foals to test for spreading. After the vaccinations the foals were daily observed for clinical signs. Rectal and nose swabs were regularly sampled to test for shedding (swabs from vaccinated foals) and spreading (swabs from sentinel foals). Two weeks after repeated dose vaccination the treated foals were killed and necropsied. The two contact foals were kept for another week and then necropsied. From all foals swabs were sampled from a gut lymphnode, lung, lung lymphnode, trachea, guttural pouch and liver. In case of any abnormalities, especially those suspected to be of infectious nature, additional swab samples and/or tissue samples were taken for bacteriological and/or histopathological examination, respectively.
From the results it can be concluded that a 10x overdose ($2x10^{10}$ CFU) followed by a normal single dose ($2x10^{9}$ CFU) can be safely administered to foals less than one week of age. No vaccine related abnormalities were observed. In addition vaccinated foals show a transient shedding of low amounts of vaccine strain. The vaccine strain did not spread to contact animals. In addition, the vaccine strain remained in the gut and did not disseminate to internal organs.

Equilis RhodE = RG2837 = double deletion mutant RE1Δ*ABAB2*

BIJLAGE VI

INTERVET - BOXMEER
R & D Laboratories

SUBJECT REPORT

Release nr. : 09R/0226
Name of project : Rhodococcus Equi Vaccine **(REV)**
Date : 01-10-2009
Pages : 1 - 35

TITLE: EFFICACY AND ONSET OF IMMUNITY STUDY OF EQUILIS RHODE IN FOALS AGAINST INTRATRACHEAL CHALLENGE.

SUMMARY

The objective of this experiment was to investigate the efficacy and onset of immunity of Equilis RhodE vaccine in foals after one or two vaccinations (2-week interval). The foals were challenged three or two weeks after (last) vaccination, respectively.
Twenty-four foals (age range 2-9 days) were used for the experiment. Eight foals (group 1) were vaccinated twice orally (2-week interval) with Equilis RhodE containing $1x10^7$ CFU of strain RG2837 per dose. Eight foals (group 2) were vaccinated once and another eight foals (group 3) were left unvaccinated. Two weeks after repeated dose vaccination or three weeks after single dose vaccination the foals were challenged intratracheally with virulent *Rhodococcus equi* strain 85F. During a period of 3 weeks after challenge the foals were clinically evaluated. At 3 weeks after challenge (or earlier in case of severe clinical signs) the foals were euthanized and a complete post-mortem examination was performed with special attention to the lungs and respiratory lymph nodes. From the results it can be concluded that the vaccine, at the tested dose, was safe for 2-9 day-old foals.
After challenge 4/16 vaccinated foals were completely protected whereas none (0/8) of the controls was protected. The reason for the low number of responders remains to be determined and could be related to dose, dose-volume, vaccination route/method, pH of stomach, age, breed or a combination of these factors.
Taking all oral vaccination studies, done so far, together, a 100% correlation between serum titres >5.0 and protection was found which makes future challenge studies redundant.

Equilis RhodE = RG2837 = double deletion mutant RE1Δ*ABAB2*

Bijlage * KOPIE *

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Review article

Rhodococcus equi

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Abstract – *Rhodococcus equi* is an important cause of subacute or chronic abscessating bronchopneumonia of foals up to 3–5 months of age. It shares the lipid-rich cell wall envelope characteristic of the mycolata, including *Mycobacterium tuberculosis*, as well as the ability of pathogenic members of this group to survive within macrophages. The possession of a large virulence plasmid in isolates recovered from pneumonic foals is crucial for virulence. The plasmid contains an 27 kb pathogenicity island (PI) that encodes seven related virulence-associated proteins (Vaps), including the immunodominant surface-expressed protein, VapA. Only PI genes are differentially expressed when the organism is grown in macrophages in vitro. Ten of the PI genes, including six Vap genes, have signal sequences, suggesting that they are exported from the cell to interact with the macrophage. Different PI genes are regulated by temperature, pH, iron, oxidative stress and probably also by magnesium, all environmental changes encountered after environmental *R. equi* are inhaled in dust and are ingested into macrophages in the lung. The basis of pathogenicity of *R. equi* is its ability to multiply in and eventually to destroy alveolar macrophages. Infectivity is largely or exclusively limited to cells of the monocyte-macrophage lineage. Current evidence suggests that infection of foals with virulent *R. equi* results in some foals in subversion of cell-mediated immunity and development of an ineffective and sometimes lethal Th2-based immune response. Significant progress has been made recently in the development of *R. equi-E. coli* shuttle vectors, transformation and random and site specific mutagenesis procedures, all of which will be important in molecular dissection of the mechanisms by which *R. equi* subverts normal macrophage killing mechanisms and cell-mediated immunity.

***Rhodococcus equi* / virulence / cell biology / immunity / genetic tools**

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1. INTRODUCTION

Rhodococcus equi is an important pathogen of young foals aged up to 3–5 months old [53]. Infection causes a subacute or chronic abscessating bronchopneumonia, sometimes with ulcerative typhlocolitis, and may include mesenteric lymphadenitis, osteomyelitis, purulent arthritis, reactive arthritis, and ulcerative lymphangitis. Tuberculosis-like lesions caused by *R. equi* may also occur in the submandibular and other lymph nodes of cattle and pigs and, in young goats, granulomatous lesions in the liver are associated with wasting and death. Infection in other animal species is rare and usually the result of immunosuppression. *Rhodococcus equi* is an important cause of AIDS-associated pneumonia in HIV-infected humans.

The distribution of *R. equi* is highly variable, being endemic on some horse farms and only occuring sporadically or not at all on others. This seems to be due to differences in foal population density, farm management and environmental factors, such as temperature dust and soil pH, as well as the presence of a high proportion of virulent strains among *R. equi* in the soil. Manure is an important reservoir of infection as this pathogen rapidly grows on volatile organic acids contained within it [38]. The majority of cases of *R. equi* infection are diagnosed during dry, warm summers; not only are these conditions optimal for bacterial multiplication but also give rise to a dusty environment causing foals to inhale contaminated dust particles.

