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

R. van der Geize[1],*, W. de Jong[1], G. I. Hessels[1], A. W. F. Grommen[2], A. A. C. Jacobs[2] and L. Dijkhuizen[1]

[1]Groningen Biomolecular Sciences and Biotechnology Institute (GBB), Department of Microbiology, University of Groningen, Kerklaan 30, 9751 NN Haren and [2]Intervet Schering-Plough Animal Health, Bacteriological R&D, Wim de Körverstraat 35, Postbus 31, 5830 AA Boxmeer, The Netherlands

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 ΔsupAB mutant confirmed that *supAB* are essential for growth of *R. equi* on cholesterol. Macrophage survival assays revealed that the ΔsupAB 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

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), $(NH_4)_6 Mo_7O_{24} \cdot 4 H_2O$ (0.22 g/l), $CuSO_4 \cdot 5H_2O$ (0.315 g/l) and $CoCl_2 \cdot 6 H_2O$ (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) 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 000g) 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 $14\,000g$). 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 $4500g$) 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 *Sal*I/*Not*I restriction released a 431 bp fragment comprising the *aphII* promoter which was then cloned into *Sal*I/*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 $200g$) 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 $200g$) 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$_2$SO$_4$ (0.25 g/l), (NH$_4$)$_2$SO$_4$ (2 g/l), MgCl$_2$·6H$_2$0 (0.6 g/l), 2-[(hydroxyl-1, 1-bis (hydroxymethyl)ethyl)amino] ethanesulfonic acid; TES (5.73 g/l) and trace elements (39). Sucrose (10.3% w/v) and L-proline (0.3% w/v) were added to MRM medium for *Streptomyces* strains, but omitted for all other strains. Glucose (20 mM) was used as carbon and energy source, except for all *Rhodococcus* strains which were grown in the presence of acetate (2 g/l). Autoclaved MRM was supplemented with KH$_2$PO$_4$ (0.1 g/l), CaCl$_2$·2H$_2$0 (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
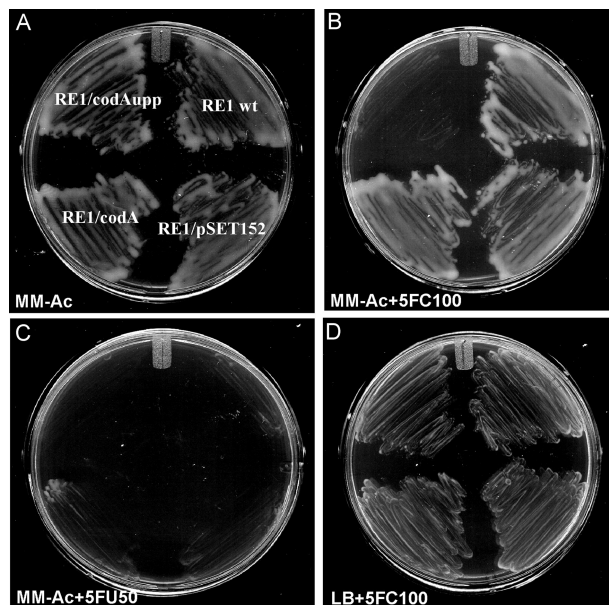

Figure 1. Introduction of *E. coli codA*::*upp* cassette, encoding CD and UPRT, confers 5-FC sensitivity to *R. equi* strain RE1. Panels show wild type strain RE1, recombinant strain RE1 containing pSET152, recombinant strain RE1 containing plasmid pSET-codA::upp streaked on mineral acetate agar (MM-Ac) 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).
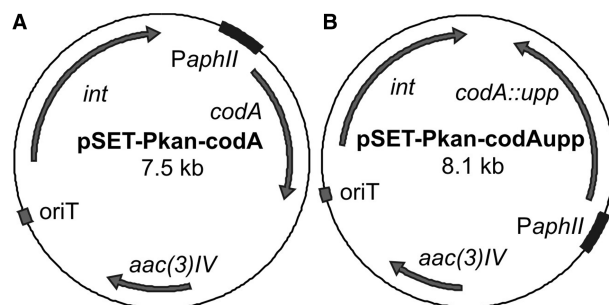

