Identification of virulence determinants of *Mycobacterium avium* that impact on the ability to resist host killing mechanisms

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Mycobacterium avium is an opportunistic pathogen associated with pulmonary disease in non-AIDS patients and disseminated infection in patients with AIDS. The chief route of infection is by colonization and invasion of the mucosa of the gastrointestinal tract, but infection through the respiratory route also occurs. After crossing the mucosa, *M. avium* infects and replicates within tissue macrophages. To identify *M. avium* genes required for survival *in vivo*, a library of signature-tagged transposon mutants was constructed and screened for clones attenuated in mice. Thirty-two clones were found to be attenuated for their virulence, from which eleven were sequenced and tested further. All the mutants studied grew similarly *in vitro* to the wild-type MAC104. Ten mutants were tested individually in mice, confirming the attenuated phenotype. MAV_2450, a polyketide synthase homologue to *Mycobacterium tuberculosis* pks12, was identified. STM5 and STM10 genes (encoding two hypothetical proteins MAV_4292 and MAV_4012) were associated with susceptibility to oxidative products. Mutants MAV_2450, MAV_4292, MAV_0385 and MAV_4264 live in macrophage vacuoles with acidic pH (below 6.9). Mutants MAV_4292, MAV_0385 and MAV_4264 were susceptible to nitric oxide *in vitro*. The study of individual mutants can potentially lead to new knowledge about *M. avium* pathogenic mechanisms.

INTRODUCTION

Mycobacterium avium is an opportunistic pathogenic bacterium that infects many host cell types, although the mononuclear phagocyte is the primary bacterial target. The bacterium is taken up by macrophages and lives inside a cytoplasmic vacuole, which does not acidify (Inderlied *et al.*, 1993; Sturgill-Koszycki *et al.*, 1994). The *M. avium* vacuole also does not follow the normal course of maturation, such as excluding proteins like Rab7 (Via *et al.*, 1997), which has a role in the docking of the late phagosome to lysosome. It is clear that *M. avium*, although not as virulent as *Mycobacterium tuberculosis*, is capable of surviving within host macrophages by subverting the phagocytic cell functions (Bermudez *et al.*, 2004; Crowle *et al.*, 1991). To do so, the bacterium has an array of virulence factors, a few of them already described (Honer Zu Bentrup *et al.*, 1999; Krzywinska *et al.*, 2005; Li *et al.*, 2005), but the majority are still unknown. It has also been shown that the virulence of *M. avium*, both in macrophages as well as in mice, depends on the bacterium morphotype. Smooth, rough, transparent or opaque, represent the bacterial morphotypes encountered. Although the smooth transparent morphotype is associated with virulence in mouse, the opaque and even the rough (without glycopeptidolipid) morphotypes have been isolated from patients with AIDS (Falkinham, 1996; Inderlied *et al.*, 1993).

Several studies have identified *M. avium* genes expressed within the macrophages. Hou and colleagues reported on *M. avium* genes upregulated in macrophages using selective capture of transcribed sequences (Hou *et al.*, 2002). More recently, Danelishvili *et al.* (2004) identified *M. avium* genes expressed in macrophages by differential fluorescence

Abbreviations: IFN-γ, gamma interferon; ROI, reactive oxygen intermediate; SOD, superoxide dismutase; STM, signature-tagged mutagenesis; WT, wild-type.

Tables of growth and susceptibility data for the wild-type and mutant isolates are available as supplementary data with the online version of this paper.

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induction, using a green fluorescent protein promoter. Still other virulent genes were identified by screening of a transposon library, such as the PPE-encoding gene described by Li *et al.* (2005).