The pyogranulomatous lung lesions characteristic of *R. equi* infections reflect its ability to survive in macrophages, a characteristic also of *Mycobacterium tuberculosis*, to which it is closely related. The latter is highlighted by a partial genome sequence of *R. equi* which showed that the majority of *R. equi* genes are most similar to those of *M. tuberculosis* [55]. *R. equi* and *Mycobacterium* species belong to the Mycolata, a phylogenetically distinct group of high G+C Gram-positive bacteria that contains a number of pathogens, including species of the genera *Rhodococcus, Nocardia, Corynebacterium* and *Mycobacterium* [26]. Mycolata are characterised by a unique cell envelope that consists of mycolic acids linked to arabinogalactan wall polysaccharide and (glyco)lipids. The lipoarabinomannan (LAM) of *R. equi* is smaller than mycobacterial LAM, and does not display the extensive branching seen in the latter. But as is the case in mycobacterial LAM, it displays the terminal mannose-containing side chains [22]. The unique cell envelope of the Mycolata is completely different from those of Gram-negative and other Gram-positive bacteria. It forms a permeability barrier to hydrophilic compounds, resulting in the formation of a periplasmic space [9, 13, 63]. Obviously this has consequences for the import of small hydrophilic molecules, which is highlighted by the presence of porins which have been identified in the cell walls of *Mycobacterium, Corynebacterium, Rhodococcus* and *Nocardia* [42, 45, 46, 58]. The mycolic acid containing cell wall is likely to be of importance for survival of these bacteria under harsh enviromental conditions as occur for example within macrophages. It has been shown that *R. equi* is extremely resistant to environmental stress conditions, such as oxidative stress and low pH [6, 7].

2. VIRULENCE PLASMID

Although *R. equi* has been recognised as an important foal pathogen since the early 1920's, it was not until 1991 that the first major breakthroughs in identifying the virulence mechanisms were made. Takai et al. showed that serum of foals infected with *R. equi* invariably has high levels of antibodies directed against a highly immunogenic protein of approximately 15–17 kDa [71]. This Virulence Associated Protein (VapA) is susceptible to trypsin digestion and accessible to biotin labeling, suggesting a surface localization for the protein. In addition, VapA could be radiolabeled with

[9,10-^{3}H] palmitate, showing that the protein is lipid modified, a feature that may serve to anchor the protein to the cell wall [69, 73]. Lipoproteins, which are abundant in Gram-positive bacteria are generally involved in transport of solutes into the cytosol, adherence to surfaces, or have enzymatic activity [64]. The signal sequence of lipoproteins contains a "lipobox", which consists of the amino acid sequence (S/T/G/L/A/V)L(A/S)(G/A)C. The cysteine residue is the first amino acid of the mature protein and is lipid modified. Analysis of the genomic sequence of *Mycobacterium tuberculosis* revealed the presence of 65 putative lipoproteins containing a lipobox, suggesting that mycolic acid containing actinomycetes employ a conventional lipid modification system as found in other bacteria [64]. However, the VapA protein does not contain cysteine nor a lipobox, suggesting that *R. equi* may employ an additional mechanism to lipid modify proteins.

Following the identification of VapA as a virulence associated protein, a second major breakthrough it was shown that the *vapA* gene is located on an indigenous *R. equi* plasmid of approximately 80 to 85 kb [72, 76]. The third major breakthrough was the discovery that *R. equi* strains cured of these plasmids are unable to survive and replicate in macrophages, and are avirulent for foals and mice [23, 35]. Isolates from the submaxillary lymph nodes of infected pigs also contain a large plasmid. However, these plasmids do not encode VapA, but a related protein of 20 kDa (VapB). Interestingly, pig isolates are less virulent in mice than foal isolates harbouring the *vapA* gene, suggesting host specificity of strains harbouring different plasmids [67]. These studies clearly establish that the virulence plasmid is essential for virulence in foals and pigs, and therefore encodes one or more virulence factors. However, *R. equi* isolates from cattle and goats lack a virulence plasmid and human isolates contain either VapA or VapB encoding plasmids or lack a virulence plasmid altogether [21, 65, 70, 75]. This suggests that the pathogenesis of *R. equi* infection in these hosts is different than in foals or pigs.

Nucleotide sequencing of the virulence plasmid of two foal isolates revealed the presence of 69 ORF [68]. Based on comparisons with genes previously identified in other organisms three functional regions of the virulence plasmid can be recognised. Two of these harbour genes that are similar to those encoding proteins involved in conjugation and in plasmid replication, stability and segregation. Interestingly, ORF 34, 38, 39 and 40 of the virulence plasmid are similar to open reading frames found on an indigenous plasmid of *Micrococcus* sp 28 (Accession number AY034092), and are organised in a similar fashion suggesting a common origin. The identification of genes that resemble genes required for conjugation suggests that the virulence plasmid can be mobilised from virulent to avirulent strains. Although this has not yet been demonstrated, it is highly significant as it would provide a mechanism for the virulence plasmid to spread through a population of avirulent strains. The third region of 27.5 kb bears the hallmarks of a pathogenicity island (Fig. 1): it is characterised by a significantly lower G+C content than the rest of the plasmid, and is flanked by genes similar to transposon resolvases. In addition, it harbours the *vapA* gene strengthening the notion that the third region is indeed a pathogenicity island. Analysis of the pathogenicity island resulted in the surprising finding that it encodes six *vapA* homologues (*vapC, D, E, F, G, H*). With exception of *vapF*, which is not functional, all *vap* genes encode proteins with a clear signal sequence, indicating that these are extracellular proteins. Byrne et al. showed that VapC, D and E are indeed secreted, but in contrast to VapA are not anchored to the cell wall [10]. In addition to the six *vap* genes, four more genes encode proteins with a clear signal sequence, bringing the total of extracellular proteins encoded by the pathogenicity island to ten. As extracellular proteins can interact with the host

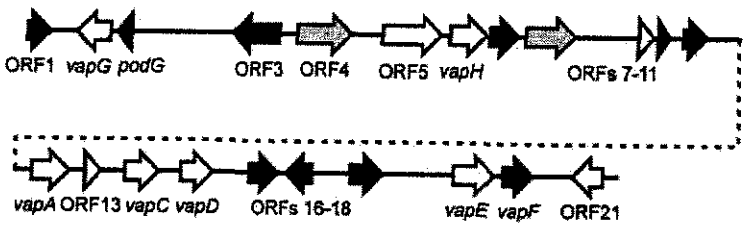

Figure1. Schematic view of the 27.5 kb pathogenicity island of the virulence plasmid of *Rhodococcus equi*. The arrows indicate the position and direction of transcription of the genes located within the pathogenicity island. White arrows indicate that the genes encode proteins with a signal sequence and may therefore be secreted. Grey arrows indicate the two transcriptional regulators encoded by the pathogenicity island. The *podG* gene was identified by Benoit et al. [5] and is not included in the numbering of the open reading frames, which is the original numbering by Takai et al. [68].

environment, these ten proteins could be *important virulence factors*.