Figure 2. The pSET152 derived integrative plasmids used to introduce the (**A**) *codA* or (**B**) *codA*::*upp* cassette into *R. equi* RE1, expressed under control of the *aphII* kanamycin resistance cassette promoter (P$_{aphII}$). The apramycin resistance cassette (*aac(3)IV*), the *Streptomyces*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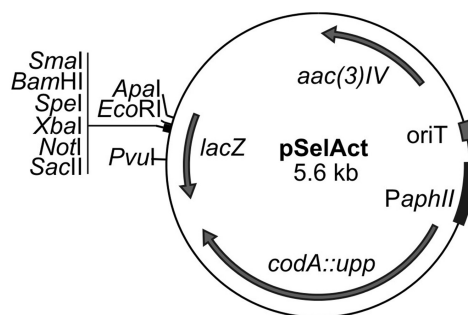

Figure 3. Schematic representation of the non-replicative plasmid pSelAct used to generate unmarked in-frame gene deletions in *R. equi* RE1. Restriction sites indicated are unique.

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
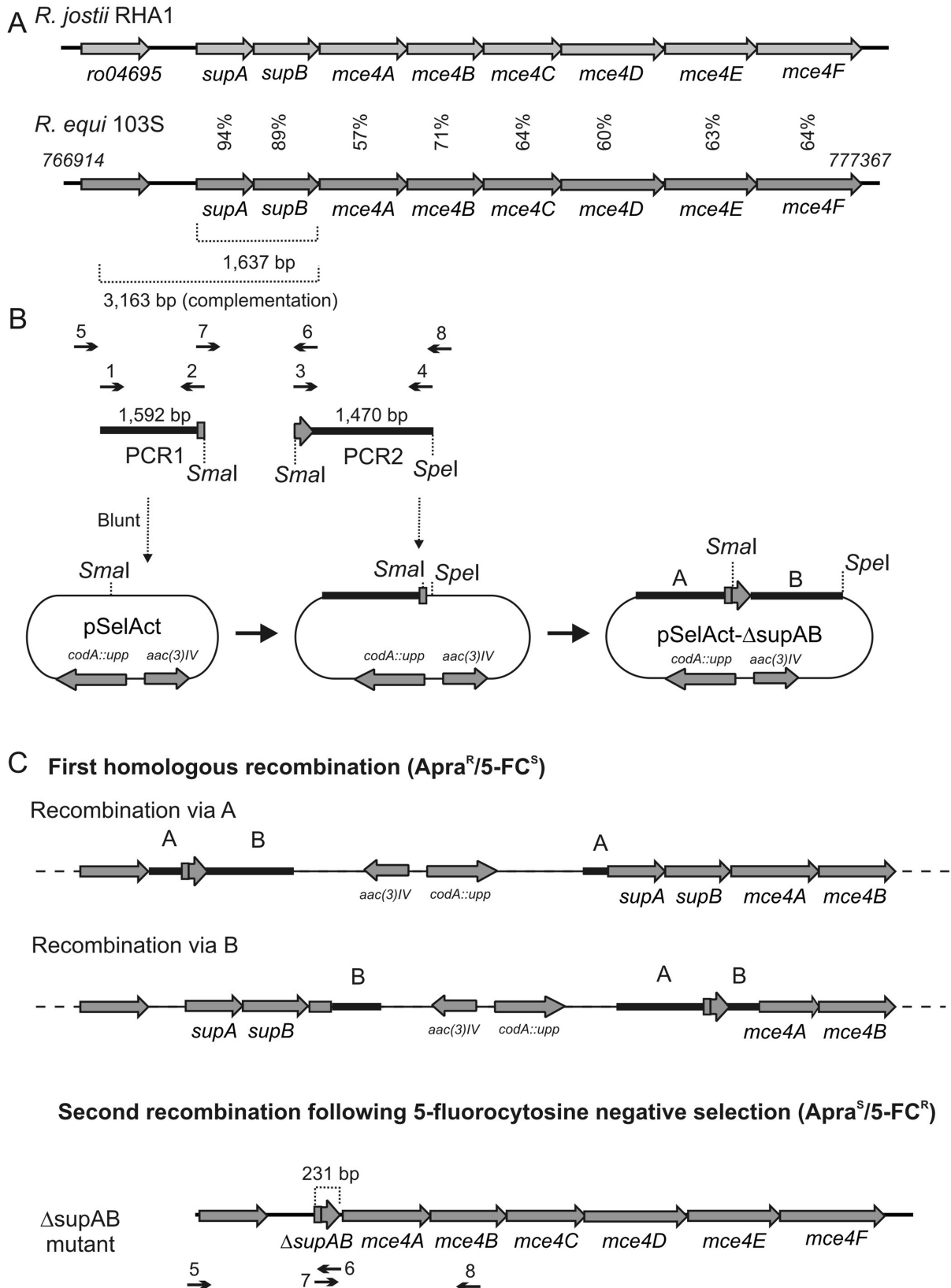
Figure 4. (**A**) Schematic overview of the molecular organization of the *mce4* gene clusters in *R. jostii* RHA1 and *R. equi* 103S. Percentages indicate amino acid sequence identities between the Mce4 proteins of *R. jostii* RHA1 and *R. equi* RE1. (**B**) Construction of mutagenic plasmid pSelAct-Δ*supAB* 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-Δ*supAB* 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.
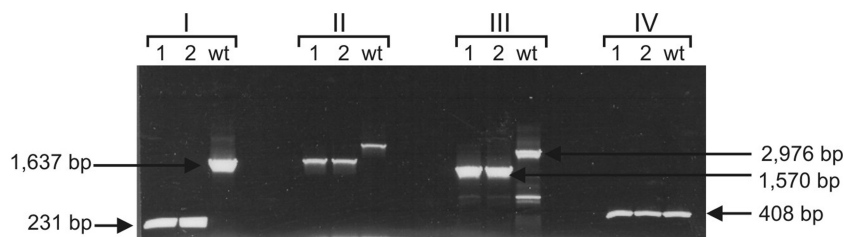