Despite the knowledge acquired regarding *M. avium* genes associated with the pathogenesis of infection, very little is known about their function. In addition, the genes important for bacterial survival *in vivo* are mostly unknown. In mice, following intravenous infection (via the tail vein), a large portion of *M. avium* organisms are located in the spleen and liver (Bermudez *et al.*, 1999). Oral infection is also associated with disseminated disease (Bermudez *et al.*, 1992). Bacteria cross the intestinal mucosa through enterocytes, and many of them survive in a latency-like state in mesenteric lymph nodes (Petrofsky & Bermudez, 2005; Sangari *et al.*, 2000). Dissemination to the spleen, liver and bone marrow still often occurs (Petrofsky & Bermudez, 2005). Once in the liver and spleen, *M. avium* is found within macrophages and Kupffer cells, respectively (L. E. Bermudez & M. Petrofsky, unpublished results). The spread of the infection and systemic dissemination requires escape from macrophages and infection of, presumably, another macrophage (Bermudez *et al.*, 2004). A recent study demonstrated that *M. tuberculosis* dissemination does seem to occur secondary to transport within phagocytic cells (Clay *et al.*, 2007).

Once within macrophages, *M. avium* can resist reactive oxygen intermediates (ROIs) and nitric oxide (NO) (Appelberg & Orme, 1993; Bermudez & Young, 1989). Differently from *M. tuberculosis*, the inactivation of induced NO has no effect on *M. avium* survival in mice (Cooper *et al.*, 2000; Gomes *et al.*, 1999). Therefore, thus far no mechanism of killing of intracellular *M. avium* has been identified, although it is known that macrophage activation by tumour necrosis factor alpha and gamma interferon (IFN-γ) have important roles in *M. avium* killing (Appelberg & Orme, 1993; Bermudez & Young, 1989).

Signature-tagged mutagenesis (STM) has been used by a number of groups to identify bacterial genes required for the survival and replication in the host (Camacho *et al.*, 1999; Cox *et al.*, 1999; Hensel *et al.*, 1995). It allows for large pools of bacterial clones to be screened simultaneously. Therefore, to get new insight on *M. avium* pathogenesis, we created and screened STM mutants in mice. We identified several genes that, when inactivated, resulted in attenuation of *M. avium in vivo*, and initial studies have suggested potential mechanisms of action.

METHODS

Bacterial strains and growth conditions. *Escherichia coli* XL1-blue was used for the cloning experiments. It was grown at 37 °C on Luria–Bertani broth supplemented with 50 μg kanamycin ml^{-1}. The *M. avium* strain 104, isolated from the blood of an AIDS patient and shown to be virulent in mice, as well as the mutants, were grown on Middlebrook 7H9 broth or 7H11 agar (Difco) supplemented with 0.2 % glycerol, 0.05 % Tween 80 and oleic acid, albumin glucose and catalase, as reported previously (Bermudez *et al.*, 1992, 2004).

Construction of plasmid pYJTags. The transposon Tn*5367* derived from IS*1096* was extracted from pYUB285 on a *Hin*dIII and *Eco*RI fragment, and cloned into the *Hin*dIII and *Eco*RI restriction sites of the *E. coli* vector pUC19 to create pYJL1. The mycobacteria temperature-sensitive origin of replication was amplified from pYUB285 by using primers with *Hin*dIII restriction site, and was cloned into pYJL1. The resulting plasmid was named pYJL2. Double-stranded DNA tags were produced by PCR amplification using a variable oligonucleotide pool RTCG as template DNA that was 5′-AAACTAACTAGTTACAACCTCAAGCTT-(NK)$_{20}$-AAGCTTGGTT-AGATGACTAGTATTAAA-3′. The primers used to amplify the tags were pTag1 (5′-AAACTAACGCGTTACAACCTC-3′), pTag2 (5′-TTTAATACGCGTCATTCTAACC-3′), pTag3 (5′-TAGTTACAACC-TCAAGCTT-3′) and pTag4 (5′-TAGTCATTCTAACCAAGCTT-3′). For the cloning, the tags were amplified using the RTCG template, and pTag1 and pTag2 as primers. The PCR was performed using an AmpliTaq kit (Perkins-Elmer) with a 50 μl reaction containing 200 pg RTCG, pTag1 and pTag2 (100 pg), 1.5 M $MgCl_2$ and 250 μM each of dATP, dGTP, dCTP dTTP, 1 × *Taq* buffer and 5 U AmpliTaq. The cycle conditions used were the same as described by Hensel *et al.* (1995). PCR products were purified using a PCR purification kit (Qiagen), digested with *Mlu*I, dephosphorylated and ligated into the vector pYJL2. The ligation mixture was used to transform *E. coli*, and the transformants were selected on Luria–Bertani agar plate containing 50 μg kanamycin ml^{-1}. The plasmids were analysed by restriction enzyme digestion to verify that tags had been incorporated into pYJL2. The resulting plasmid with a mixture of tags was named pYJTags.