Although the discovery that virulence of *R. equi* is dependent on a large plasmid, the subsequent determination and analysis of the nucleotide sequence did not provide the immediately expected insight into the virulence mechanisms of *R. equi*. The reason is that the majority of the proteins encoded within the pathogenicity island do not share any similarity with proteins from other organisms, suggesting that *R. equi* employs a novel virulence mechanism. Two of the proteins that do have homologues in other bacteria are transcriptional regulators.

ORF8 encodes a response regulator, which usually interact with a sensor kinase protein to regulate gene expression. The latter autophosphorylates a conserved histidine residue in response to an environmental signal; the phosphate is transferred to a conserved aspartate residue in the response regulator resulting in transcriptional activation. Although the virulence plasmid encodes a response regulator, it does not encode a sensor kinase, indicating that the ORF8 gene product interacts with a chromosomally encoded sensor kinase protein. The PhoP/PhoQ system is an example of a two-component system in *Salmonella typhimurium* that is induced within the phagosome of the macrophage [2]. PhoQ, an inner membrane sensor kinase, responds to changes in cation concentration and transduces this signal to the cytoplasmic response regulator, PhoP. The latter regulates invasion genes on the pathogenicity island I, SPI-1 [29]. This regulatory system has also been identified in *Yersinia pestis* and was shown to be important for survival under conditions of macrophage-induced stress [51].

ORF4 encodes a protein homologous to the LysR type transcriptional regulators (LTTR). LTTR are, after the two component response regulators, the second largest class of bacterial regulatory proteins [61]. The more than 100 proteins assigned to this family control a wide range of bacterial processes, for example bacterial carbon dioxide fixation (CbbR) and oxygen stress response in pathogens (OxyR). A number of LTTR have been identified which are associated with virulence gene regulation. For example, SpvR encoded by the 90 kb virulence plasmid of *Salmonella typhimurium*, regulates the *spv* operon in response to conditions such as late growth phase [11]. Interestingly, the *spv* operon has been shown to be induced in bacteria within macrophages [57].

Three other genes, ORF 3, 5 and 21, encode proteins with clear homologues in other bacteria. The protein encoded by

ORF21, which has a *M. tubercolosis* homologue (Rv1885c), is a secreted chorismate mutase, an enzyme of the shikimate pathway for biosynthesis of aromatic amino acids. The extracellular location of this protein is enigmatic, as this pathway is usually located inside the cytoplasm. Secreted chorismate mutase proteins have also been encountered in *S. typhimurium*, *Erwinia herbicola*, and *Pseudomonas fluorescens* [12]. The nematode plant pathogen *Meloidogyne javanica* produces an extracellular chorismate mutase that appears to interfere with the shikimate pathway of plants, which is required for plant growth and defense [44]. Interestingly, overexpression of this nematode enzyme in plants alters plant cell development, leading the authors to speculate that it is involved in allowing nematodes to establish a parasitic relationship with the host plant [20]. An alternative possibility is that the protein encoded by ORF21 is not involved in the biosynthesis of aromatic amino acids, but may catalyse a reaction similar to the chorismate mutase reaction using a substrate structurally related to chorismate. The protein encoded by ORF5 is predicted to be an integral membrane protein, displaying a strong similarity to sugar permeases (Pfam 00083). ORF3 encodes an O-methyl-transferase protein, that lacks a signal sequence and is therefore likely to be located in the cytoplasm. Although it has not yet been shown whether these genes are required for virulence, their presence in the pathogenicity island might suggest that virulent *R. equi* either produces a metabolite that is secreted by the cell or that *R. equi* takes up a modified host metabolite. The former could serve to alter the macrophage response, the latter could be involved in signaling.

3. REGULATION OF VIRULENCE PLASMID GENE EXPRESSION

Successful invasion and survival within macrophages are totally dependent on an adequate response to environmental signals. Bacteria therefore employ complex regulatory networks to perceive and integrate environmental signals which ultimately leads to a pattern of gene expression uniquely adapted to meet environmental challenges. The first gene for which it was shown that its expression is indeed controlled by environmental parameters is *vapA*. The expression of this gene was shown to respond to temperature and pH, with maximal expression occuring at high temperature (37 °C) and low pH (6.5) [66, 69]. It was subsequently shown that the *vapA* homologues, *vapC, D, E*, are also thermoregulated in a similar fashion as *vapA* [10]. Benoit et al. demonstrated that *vapA* and *vapG* are upregulated under oxidative stress conditions, by exposing *R. equi* to H_2O_2 [7]. Following uptake of *R. equi* by its host it is subjected to oxidative stress, a decrease in pH, an increase in temperature, signaling that virulence factors must be induced. Some of these factors are discussed under Section 4. Cell biology. The pattern of gene regulation of the *vap* genes is therefore consistent with their presumed role as virulence factors.

Recently Ren and Prescott [56] constructed a DNA microarray to analyse virulence plasmid gene expression. Using this array they identified the concentration of iron and magnesium as additional environmental parameters influencing the expression of genes within the pathogenicity island. Of the genes that were up-regulated by an increase of growth temperature from 30 to 37 °C, twelve genes, including the six *vap* genes were further up-regulated under low-iron conditions, and were down-regulated at decreased magnesium concentrations. A second group displayed the converse regulatory pattern in response to iron and magnesium: ORF 3, 9 and 10 were down-regulated by low-iron and up-regulated by low magnesium concentrations. A key question is whether virulence plasmid genes are transcribed following internalization by macrophages. Using the virulence plasmid DNA array, it was shown that only the genes within the pathogenicity island

were differentially transcribed inside equine macrophages compared to growth of *R. equi* under non-limiting growth conditions at 30 °C. Not surprisingly these included the *vap* genes. Two genes unique to *R. equi* and of unknown function, ORF 9 and 10, were the most abundantly expressed.