Figure 5. PCR analysis of wild type *R. equi* strain RE1 (wt) and two Δ*supAB* gene deletion mutants of *R. equi* strain RE1 (1 and 2). Panels show amplification of (I) the *supAB* locus [6 + 7], (II) the upstream region of *supAB* [5 + 6], (III) the downstream region of *supAB* [7 + 8], (IV) the *vapA* 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
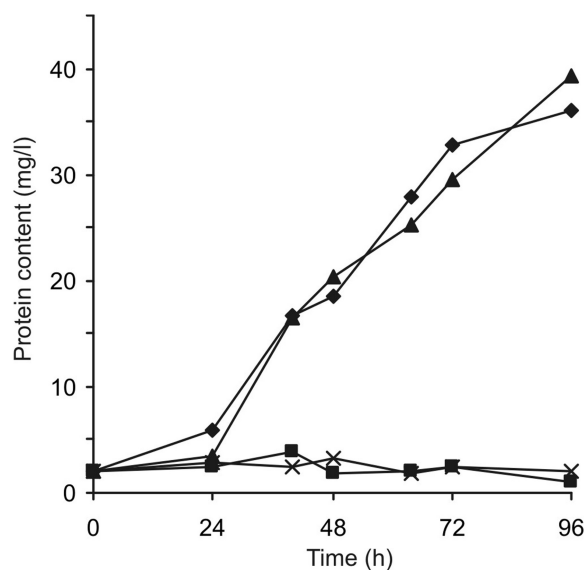

Figure 6. Growth curves in mineral medium supplemented with cholesterol (0.5 g/l) of *R. equi* RE1 wild-type (filled diamond), RE1 Δ*supAB* mutant strain (filled square), RE1 Δ*supAB* + pSET-supAB complemented strain (filled triangle), and RE1 Δ*supAB* + pSET152 control strain (x). Protein content (mg/l) 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).
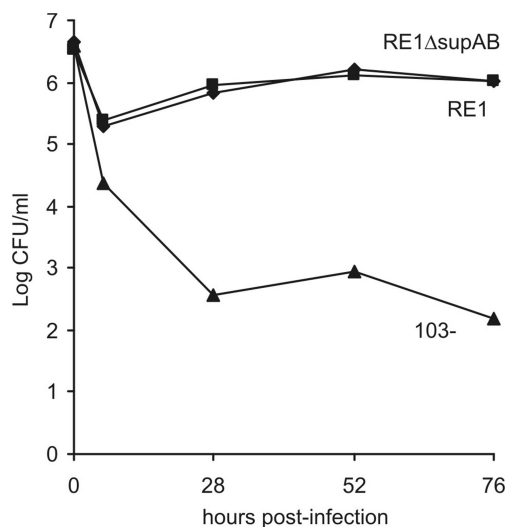

Figure 7. Survival and proliferation of *R. equi* strains in the human monocyte cell line U937. Macrophage cell suspensions were infected with wild type virulent strain *R. equi* RE1 (filled diamond), mutant strain *R. equi* RE1Δ*supAB* (filled square) and non-virulent (control) strain *R. equi* 103⁻ (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.