Construction of the *M. avium* mutant library. A pool of 5000 *E. coli* transformants was used to prepare plasmid pYJTags DNA (Fig. 1). These plasmids were electroporated into *M. avium* 104 strain, as described previously (Dam *et al.*, 2006; Li *et al.*, 2005), and transformants were selected on 7H11 medium containing 200 μg kanamycin ml^{-1}. A total of 50 transformants containing pYJTags were obtained and used to produce 50 different mutant banks. A library of 2064 mutants picked randomly from 50 banks was organized and grown in microtitre plates containing 7H9 medium supplemented with 400 μg kanamycin ml^{-1} at 30 °C. After shifting the temperature to 41 °C for 3 days, the suspension was plated onto 7H11 agar plates with 400 μg kanamycin ml^{-1}. Hundreds of mutants were obtained from each well. One colony was picked up from each of 50 individual plates to form 48 pools of 50 clones, each containing 50 different tags. A series of membranes containing the 50 different tags was produced by amplifying the different tags using pTag3 and pTag4 primers, and transferring them onto nylon N^{+} membrane using a manifold apparatus.

Labelling of input and output probe, and hybridization. For amplification of the output probes from the spleens of the infected mice, homogenized spleen was plated onto solid medium supplemented with 400 μg kanamycin ml^{-1}. After 7 to 10 days, mutant pools were recovered from the plates as output probes. For amplifying tags from the input of *M. avium* mutants, the pool containing 50 different tags was grown in 7H9 broth with 400 μg kanamycin ml^{-1}, and bacterial cells were lysed using a high temperature incubation protocol (100 °C for 10 min and 4 °C for 1 min, 4 cycles). A 20 μl aliquot of supernatant was used for the 50 μl PCR; the PCR product was purified with a PCR purification kit (Qiagen), and labelled with non-radioactive digoxin with a DIG-Chem-Link labelling and detection set (Roche). The probe was digested with *Hin*dIII and purified to remove the non-specific arms consisting of the tags. Prehybridization, hybridization and washes were carried out at 68 °C using the DIG Wash and Block Buffer set hybridization kit from Roche. CDP-*Star* chemiluminescent substrate for alkaline phospha-

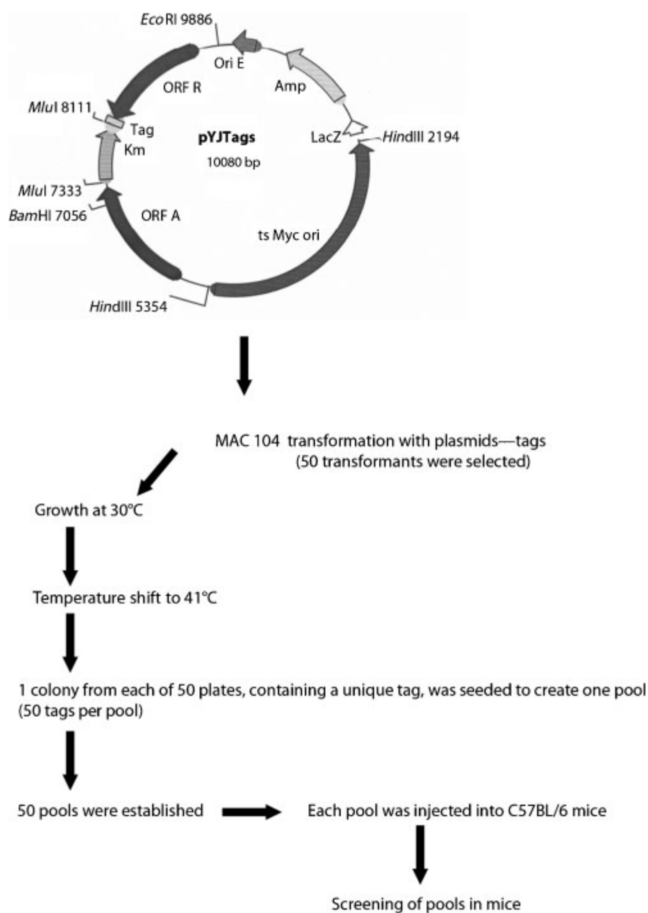

Fig. 1. Schematic representation of the approach used. The pYJTags was created from pUC19 with the insertion of a temperature-sensitive (ts) Myc ori. It allowed for the integration of the transposon once the environmental temperature was elevated to 41 °C.