These studies show that the regulation of expression of genes within the pathogenicity island is complex, and depends on at least five environmental signals: temperature, pH, oxidative stress, magnesium and iron. It seems very likely that the two transcriptional regulators encoded by ORF4 and ORF8 play an important role in transducing these environmental signals to the transcription apparatus, although this has not yet been established. To date two transcriptional repressors, Fur and IdeR, controlling iron dependent gene expression have been identified [31]. Although these proteins are unrelated, they operate in a similar manner. Both bind to their cognate binding sites in the presence of Fe^{2+}, and repress transcription. In the absence of Fe^{2+}, the proteins release from their binding sites and repression is alleviated. An *ideR* homologue of *R. equi* was recently cloned and over-expressed in *E. coli*. It was shown that the *R. equi* IdeR protein is functional; it binds to a consensus IdeR recognition sequence and regulates gene expression in an Fe^{2+} dependent manner [8]. Interestingly the promoter region of the *vapA* gene contains an IdeR consensus binding site [56], suggesting that the iron dependent expression of this gene may be controlled by IdeR.

4. CELL BIOLOGY

The basis of pathogenicity of *R. equi* is its ability to multiply in and eventually to destroy alveolar macrophages. Infectivity is largely or exclusively limited to cells of the monocyte-macrophage lineage, whereas neutrophils from foals are fully bactericidal. How virulent *R. equi* survive and replicate in macrophages offers fertile ground for future study.

Since opsonisation of *R. equi* with specific antibody is followed by increased phagosome-lysosome fusion and by significantly enhanced *R. equi* killing by equine macrophages [32, 79], macrophage entry through non-Fc receptors may be important in determining the fate of the bacteria. The general limitation of the disease in foals to the period between 4 and 12 weeks after birth coincides with the time of major decline of maternally-derived antibodies, supporting the concept that entry of *R. equi* into macrophages by non-Fc receptors may be important in allowing it to avoid antibody-associated macrophage killing pathways. The mechanisms of bacterial ingestion vary with the phagocyte receptor involved, which in turn also affects activation of the macrophage.

Complement receptor (CR)-mediated phagocytosis of bacteria involves several macrophage receptors (CR1, CR3, CR4) which bind one or more of C3b, C3bi, and C4b deposited on the bacterial surface. Whereas in antibody (FcR)-mediated phagocytosis, pseudopodia tightly engulf opsonized bacteria before drawing them into the macrophage, in CR-mediated phagocytosis, opsonized bacteria sink into the macrophage without marked pseudopodia [1]. FcR-induced phagocytosis is strongly associated with production of reactive oxygen intermediates (oxidative burst) and pro-inflammatory mediators, whereas CR-mediated phagocytosis does not induce this effect [1]. The effect of the phagocyte receptor involved in mediating differences in internalization mechanisms is illustrated also by the mannose receptor (MR), which recognizes mannose and fucose on bacterial surfaces. Unlike FcR and CR-mediated phagocytosis, MR-mediated phagocytosis is not associated with accumulation of the cytoskeletal proteins vinculin and paxillin around the phagosomes [1].

Optimal binding of *R. equi* to macrophages in vitro requires complement and is

mediated by Mac-1, a leukocyte complement receptor type 3 (CR3) [35]. The lipoarabinomannam of *R. equi* (ReqLAM) binds to recombinant mannose-binding protein, which may activate complement C3b deposition onto *R. equi* via the lectin pathway, and thus may also promote Mac-1 mediated uptake into macrophages [22]. ReqLAM may bind other collectins, since *M. tuberculosis* lipoarabinomannam is a ligand for human pulmonary surfactant protein A, and equine surfactant proteins bind mannose [22]. In addition, entry to macrophages through ReqLAM binding to the mannose receptor may occur.

Once bacteria are internalized, the phagosome undergoes a series of fusion and fission events with vesicles from the endocytic pathway, a complex maturation process which leads to the formation of phagolysosomes. The usual progressive movement of the phagosome on microtubules into the cell during maturation allows the phagosome to interact with the endosomal system. During maturation, the phagosome membrane increasingly changes to resemble that of late endosomes and lysosomes. This follows a regulated and sequential pattern in which phagosomes change from ready fusion with both early and late endosomes, to fusion most readily with lysosomes, and finally to failure to fuse with any endocytic organelle [74]. The evidence supports phagosome-endosome fusion as occurring as brief exchanges ("kiss-and-run") of content and membranes [18, 19]. This maturation process is characterized by changes in the molecules associated with the phagosomal membrane from those of early endosomes (Rab5, annexin, NSF, SNAP [soluble NSF-attachment proteins], transferrin receptor, mannose receptor) to those of late endosomes and lysosomes (Rab5, Rab7, mannose-6-phosphate receptor, cathepsin D, LAMP1 [lysosome-associated membrane-protein1], LAMP2). One or more proteins involved in budding from endosomal vesicles and their fusion with the phagosome may be important targets for *R. equi* but this requires to be investigated.

The presence of transferrin in early endosomes likely explains the importance of iron restriction in regulating transcription of some virulence plasmid pathogenicity island genes. NRAMP1 (natural-resistance-associated macrophage protein 1) confers innate resistance to macrophages against the growth of certain intracellular pathogens [60]. A divalent cation transporter that shows preference for iron or manganese, and pumps iron into the phagosome [80], NRAMP1 is associated with efficient acidification of the phagocytic vacuole, although details of its mechanism of action are unclear [60]. Once inside the phagosome, iron stimulates formation of biotoxic reactive oxidative species via the Fenton/Haber-Weiss reactions [52]. The limited quantities of iron transported into the phagosome by NRAMP1 to catalyze the generation of antimicrobial oxidative radicals do not increase iron availability for bacteria since iron in the phagosome is removed during the process of nitric oxide (NO) formation. Interestingly, dramatic down-regulation of NRAMP1 transcription was observed in equine macrophages following infection with *R. equi* [77], but whether this is different between plasmid positive and negative strains was not explored. In *M. tuberculosis*, IdeR controls the transcription of genes involved in macrophage survival, as well as in iron acquisition and iron storage [25].