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 mc²155	+ +	+	–	–	–	–	–	(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.

REFERENCES

1. Ried,J.L. and Collmer,A. (1987) An *nptI-sacB-sacR* cartridge for constructing directed, unmarked mutations in gram-negative bacteria by marker exchange-eviction mutagenesis. *Gene*, **57**, 239–246.

2. Reyrat,J.M., Pelicic,V., Gicquel,B. and Rappuoli,R. (1998) Counterselectable markers: untapped tools for bacterial genetics and pathogenesis. *Infect. Immun.*, **66**, 4011–4017.

3. Van der Geize,R., Hessels,G.I., Van Gerwen,R., Van der Meijden,P. and Dijkhuizen,L. (2001) Unmarked gene deletion mutagenesis of kstD, encoding 3-ketosteroid Δ^1-dehydrogenase, in *Rhodococcus erythropolis* SQ1 using *sacB* as counter-selectable marker. *FEMS Microbiol. Lett.*, **205**, 197–202.

4. Nga,D.P., Altenbuchner,J. and Heiss,G.S. (2004) NpdR, a repressor involved in 2,4,6-trinitrophenol degradation in *Rhodococcus opacus* HL PM-1. *J. Bacteriol.*, **186**, 98–103.

5. Sharp,J.O., Sales,C.M., LeBlanc,J.C., Liu,J., Wood,T.K., Eltis,L.D., Mohn,W.W. and Alvarez-Cohen,L. (2007) An inducible propane monooxygenase is responsible for N-nitrosodimethylamine degradation by *Rhodococcus* sp. strain RHA1. *Appl. Environ. Microbiol.*, **73**, 6930–6938.

6. Van der Geize,R., Yam,K., Heuser,T., Wilbrink,M.H., Hara,H., Anderton,M.C., Sim,E., Dijkhuizen,L., Davies,J.E., Mohn,W.W. *et al.* (2007) A gene cluster encoding cholesterol catabolism in a soil actinomycete provides insight into *Mycobacterium tuberculosis* survival in macrophages. *Proc. Natl Acad. Sci. USA*, **104**, 1947–1952.

7. Jäger,W., Schäfer,A., Pühler,A., Labes,G. and Wohlleben,W. (1992) Expression of the *Bacillus subtilis sacB* gene leads to sucrose sensitivity in the gram-positive bacterium *Corynebacterium glutamicum* but not in *Streptomyces lividans*. *J. Bacteriol.*, **174**, 5462–5465.

8. Zhang,X.Z., Yan,X., Cui,Z.L., Hong,Q. and Li,S.P. (2006) *mazF*, a novel counter-selectable marker for unmarked chromosomal manipulation in *Bacillus subtilis*. *Nucleic Acids Res.*, **34**, e71.

9. Van Wezel,G.P. and Bibb,M.J. (1996) A novel plasmid vector that uses the glucose kinase gene (*glkA*) for the positive selection of stable gene disruptants in *Streptomyces*. *Gene*, **182**, 229–230.

10. Hosted,T.J. and Baltz,R.H. (1997) Use of *rpsL* for dominance selection and gene replacement in *Streptomyces roseosporus*. *J. Bacteriol.*, **179**, 180–186.

11. Knipfer,N., Seth,A. and Shrader,T.E. (1997) Unmarked gene integration into the chromosome of *Mycobacterium smegmatis* via precise replacement of the *pyrF* gene. *Plasmid*, **37**, 129–140.

12. Fabret,C., Ehrlich,S.D. and Noirot,P. (2002) A new mutation delivery system for genome-scale approaches in *Bacillus subtilis*. *Mol. Microbiol.*, **46**, 25–36.

13. Gust,B., Challis,G.L., Fowler,K., Kieser,T. and Chater,K.F. (2003) PCR-targeted *Streptomyces* gene replacement identifies a protein domain needed for biosynthesis of the sesquiterpene soil odor geosmin. *Proc. Natl Acad. Sci. USA*, **100**, 1541–1546.

14. Fedoryshyn,M., Petzke,L., Welle,E., Bechthold,A. and Luzhetskyy,A. (2008) Marker removal from actinomycetes genome using Flp recombinase. *Gene*, **419**, 43–47.

15. Andersen,L., Kilstrup,M. and Neuhard,J. (1989) Pyrimidine, purine and nitrogen control of cytosine deaminase synthesis in *Escherichia coli* K 12. Involvement of the *glnLG* and *purR* genes in the regulation of *codA* expression. *Arch. Microbiol.*, **152**, 115–118.