tase was used for visualization. X-ray films (Kodak) were exposed for 1 to 10 min at room temperature, depending on the signal strength.

Mapping of transposon insertion and sequence analysis. To identify the transposon insertion site, chromosomal DNA from the mutant isolates was prepared. The interrupted unknown sequence was amplified using non-specific PCR as described previously (Danelishvili *et al.*, 2004). The product was purified and directly sequenced using an ABI 373 DNA sequencer with a *Taq* dideoxy terminator cycle sequencing kit (Perkin-Elmer Cetus). Only sequences containing the transposon were considered. The sequences were mapped on the *M. avium* genome sequence [The Institute for Genomic Research (TIGR) database] and *M. tuberculosis* H37Rv genome sequence using the BLAST program.

Complementation of genes. To address the importance of the genes inactivated on *M. avium* virulence, complementation of the genes MAV_2450, MAV_4292, MAV_1346, MAV_4012 and MAC_4264 was carried out using a recently published strategy (Li *et al.*, 2005). Specific primers were designed and used to amplify the four genes from genomic DNA using PCR as described previously (Li *et al.*, 2005). The amplification product was then cloned in pMV261-Apr plasmid and used to transform the mutants STM1 (STM1 comp), STM5 (STM5 comp), STM8 (STM8 comp), STM10 (STM10 comp) and STM11 (STM11 comp). Clones containing the plasmid were selected in presence of 200 μg apramycin ml^{-1}. Selected clones were confirmed for the presence of the plasmid.

Mice infection studies. In order to screen clones for attenuated mutants, 0.2 ml (1×10^8 c.f.u.) each input pool were injected intravenously into C57BL/6 mice (Jackson Laboratories). Mice were observed, and 3 weeks post-infection mice were sacrificed by cervical dislocation, spleens removed aseptically and homogenized. Series dilutions of spleen homogenates were plated onto 7H11 with kanamycin at 400 μg ml^{-1}. The colonies were collected and processed, as described above, to make the output probe tags.

To prove that the strains identified in the initial screen were, in fact, attenuated mutants, mice were injected intravenously with 3×10^7 individual bacterial mutant. Twelve mice were used for each of the time points. In two independent experiments (with a total of 48 animals per time point), organs were removed 1 day, 1 week, 3 weeks and 5 weeks post-infection, homogenized, and serially diluted and plated onto 7H11 plates, as previously reported (Bermudez *et al.*, 1992, 1997). The number of c.f.u. was determined and compared with the number of colonies in the spleen of mice infected with the wild-type (WT) bacterium.

Macrophages and specimen preparation. Human monocyte-derived macrophages were isolated and prepared as previously described (Bermudez *et al.*, 1997, 2004). Cells were adjusted to 10^5 cells ml^{-1} and seeded for 18 to 24 h on sterile, Formvar-coated London finder-gold grids (Electron Microscopy Sciences) in RPMI 1640 supplemented with 10 % fetal bovine serum (Sigma Chemicals). The monolayers were infected with *M. avium* strains for 1 h with an approximate ratio of 10 bacteria per macrophage, as reported previously (Bermudez *et al.*, 1997, 2004). After infection, specimens were fixed in 1 % paraformaldehyde, pH 7.2, for 30 min. The grids were then washed and chambers were observed for identification of bacteria or phagosomes, as previously reported (Wagner *et al.*, 2005). In other studies, RAW 246.7 macrophage monolayers (5×10^5 cells), cultured in presence of Dulbecco's modified Eagle's medium supplemented with 10 % heat inactivated fetal bovine serum, were stimulated with 100 mg recombinant mouse IFN-γ ml^{-1} and subsequently infected with different *M. avium* strains for 1 h (m.o.i. of 10), and washed to remove extracellular bacteria (Bermudez *et al.*, 1992; Li *et al.*, 2005). Depending on the experiment, 2 mg bovine superoxide dismutase (SOD) ml^{-1} (Sigma Chemicals) or *N*-methylarginine (Sigma) at 100 μM concentration was added concomitantly with the bacteria. These concentrations of inhibitors are known to suppress O_2 and NO, respectively, in infected macrophages (Bermudez, 1993). Prior studies have shown that neither SOD nor *N*-methyl-arginine has any effect on monocyte and macrophage phagocytosis (data not shown). Monolayers were kept for 24 h and then lysed as previously described (Bermudez *et al.*, 1992; Li *et al.*, 2005). The macrophage lysate was serially diluted and plated onto 7H10 agar plates. Plates were incubated at 37 °C for 10 days and the number of colonies quantified (Bermudez *et al.*, 1992; Li *et al.*, 2005).