Maturation of the phagosome is also characterized by progressive acidification with delivery of the vacuolar proton pump from early and late endosomes, and removal of the Na^+/K^+-ATPase from the late endosome, so that the vacuolar pump works unopposed in the phagolysosome (pH $\leq$ 5.5) [15]. For bacteria which do not interfere with the process, phagocytosis to phagolysosome formation takes about five minutes [49]. Phagocytosed bacteria are degraded in the late phagolysosome by the same mechanisms employed by lysosomes, involving a wide range of acid-resistant hydrolases within the acid environment of the phagolysosome. Materials may also be transported for further degradation from the

phagolysosome to late endosomes or lysosomes.

Intramacrophage *R. equi* appear to be located exclusively within membrane-enclosed vacuoles and persistence correlates with the absence of phagosome-lysosome fusion [32, 79]. It has been shown that plasmid-encoded products contribute to the ability of *R. equi* to survive and replicate in macrophages. Preliminary observations by Haas et al. [30] suggest that maturation of the phagosome is more efficiently diverted in strains possessing the virulence plasmid compared to plasmid-negative isogenic strains that do not, and that cytotoxicity of *R. equi* for J774E murine macrophages is strongly up-regulated by the possession of the virulence plasmid.

Although details are sparse, *R. equi* may therefore behave like *M. tuberculosis* and related mycobacteria which interfere with phagosome maturation, residing in a vacuole which is only mildly acid (pH 6.5) and which resists lysosomal fusion [3, 16]. *M. tuberculosis* interferes with the acquisition of the fusion facilitating molecules, either by avoiding loss of Rab5 and/or early endosome SNARES (soluble N-ethylmaleimide-sensitive fusion factor attachment protein receptor), or by blocking acquisition of other facilitating molecules (such as Rab7) [16, 58]. The specific lack of the vacuolar proton pump [59] appears to be responsible for the mildly acid conditions. Inhibition of acidification blocks vesicular delivery to lysosomes [14], and the pH of the phagosome is probably important in controlling fusion events. pH in the phagosomes containing mycobacteria is maintained mildly acidic, possibly by persistence of the Na^+/K^+-ATPase or by ammonia production within the phagosome. Ammonia, which can be produced in several ways by *M. tuberculosis* inhibits phagolysosome formation [27]. Whether the prominent urease of *R. equi* has a similar role is unknown.

Besides lysosomal degradation, macrophages can inhibit or kill bacteria by producing toxic reactive oxygen and nitrogen species through the respiratory burst phagocyte oxidase and inducible nitric oxide synthase (iNOS). As noted earlier, *R. equi* may avoid the oxidative burst through its mode of entry into macrophages. Macrophage production of toxic superoxides from the relatively inert oxygen molecule involves production by a membrane-bound NADPH oxidase that is activated in the "respiratory burst" which occurs when opsonized bacteria initiate the phagocytosis process. Nitric oxide synthetases, especially iNOS, catalyse the oxidation of a guanidino nitrogen of L-arginine to NO. iNOS is usually transcribed in response to cytokines (IFN-γ, IL-1, TNF-α) released in response to microbial products or through immune cell interactions. Both IFN-γ and TNF-α are required for clearance of virulent *R. equi* from mice [43]. Besides sometimes synergizing with the oxidative burst to produce peroxynitrite, NO and its derivatives have a longer lasting bacteriostatic effect on bacteria in the phagosome. Killing of *R. equi* by murine macrophages was shown to be dependent on IFN-γ, which activated macrophages to produce reactive nitrogen and oxygen intermediates which in turn combined to peroxynitrite ($ONOO^-$), the essential bactericidal factor [17]. IFN-γ activation of macrophages prevented growth of *R. equi*, in contrast to their replication in non-IFN-γ activated macrophages, consistent with the role of IFN-γ in activating the high-output iNOS [17]. The study suggested a two-step model for efficient killing of *R. equi* in immune animals [17]. The first step, macrophage activation by IFN-γ and TNF-α results in nitric oxide production following iNOS transcription, and the second step, signalled by bacterial phagocytosis, results in the respiratory burst and the production of, and killing by, $ONOO^-$ produced by reaction between nitrogen and oxygen intermediates.

For *M. tuberculosis* and possibly for *R. equi*, once macrophages are activated by IFN-γ, these cells overcome the block that

the bacterium imposes on endosomal maturation. *R. equi* is highly resistant to hydrogen peroxide, an oxidative stress which reflects the production of reactive oxygen intermediates effect during the oxidative burst of phagocytes following bacterial phagocytosis [7]. This resistance may result from its catalase activity. Although resistance to hydrogen peroxide is not a function of the virulence plasmid, treatment with hydrogen peroxide resulted in enhanced *vapA* and *vapG* transcription [7], supporting a role for VapA and VapG in survival of *R. equi* under the oxidative stress imposed by macrophages.

5. IMMUNITY

Current evidence suggests that infection of foals with virulent *R. equi* may result in subversion of cell-mediated immunity and development of an ineffective and sometimes lethal immune response. Although details are not clear, the pathogenicity island on the virulence plasmid must play a crucial role in this immunomodulatory process.

Immunity to *R. equi* pneumonia in foals likely depends on both the antibody and cell-mediated components of the immune system but its exact basis remains to be determined. Antibodies to *R. equi* are widespread in horses. Evidence for a role of antibody in protection against *R. equi* is the protective effect of passively transferred anti-*R. equi* hyperimmune equine plasma which is used extensively on endemically affected farms to reduce morbidity and mortality. Hooper-McGrevy et al. [36] showed that purified VapA- and VapC-antibody provided protection of foals against experimental pneumonic infection equivalent to that obtained with hyperimmune plasma. Interestingly, however, foals born to vaccinated mares are not protected against infection despite passive transfer of *R. equi* antibody. This failure may relate to the isotype of antibody produced by the vaccines used experimentally but this has not been investigated in detail.