16. Danielsen,S., Kilstrup,M., Barilla,K., Jochimsen,B. and Neuhard,J. (1992) Characterization of the *Escherichia coli codBA* operon encoding cytosine permease and cytosine deaminase. *Mol. Microbiol.*, **6**, 1335–1344.

17. Heidelberger,C., Danenberg,P.V. and Moran,R.G. (1983) Fluorinated pyrimidines and their nucleosides. *Adv. Enzymol. Relat. Areas Mol. Biol.*, **54**, 58–119.

18. Mullen,C., Kilstrup,M. and Blaese,R.M. (1992) Transfer of the bacterial gene for cytosine deaminase to mammalian cells confers lethal sensitivity to 5-fluorocytosine: A negative selection system. *Proc. Natl Acad. Sci. USA*, **89**, 33–37.

19. Tiraby,M., Cazaux,C., Baron,M., Drocourt,D., Reynes,J.P. and Tiraby,G. (1998) Concomitant expression of *E. coli* cytosine deaminase and uracil phosphoribosyltransferase improves the cytotoxicity of 5-fluorocytosine. . *FEMS Microbiol. Lett.*, **167**, 41–49.

20. Hughes,L.E., Beck,D.A. and O'Donovan,G.A. (2005) Pathways of pyrimidine salvage in *Streptomyces*. *Curr. Microbiol.*, **50**, 8–10.

21. Tsukamura,M. (1981) Differentiation between genera *Mycobacterium*, *Rhodococcus* and *Nocardia* by susceptibility to 5-fluorouracil. *J. Gen. Microbiol.*, **125**, 205–208.

22. Tsukamura,M., Komatsuzaki,C., Sakai,R., Kaneda,K., Kudo,T. and Seino,A. (1988) Mesenteric lymphadenitis of swine caused by *Rhodococcus sputi*. *J. Clin. Microbiol.*, **26**, 155–157.

23. Prescott,J.F. (1991) *Rhodococcus equi*: an animal and human pathogen. *Clin. Microbiol. Rev.*, **4**, 20–34.

24. Meijer,W.G. and Prescott,J.F. (2004) Rhodococcus equi. *Vet. Res.*, **35**, 383–396.

25. Ahmad,S., Roy,P.K., Basu,S.K. and Johri,B.N. (1993) Cholesterol side-chain cleavage by immobilized cells of *Rhodococcus equi* DSM89-133. *Indian J. Exp. Biol.*, **31**, 319–322.

26. Murohisa,T. and Iida,M. (1993) Some new intermediates in microbial side chain degradation of β-sitosterol. *J. Ferment. Bioeng.*, **76**, 174–177.

27. Navas,J., González-Zorn,B., Ladrón,N., Garrido,P. and Vázquez-Boland,J.A. (2001) Identification and mutagenesis by allelic exchange of choE, encoding a cholesterol oxidase from the intracellular pathogen *Rhodococcus equi*. *J. Bacteriol.*, **183**, 4796–4805.

28. Pei,Y., Dupont,C., Sydor,T., Haas,A. and Prescott,J.F. (2006) Cholesterol oxidase (ChoE) is not important in the virulence of *Rhodococcus equi*. *Vet. Microbiol.*, **118**, 240–246.

29. McLeod,M.P., Warren,R.L., Hsiao,W.W., Araki,N., Myhre,M., Fernandes,C., Miyazawa,D., Wong,W., Lillquist,A.L., Wang,D. *et al.* (2006) The complete genome of *Rhodococcus* sp. RHA1 provides insights into a catabolic powerhouse. *Proc. Natl Acad. Sci. USA*, **103**, 15582–15587.

30. Rengarajan,J., Bloom,B.R. and Rubin,E.J. (2005) Genome-wide requirements for *Mycobacterium tuberculosis* adaptation and survival in macrophages. *Proc. Natl Acad. Sci. USA*, **102**, 8327–8332.

31. Joshi,S.M., Pandey,A.K., Capite,N., Fortune,S.M., Rubin,E.J. and Sassetti,C.M. (2006) Characterization of mycobacterial virulence genes through genetic interaction mapping. *Proc. Natl Acad. Sci. USA*, **103**, 11760–11765.

32. Pandey,A.K. and Sassetti,C.M. (2008) Mycobacterial persistence requires the utilization of host cholesterol. *Proc. Natl Acad. Sci. USA*, **105**, 4376–4380.