In some experiments, intracellular bacteria were allowed to grow inside macrophages for 4 days before lysing. Macrophage viability was monitored during the experiments to evidence differential detachment of macrophages from monolayers (Bermudez *et al.*, 1992; Li *et al.*, 2005).

Vacuole pH. Measure of the vacuole pH was performed as previously described (Li *et al.*, 2005). Briefly, to label bacteria, mid-exponential growth phase *M. avium*, WT and mutants, were washed in PBS and resuspended in PBS with 0.05 % Tween 80. Bacteria were labelled with fluorescent probes NHS-CF and NHS-Rho (10 μg each). Measurements were made in a fluorimeter (CytoFluor II;

Biosearch). The filters used consisted of an excited band pass at 465–505 nm and emission at 505–555 nm. Calculation of phagosome pH for each well was carried out according to the standard calibration curve based on CF/Rho ratio versus pH, according to the method previously reported (Li *et al.*, 2005).

Susceptibility to NO. The NO donor NOR-3 was used to determine the effects of reactive nitrogen substances on *M. avium* strains. NOR-3 was dissolved in 15 mM Tris/HCl, pH 7.4, and was quantified spectrophotometrically. The NOR-3 (NO donor) was added to the culture of *M. avium*, WT or mutants, for 60 min. Afterwards, bacteria were quantified by plating the diluted suspension to 7H10 agar medium.

Statistical analysis. Results of experimental studies in mice were analysed by Student's *t*-test. Each number represented the total number of mice divided into two different experiments. Experiments *in vitro* were analysed using ANOVA. The P values <0.05 were considered significant.

RESULTS AND DISCUSSION

M. avium is an intracellular opportunistic pathogen that infects and survives within host macrophages. *M. avium* is acquired from the environment, and its ability to cross the mucosal barrier and infect submucosal macrophages appears to be key for the establishment of infection (Bermudez *et al.*, 1997; Inderlied *et al.*, 1993; Sangari *et al.*, 2000). Several studies have identified virulence determinants *in vitro* that allow the bacterium to enter (Dam *et al.*, 2006; Miltner *et al.*, 2005) and survive within macrophages (Honer Zu Bentrup *et al.*, 1999; Hou *et al.*, 2002; Krzywinska *et al.*, 2005; Li *et al.*, 2005). Although some other studies have determined the expression of *M. avium* genes in mononuclear phagosomes *in vitro* (Danelishvili *et al.*, 2004; Hou *et al.*, 2002) and *in vivo* (Li *et al.*, 2005), very little has been performed in an attempt to identify virulence genes important for the survival in the host using an *in vivo* model.