Because of the facultative intracellular nature of *R. equi*, cell-mediated immune mechanisms are thought to be of major importance in resistance. Key studies by Kanaly et al. [39–41] have confirmed this in mice. For example, immunocompetent BALB/c mice experimentally infected with virulent *R. equi* developed a Th1 cytokine response and progressively cleared the infection. Mice in which a Th2 response was induced by administration of monoclonal antibodies against IFN-γ, failed to clear the infection and developed pulmonary granulomas. The two major mechanisms by which T lymphocytes mediate clearance of intracellular pathogens are secretion of cytokines and direct cytotoxicity (usually mediated by MHC class I restricted $CD8^+$ T lymphocytes). Kanaly et al. [39] conclusively demonstrated the central role of $CD4^+$ T-cells since MHC class I transgenic mice deficient in $CD8^+$ T-lymphocytes cleared virulent *R. equi* from the lungs whereas infection persisted in MHC class II transgenic mice deficient in $CD4^+$ T-lymphocytes and led to the formation of granulomas.

It is apparent that infection of foals by virulent *R. equi* can result in an immunomodulatory effect which drives an ineffective, Th2-like, rather than an effective, Th1-like, response. For example, Giguère et al. [24] found that foals experimentally infected with a virulent *R. equi* strain showed marked reduction in IFN-γ production by $CD4^+$ lymphocytes isolated from bronchial lymph nodes compared to $CD4^+$ lymphocytes similarly isolated from foals infected with an avirulent, plasmid-cured derivative of the same strain and that concentrations of IL-10, a Th1-immune response down-regulatory cytokine, were significantly higher in the lungs of foals infected with the virulent strain. The conclusion was that virulent *R. equi* have an immunomodulating effect which drives an ineffective, Th2-like, immune response. Interestingly, Hines et al. [34] observed that clearance of virulent *R. equi* from the lung of experimentally-infected adult horses was associated

with increased numbers of IFN-γ producing $CD4^+$ and $CD8^+$ lymphocytes. This difference between immune adults and non-immune foals may reflect unique features of the foal's immune system, differences between immune and non-immune animals, or differences in the experimental procedures.

Hooper-McGrevy et al. [37] observed an IgGb- and IgGT-dominant response to Vap proteins in the serum of foals sick with *R. equi* compared to healthy, immune, foals and to adults, both of which had an IgGa-dominant response. This finding was interpreted as supporting the concept that foals that become sick with *R. equi* do so because of an ineffective, Th2-dominated, immune response and that this is the reason for the IgGb and IgGT dominant response in these foals. Because of the reciprocal relationship between these two types of immune response, or perhaps also because of an interference effect of IgGT on complement activation by IgGa, immunization with VapA in an adjuvant which drives a Th2-response would be expected to result in development of disease. In support of this concept, immunization of foals under natural settings with a VapA extract in aluminium hydroxide adjuvant resulted in development of pneumonia in immunized but not in nonimmunized foals, and a IgGb- and IgGT-dominated isotype response compared to an IgGa response in immune foals [54].

Adult immune horses showed marked lymphoproliferative responses to recombinant VapA following intrabronchial challenge with virulent *R. equi* [33], as well as a marked $CD4^+$ and $CD8^+$ T-lymphocyte response, suggesting that both subsets play a role in clearance of infection from the lung, reinforcing also earlier conclusions of the importance of VapA as an immunodominant antigen. Stimulated cells showed significant increases in antigen-specific IFN-γ but not in IL-4 expression, and local levels of IgGa and IgGb isotypes against VapA were dramatically enhanced after challenge, suggesting that these are important correlates of protective immunity. Interestingly, IgGa and IgGb fix complement whereas IgGT does not, and indeed IgGT may inhibit complement fixation by IgGa and IgGb [47].

6. DEVELOPMENT OF GENETIC TOOLS

Although considerable progress has been made in understanding important aspects of virulence of *R. equi*, there is an almost complete lack of genetic tools for this bacterium that will be required to make further progress. Recently, this problem has been addressed, and significant progress has been made in the development of *R. equi–E. coli* shuttle vectors, transformation and random and site specific mutagenesis procedures. To date three plasmids have been described that efficiently replicate in *R. equi*. As these are based on different replicons, they are compatible, allowing stable maintenance of these plasmids in the same cell. pRE7 is a shuttle vector based on the origin of replication of the *R. equi* virulence plasmid, and is therefore not maintained in virulent *R. equi* strains [78]. A shuttle vector based on an indigenous plasmid from the plant pathogen *R. fascians* containing a chlorampenicol resistance marker was shown to efficiently replicate in *R. equi* [62]. Plasmids containing an origin based on the pAL5000 plasmid of *Mycobacterium fortuitum* subsp. *fortuitum* were also shown to replicate in *R. equi* [23]. All plasmids are introduced into *R. equi* by electroporation [62, 78], however, conjugation using the *Escherichia coli* strain S17-1 has also been shown to be an efficient method to introduce plasmids (Boland and Meijer, unpublished results).

An essential technique to identify virulence factors is random mutagenesis, in which a selectable marker is randomly integrated into the genome. A mutagenesis system based on the insertion of a protein-DNA complex of transposase and a DNA fragment harbouring a kanamycin resistance gene flanked by transposase binding

sites [28] was shown to integrate efficiently and randomly into the genome of *R. equi* [48]. Transposome mutagenesis therefore allows the generation of mutant libraries which can be screened for virulence. This approach led to the isolation of an *R. equi* mutant defective in haem utilisation (Graham and Meijer, unpublished results). Recently, a random mutagenesis system for *R. equi* based on the Himar1 transposon, which belongs to the Mariner family of transposable elements, was described. This highly efficient system was used to isolate an attenuated mutant of *R. equi*, underscoring the usefulness of transposon mutagenesis [4]. Navas et al. [50] developed a highly efficient system based on homologous recombination to disrupt the *choE* gene encoding cholesterol oxidase of *R. equi*. The development of random and site directed mutagenesis procedures for *R. equi* was a major development which will facilitate a detailed analysis of *R. equi* virulence.

7. CONCLUSION

The last decade has seen a number of important breakthroughs in understanding the mechanisms used by *R. equi* to allow it to proliferate in the host. Current work is focusing on identifying the molecular basis of how *R. equi* subverts the macrophage and subverts the foal's immune response, as well as on how to develop an effective vaccine for foals based on forcing a Th1-like immune response to Vap proteins.