33. Jain,S., Bloom,B.R. and Hondalus,M.K. (2003) Deletion of *vapA* encoding Virulence Associated Protein A attenuates the intracellular actinomycete *Rhodococcus equi*. *Mol. Microbiol.*, **50**, 115–128.

34. Pei,Y., Nicholson,V., Woods,K. and Prescott,J.F. (2007) Immunization by intrabronchial administration to 1-week-old foals of an unmarked double gene disruption strain of *Rhodococcus equi* strain 103+. *Vet. Microbiol.*, **125**, 100–110.

35. Pei,Y., Parreira,V., Nicholson,V.M. and Prescott,J.F. (2007) Mutation and virulence assessment of chromosomal genes of *Rhodococcus equi* 103. *Can. J. Vet. Res.*, **71**, 1–7.

36. Vishniac,W. and Santer,M. (1957) The thiobacilli. *Bacteriol. Rev.*, **21**, 195–213.

37. Van der Geize,R., Hessels,G.I., Van Gerwen,R., Van der Meijden,P. and Dijkhuizen,L. (2002) Molecular and functional characterization of *kshA* and *kshB*, encoding two components of 3-ketosteroid 9α-hydroxylase, a class IA monooxygenase, in *Rhodococcus erythropolis* SQ1. *Mol. Microbiol.*, **45**, 1007–1018.

38. Sundstrom,C. and Nilsson,K. (1976) Establishment and characterization of a human histiocytic lymphoma cell line (U937). *Int. J. Cancer*, **17**, 565–577.

39. Kieser,T., Bibb,M.J., Buttner,M.J., Chater,K.F. and Hopwood,D.A. (2000) *Practical Streptomyces Genetics*, John Innes Foundation, Norwich, UK.

40. Bierman,M., Logan,R., O'Brien,K., Seno,E.T., Rao,R.N. and Schoner,B.E. (1992) Plasmid cloning vectors for the conjugal transfer of DNA from *Escherichia coli* to *Streptomyces* spp. *Gene*, **116**, 43–49.

41. Hong,Y. and Hondalus,M.K. (2008) Site-specific integration of *Streptomyces* PhiC31 integrase-based vectors in the chromosome of *Rhodococcus equi. FEMS Microbiol. Lett.*, **287**, 63–68.

42. Jäger,W., Schäfer,A., Kalinowski,J. and Pühler,A. (1995) Isolation of insertion elements from gram-positive *Brevibacterium*, *Corynebacterium* and *Rhodococcus* strains using the *Bacillus subtilis sacB* gene as a positive selection marker. *FEMS Microbiol. Lett.*, **126**, 1–6.

43. Takai,S., Hines,S.A., Sekizaki,T., Nicholson,V.M., Alperin,D.A., Osaki,M., Takamatsu,D., Nakamura,M., Suzuki,K., Ogino,N. *et al.* (2000) DNA sequence and comparison of virulence plasmids from *Rhodococcus equi* ATCC33701 and 103. *Infect. Immun.*, **68**, 6840–6847.

44. De Boer,L., Dijkhuizen,L., Grobben,G., Goodfellow,M., Stackebrandt,E., Parlett,J.H., Whitehead,D. and Witt,D. (1990) *Amycolatopsis methanolica* sp. nov., a facultative methylotrophic actinomycete. *Int. J. Sys. Bacteriol.*, **40**, 194–204.

45. Snapper,S.B., Melton,R.E., Mustafa,S., Kieser,T. and Jacobs,W.R. Jr. (1990) Isolation and characterization of efficient plasmid transformation mutants of *Mycobacterium smegmatis. Mol. Microbiol.*, **4**, 1911–1919.

46. Quan,S. and Dabbs,E.R. (1993) Nocardioform arsenic resistance plasmid characterization and improved *Rhodococcus* cloning vectors. *Plasmid*, **29**, 74–79.

47. Chater,K.F. and Wilde,L.C. (1980) *Streptomyces albus* G mutants defective in the *Sal*GI restriction-modification system. *J. Gen. Microbiol.*, **116**, 323–334.

48. Richter,M., Willey,J.M., Süssmuth,R., Jung,G. and Fiedler,H.-P. (1998) Streptofactin, a novel biosurfactant with aerial mycelium inducing activity from *Streptomyces tendae* Tь 901/8c. *FEMS Microbiol. Lett.*, **163**, 165–171.