Identification of attenuated mutants

To identify *M. avium* mutants attenuated *in vivo*, we partially screened an STM library in mice (2064 clones). Thirty-two clones with significant attenuation *in vivo*, in comparison to the WT bacterium, were identified by comparing the hybridization of the pool of DNA from bacteria in the spleen after 3 or 5 weeks of infection with the pool of DNA of the bacterial inoculum before administration (Fig. 1). Eleven clones were sequenced and further tested individually. The clones STM1 and STM7 had the transposon inserted in the same gene. The inactivated genes were sequences as described in Methods and elsewhere (Danelishvili *et al.*, 2004; Li *et al.*, 2005). The genes inactivated in the sequenced mutants are listed in Table 1. To confirm the phenotype of the isolated mutants, we infected C57BL/6 mice intravenously with the individual mutants (except for STM7 and STM9), and the number of bacteria in the spleen (compared with the WT MAC 104) was determined at 24 h, 1 week, 3 weeks and 5 weeks. STM7, as for STM1, had the *pks12* gene inactivated, and STM9 had a transposon in a chaperonin and was considered less relevant than other mutants. As shown in Table 2, some mutants were attenuated at the first week of infection (STM5, STM6 and STM10), some were attenuated after 3 weeks of infection (STM1, STM3, STM4, STM8 and STM11) and only one clone showed attenuation in mice only after 5 weeks of infection (STM2).

In vitro growth

To determine whether or not the identified mutants had impaired growth ability *in vitro*, growth curves were established in 7H9 broth. STM1, STM7, STM5–6 and STM8–11 were grown in parallel to *M. avium* 104, and the growth curve determined over 10 days. Growth of all mutant strains was similar to the WT bacterium.

In the current study, we identified a number of *M. avium* clones that were attenuated *in vivo* by using an STM system. The attenuation of the individual mutants was then confirmed by challenge experiments, and some of the genes were complemented, leading to the recovery of the virulent phenotype. Some of the genes associated with attenuation *in vivo* were those encoding the polyketide synthases 11

Table 1. Genes inactivated in the identified mutants

Attenuated mutants were identified by screening an STM library. The mutants were tested individually for attenuation.

Clone	*M. avium* gene	Function
STM1/STM7	MAV_2450	Polyketide synthase 12
STM2	MAV_3321	ABC transporter, ATP-binding subunit
STM3	MAV_3105	Polyketide synthase 11
STM4	MAV_2723	PAP A2 condensation domain
STM5	MAV_4292	Hypothetical protein
STM6	MAV_0385	*N*-Acetylmuramyl-L-alanine amidase
STM8	MAV_1346	PE family protein
STM9	MAV_4366	Chaperonin GroES
STM10	MAV_4012	Conserved hypothetical protein
STM11	MAV_4264	Hypothetical protein, homology with bacterial regulatory protein TetR domain

Table 2. Comparison of the number of organisms in the WT strain MAC 104 and the other STM clones at several time points following infection of mice

C57BL/6 mice were infected intravenously and their spleens were harvested at 24 h, and at weeks 1, 3 and 5, for quantification of the bacterial load.

Clone	No. of c.f.u. per organ			
	24 h	1 week	3 weeks	5 weeks
STM1	$6.7 \pm 0.3 \times 10^{6}$	$6.7 \pm 0.4 \times 10^{6}$	$6.1 \pm 0.4 \times 10^{5}$*	$5.2 \pm 0.3 \times 10^{4}$*
STM2	$6.1 \pm 0.3 \times 10^{6}$	$6.5 \pm 0.4 \times 10^{6}$	$6.0 \pm 0.2 \times 10^{6}$	$4.9 \pm 0.2 \times 10^{3}$*
STM3	$5.5 \pm 0.5 \times 10^{6}$	$2.3 \pm 0.2 \times 10^{6}$	$1.2 \pm 0.1 \times 10^{6}$*	$8.6 \pm 0.4 \times 10^{5}$*
STM4	$5.6 \pm 0.4 \times 10^{6}$	$3.2 \pm 0.3 \times 10^{6}$	$2.0 \pm 0.2 \times 10^{6}$*	$1.4 \pm 0.3 \times 10^{6}$*
STM5	$3.8 \pm 0.2 \times 10^{6}$	$1.1 \pm 0.2 \times 10^{6}$*	$1.5 \pm 0.1 \times 10^{6}$	$9.4 \pm 0.7 \times 10^{5}$*
STM6	$2.3 \pm 0.9 \times 10^{6}$	$1.1 \pm 0.7 \times 10^{6}$*	$8.3 \pm 0.6 \times 10^{5}$*	$8.1 \pm 0.5 \times 10^{5}$*
STM8	$1.6 \pm 0.2 \times 10^{6}$	$1.8 \pm 0.6 \times 10^{6}$*	$1.8 \pm 0.4 \times 10^{6}$	$1.9 \pm 0.3 \times 10^{5}$*
STM10	$3.2 \pm 0.4 \times 10^{6}$	$1.1 \pm 0.3 \times 10^{6}$*	$2.0 \pm 0.4 \times 10^{6}$	$7.3 \pm 0.3 \times 10^{4}$*
STM11	$5.1 \pm 0.3 \times 10^{6}$	$2.7 \pm 0.4 \times 10^{6}$	$8.5 \pm 0.3 \times 10^{5}$*	$5.3 \pm 0.6 \times 10^{4}$*
WT (MAC 104)	$1.9 \pm 0.2 \times 10^{6}$	$3.8 \pm 0.2 \times 10^{6}$	$1.3 \pm 0.2 \times 10^{7}$	$4.6 \pm 0.4 \times 10^{7}$