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Havemeyer Workshop Report

Rhodococcus equi comes of age

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Introduction

An international Workshop on *Rhodococcus equi*, an important bacterial pathogen of foals and of immunocompromised human subjects, was held in Edinburgh 13–16th July 2008. Funded by the Havemeyer Foundation as the 4th in a series held over the last 21 years, the Workshop celebrated the determination of the complete genome sequence of *R. equi*. Among the 80 scientists from 14 countries were epidemiologists, immunologists, microbiologists, molecular biologists and veterinary clinicians, providing a broad-ranging forum for discussion of how to control this unusual pathogen now that this field is entering the post genomic era.

The Workshop begun with a Plenary Lecture by Shinji Takai (Kitasato University, Japan) on the virulence plasmids of *R. equi*. These plasmids carry a pathogenicity island (the *vap* PAI) that is essential for virulence. The virulence factors encoded in this PAI, called Vap proteins, are up-regulated by the temperature and acidity found in the macrophage vacuole where the pathogen survives and replicates within the host. Speculatively, the virulence plasmid may have evolved to resist killing by the intestinal protozoa, which share many of the phagocytic and killing processes of the macrophages in which virulent *R. equi* thrive.

The *Rhodococcus equi* genome: versatile and adaptable

Michal Letek (University of Edinburgh) gave an overview of the features of the first *R. equi* strain to be sequenced, 103S, a genetically-manipulatable isolate from a foal with bronchopneumonia. The sequencing project was an international collaborative venture coordinated by José Vazquez-Boland (initially University of Bristol, since 2008 University of Edinburgh) and co-funded by Ireland's National Development Plan through the Irish Equine Centre and grants from the Horserace Betting Levy Board (UK) and the Grayson-Jockey Club Research Foundation (USA). Gap closure of this GC-rich bacterium occupied a year following 99.97% genome coverage by shotgun sequencing at the Sanger Centre, with manual annotation completed by a team of 14 scientists from 6 institutions from 4 countries (UK, Ireland, Canada and Spain) in June 2008.

The genome is just over 5 million base pairs and encodes 4594 genes of average length 1008 base pairs. It has a relatively large number of regulatory genes including 21 sigma factors, more typical of a versatile saprophytic bacterium such as *Pseudomonas aeruginosa* than of a host-adapted pathogen such as *Mycobacterium tuberculosis*. Interestingly, 25% of the genome involves surface proteins, and genes involved with lipid metabolism are extensively represented, in contrast to the absence of genes involved in transport of sugars. The comparative analysis of the genome showed *Rhodococcus jostii* (RHA1), the only other *Rhodococcus* sp. sequenced to date, to be the most closely related species. It also confirmed the close phylogenetic relatedness with *M. tuberculosis*, which is one reason for the increasing interest in *R. equi*. The overall features of the organism appear to be versatility and capacity to adapt to environments as diverse as soil and the mammalian body.

Delegates at the fourth Havemeyer Workshop in Edinburgh, July 2008.

An *R. equi* microarray has been developed by the Edinburgh team based on the genome sequence and this resource will be very useful to gain insight into *R. equi* biology and virulence mechanisms.

Technical advances in the genetic analysis of *R. equi*

Robert van der Geize (University of Groningen) reported that the *R. equi* genome was unusually rich in steroid degradation genes, which may be a reflection of use of plant derived steroids during saprophytic growth of this soil organism. He described the development of a counter-selectable marker based on 5-fluorocytosine for use in suicide-vectored unmarked in-frame gene deletion in *R. equi*, a breakthrough warmly greeted by Workshop participants, several of whom had struggled to develop counter-selectable markers. Both he and Raul Miranda Casoluengo (University College Dublin) who discussed a novel IupIR iron-regulated signal transduction system, recognised the value of use of the genome to assess novel drugs in *R. equi* as a surrogate for *M. tuberculosis*. Mary Hondalus (University of Georgia) described

how a ϕc31 phage-based integrative plasmid could mobilise genes into *R. equi*. This was another novel molecular tool welcomed by the Workshop for its value in mutant complementation or production of novel bacterial and potential vaccine constructs.

Pathogenesis

In her overview of *R. equi* pathogenesis, Mary Hondalus (University of Georgia, USA) suggested that one advantage of working with *R. equi* as a pathogen was that foals, the natural host, could be used. Since foals often behave like immunocompromised human subjects in relation to *R. equi* infection, she promoted the use of immunocompromised (SCID) rather than immunocompetent mice in assessing the role of different genes in the virulence of the organism.

Neil Stoker (Royal Veterinary College, London) enlightened the audience with his experience on microarray-based analysis as applied to understanding *M. tuberculosis* pathogenesis. He warned of the difficulty and care required to obtain high quality, reproducible data, the difficulties of their use in defining genes regulated by 2-component regulatory systems (TCS), the dangers of comparison of gene transcription to broth cultures, and the need to take a variety of approaches in analysis of data. Microarrays rarely yield clear or complete data and it is therefore usually necessary to correlate results with data obtained in other ways, such as protein expression.

Wim Meijer (University College Dublin) described his laboratory's work to determine how the *vap* PAI worked and, in particular, the synergism between its multiple components. Three operons had been identified on the PAI: *virR* (ORF 4-8) that controls *vapA*, ORF9 (ORF9-11) and *vapA* (ORF12-14). The mechanism of expressional crosstalk, the dynamics of interaction of the encoded proteins, and the structure and purpose of function of the Vap protein network remain to be determined. One of the 2 promoters in the *virR* operon responds to a temperature-dependent alternative sigma factor, partially explaining how temperature regulates *vapA* expression and virulence. Although his laboratory identified a sensor kinase interacting with the orphan response regulator VirS (ORF8), disruption of this sensor kinase gene did not abolish VapA expression, demonstrating that VirS interacts with more than one sensor kinase.

Albert Haas (University of Bonn) discussed how *R. equi* interferes with the phagosome of macrophages, so that the early endosome stops maturing midway into a late endosome. The biochemical basis of this maturation failure, particularly the role of *R. equi* glycolipids and of the *VapA* protein network in this pathology, is one of the crucial questions in *R. equi* pathogenesis.

Immunity to *R. equi* in foals: muddier than ever or poised for breakthrough?