*$P<0.05$ compared with the WT MAC 104 at the same time point.

and 12. Recent studies showed that genes involved in the synthesis and transport of polyketides are required for *M. tuberculosis* growth and virulence in mice (Appelberg & Orme, 1993; Hahn *et al.*, 2005; Jain & Cox, 2005; Wagner *et al.*, 2005). In addition, the work by Hou *et al.* (2002) demonstrated that the polyketide synthase 11 encoding gene was upregulated upon uptake of *M. avium* by macrophages. Polyketides are involved in cell-wall-lipid synthesis and the integrity of the outer surface of mycobacteria. They participate in the synthesis of dimycocerosyl phthiocerol, which has been suggested as playing a role in the permeability of the cell wall (Camacho *et al.*, 2001). In *M. tuberculosis*, 24 polyketide synthases have been annotated (Cole *et al.*, 1998); while in *M. avium*, there are at least 22 polyketide synthases, all with significant homology to the ones in *M. tuberculosis* (L. E. Bermudez & Y. Li, unpublished observation). Polyketides were also associated with modulation of the host immune response to infection by impacting on cytokine production by infected macrophages (Converse *et al.*, 2003). Polyketides are transported to the surface of *M. tuberculosis* by a unique transport mechanism involving several genes of the *mmpL* family (Camacho *et al.*, 1999; Converse *et al.*, 2003; Domenech *et al.*, 2004). In *M. avium*, the role of *mmpL* genes has not yet been defined, but infections of macrophages have been associated with the expression of the MmpL transport proteins (Danelishvili *et al.*, 2004; Hou *et al.*, 2002), and it is likely to be somewhat similar to the role in *M. tuberculosis*.

The identification of an ABC transporter subunit as virulence factor points to the importance of secreted proteins. ABC transporters are two-protein complexes, one protein involved in transport and the other providing energy for the process. One recent ABC transporter linked with virulence in mycobacteria is involved in the transport of oxidoreductase associated with the invasion of epithelial cells (Patel *et al.*, 2006). Other ABC transporters have been identified in screening for mycobacterial virulence genes using several methods, but the functions have not been characterized.

Our screen also identified a PE protein (mycobacterial protein rich in proline and glutamic acid), GroES, a polyketide synthase-associated protein and many proteins of unknown function, some of them not present in *M. tuberculosis* but with homology to *Mycobacterium paratuberculosis*. PE, PPE and PE-PGRS are families of proteins that have been linked with virulence of *Mycobacterium marinum* and *M. tuberculosis* (Dubnau *et al.*, 2002; Ramakrishnan *et al.*, 2000). Some PE proteins have more recently been described as exported proteins, with expression on the surface of the bacterium (Delogu & Brennan, 2001) and are expressed differently in host tissues (Delogu *et al.*, 2006). In fact, PE–PGRS proteins apparently have different patterns of expression, as evidenced by studies with *M. tuberculosis*, *M. marinum* and *M. avium* (Dheenadhayalan *et al.*, 2006b; Li *et al.*, 2005; Ramakrishnan *et al.*, 2000). Initially, PE, PPE and PE–PGRS groups of proteins were suggested as being involved in the interaction with the host immune system, and being important antigenic determinants of *M. tuberculosis* (Cole *et al.*, 1998). Although these families of proteins may be important to the immune response of the host, it is increasingly evident that several of them are involved in the pathogenesis of the infection, as shown by the requirement for survival in granulomas (Ramakrishnan *et al.*, 2000) and within macrophages (Li *et al.*, 2005). Our current work identified another PE protein in *M. avium* important for full virulence *in vivo*. Its function is unknown at this point, but recent work demonstrates that PE–PGRS 33 of *M. tuberculosis*, when expressed in *Mycobacterium smegmatis*,