In a keynote overview introducing the Immunology session, Steve Hines (Washington State University) suggested that understanding of immunity of horses to *R. equi* may be "muddier than ever". He questioned the relevance of the mouse in assessing immunity to *R. equi* and discussed use of immune mature horses as a way of teasing out the correlates of immunity, knowledge that is essential for vaccine development and refinement. Lack of a suitable model, other than the foal, for studying *R. equi* immunity and pathogenesis, is holding back the field. Although the Th1-Th2 paradigm has been critical to understanding the basis of immunity to *R. equi*, the contributions of Th17 and T regulatory cells have yet not been investigated. The relative importance of antibody in opsonisation and immunoregulation, especially in naïve neonates, also needs to be understood.

Although there is a general consensus that neonatal animals are more susceptible to intracellular infections, such as *R. equi*, there is contradictory evidence as to whether foals have a defective ability to produced interferon-γ. The apparent Th2 bias of neonates, coupled with defects in or the immaturity of antigen-presenting cells may hinder development of effective vaccines, which will be required to protect foals by 2–3 weeks of age. The critical importance of innate rather than acquired immunity in determining the direction and efficacy of adaptive immunity is now broadly recognised, and is probably a crucial factor determining the outcome of the interaction of the foal with virulent *R. equi*. In this context, understanding the response of equine dendritic cells to *R. equi* is underway in the laboratories of Julia Flaminio (Cornell University, Ithaca) and Joie Watson (University of California, Davis).

Several groups (David Horohov, University of Kentucky; Tong Liu, Texas A&M; Claire Ryan and Steeve Giguère, University of Florida, Gainesville) reported initial studies of immunostimulation of foals to enhance macrophage killing of *R. equi*. Given the apparent maturational defects of the neonatal foal's immune system, a crucial question was whether an immunisation strategy could be successful, although there is evidence that oral immunisation might be an effective approach. Data presented at the Workshop continues to support the apparent importance of VapA as a crucial protective immunogen. Despite uncertainty and some despondency, there was a belief among many participants that an immunological, particularly a vaccine-based, approach to control was both possible and tantalisingly in sight. Certainly it is an experimental question that should be further investigated.

Clinical session: is there age susceptibility to *R. equi* infection? Conflicting data

In his introduction to the Clinical session, Des Leadon (Irish Equine Centre) presented a review of the global and national horse populations, indicating that only a small minority would be of sufficient value to justify owner investment in vaccination. He warned that there would probably be only room for one vaccine in the marketplace and that vaccination would never be a substitute for environmental hygiene. He then presented Irish studies on *R. equi* clinical diagnosis and epidemiology by Mariann Klay and colleagues. These showed that the classical text-book description of *R. equi* disease is now rarely seen on well managed Irish farms and that today's clinicians in Ireland are confronted by a disease of ill-thrift, rather than the acute febrile entity and massive abscessation of the past.

One of the immunological arguments addressed in the Clinical session was whether some foals have a unique susceptibility to *R. equi* infection in their first 2 weeks of life. Keith Chaffin and colleagues (Texas A&M) described the efficacy of orally administered azithromycin to foals in the first 2 weeks *post partum* in reducing pneumonia in foals living on endemically-affected farms in the USA by 85%. By contrast, Monica Venner (University of Veterinary Medicine, Hanover) described German experience with similar administration for the first 4 weeks *post partum* in only delaying but not preventing development of pneumonia. The jury is clearly still out on the question of age susceptibility.

Some participants were critical of the use of prophylactic antibiotics to prevent infection in foals, particularly since Steeve Giguère and colleagues reported the presence of macrolide-resistance in about 3% of clinical isolates. Tom Buckley of the Irish Equine Centre endorsed this view by reporting a doubling of the mean minimum inhibitory concentration (MIC) of *R. equi* to rifampin and erythromycin over a 10 year period, suggesting a potential for future problems. Ron Martens and colleagues from Texas A&M described studies of the use of gallium maltolate in reducing *R. equi* infection in macrophages and mice, as well as its safety and pharmacokinetic behaviour in foals. The idea of using gallium, which accumulates in macrophages when transferrin is up-regulated, came from a casual conversation at a previous Workshop in a discussion of the importance of iron for virulence of *R. equi*. If gallium proves to be useful for prevention and treatment of *R. equi* in foals, this could be applied to the treatment of tuberculosis, including multi-drug resistant TB, in man.

Ursula Fogarty (Irish Equine Centre) shifted the focus from foals to adult horses. She reported an important series of cases of *R. equi* in young adult racehorses-in-training, in which an indoor environmental challenge appeared to be the primary source.

Pneumonic foals may be an underestimated source of infection

Ecological studies were reviewed in the Epidemiology session by Gary Muscatello (University of Sydney), who described recent findings that air-borne exhalation of infection by pneumonic foals was an important source of infection, suggesting that the stable environment might be more important than the more conventionally-recognised pasture contamination as a source of infection on endemic farms. Nevertheless, management environmental interventions had been shown to reduce the incidence of infection on farms. Noah Cohen (Texas A&M) reviewed epidemiological studies, emphasising the year-to-year variation of disease on farms, as well as the importance of foal density and the increasingly characterised equine genetic associations in the development of disease.

Are we too 'equino-centric'?

In the Epidemiology session, José Vazquez-Boland (University of Edinburgh) described the molecular analysis of a global collection of isolates from many sources and geographical origins. His group has shown that adaptation of *R. equi* to different hosts (horses, pigs, cattle) is driven by selection of specific plasmid-types associated with variations in *vap* genes associated with the particular sources. They had sequenced a *vapB* (pig)-type plasmid and identified 6 new *vap* genes by comparative analysis with the *vapA* (horse)-type virulence plasmid. Their findings suggest that the *vap* PAI was acquired via lateral gene transfer by a member of an ancestral family of actinobacterial conjugative replicons and subsequently evolved in *R. equi* by *vap* gene duplication and sequence diversification to give the contemporary host-adapted virulence plasmids. They also showed for the first time that cattle isolates possess a novel type of virulence plasmid characterised by the absence of *vapA* and *vapB* gene markers. The species of origin of isolates infecting people could now be suggested and the data presented suggested that *R. equi* represents another infection that can reach human subjects through food. Given this emerging understanding of *R. equi* as a zoonotic agent, he suggested that research should be less 'equino-centric' in the future in order to gain a better understanding of the biology, ecology, virulence and transmission of this multi-host pathogen.

In summary, the increasing pace of discovery and understanding about *R. equi* will gain a boost by the publication of the first genome sequence and the collaborative activities of participants working on different aspects of the host-pathogen-environment dynamic.

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