triggers necrosis of macrophages (Dheenadhayalan *et al.*, 2006a). Chaperonins, such as GroEL, have recently been associated with mycobacterial virulence (Ojha *et al.*, 2005). GroEL1 has been linked with the ability of *M. smegmatis* to form biofilms (Ojha *et al.*, 2005) by modulating the synthesis of mycolates. The role in cell-wall synthesis may impact on the ability of the bacterium to interact with the host. In the case of GroES, the association with virulence has not been established yet, but the fact that the inactivation of the genes is associated with attenuation early in the infection *in vivo*, indicates that GroES might have other, still unknown, function(s). The different functions of stress-related proteins are still not completely known.

Inactivation of the genes encoding the polyketide synthases 11 and 12 was associated with lack of growth from the beginning of the infection, but major attenuation was only observed after 3 weeks following challenge. Those results agree with what has been described for clones with the inactivation of pks12 in *M. tuberculosis* (Sirakova *et al.*, 2003). In contrast, the inactivation of the PE-encoding gene was associated with significant bacterial attenuation 5 weeks after infection, indicating that this protein may have function important for survival during the host adaptive response leading to the activation of macrophages or with the spreading phase of the infection, as recently suggested (Dheenadhayalan *et al.*, 2006a). Polyketide synthases 11 and 12, as proteins associated with surface moieties, could potentially impact on the ability of the bacterium to interact with the environment.

Attenuation in macrophages

To examine whether the STM mutants were attenuated in macrophages, RAW 246.7 mouse macrophages were infected and the growth was followed for 4 days. It was observed that all mutants but STM3 and STM4 had no significant attenuation in macrophages *in vitro*. It suggests that the attenuation of STM3 and STM4 *in vivo* is probably not related with the innate immune response (Supplementary Table S1 available with the online journal). It was also determined that attenuation was due to increased susceptibility to superoxide anion or nitric oxide. STM5 and STM10 showed increased sensitivity to ROIs (Table 3), while STM10 and STM11 were susceptible to nitric oxide (Table 4). The concentrations of inhibitors used in these assays are knows to suppress O_2 and NO in infected macrophages.

Susceptibility to NO

To examine whether STM strains are susceptible to NO *in vitro*, we incubated the strains STM1, 5, 10 and 11 in the presence of NO donor and verified the viability afterward. As shown in Supplementary Table S2 (available with the online journal), the mutants STM5 and STM10 were susceptible to NO at a concentration of 1 μM.

We identified a number of mutations in *M. avium* genes of unknown function that are associated with attenuation *in vivo*. Some of the genes inactivated, such as those homologous to MAV_4292 and MAV_4012, lead to increased susceptibility to SOD and NO (MAV_4012 and MAV_4264), raising interesting questions about the mechanisms of bacterial resistance to these toxic products of phagocytic cells. Production of superoxide anion is ineffective as a mechanism of killing of the majority of the *M. avium* strains (Bermudez & Young, 1989; Gunzel *et al.*, 2006; Sarmento & Appelberg, 1996). Likewise, the role of NO, in contrast to *M. tuberculosis*, has not been established

Table 3. Susceptibility of *M. avium* attenuated clones to superoxide anion

M. avium strain	No. of c.f.u. (ml macrophage lysate)$^{-1}$*		
	1 h	24 h	
		−SOD	+SOD
WT (MAC 104)	$2.1 \pm 0.3 \times 10^5$	$3.3 \pm 0.5 \times 10^5$	$2.1 \pm 0.4 \times 10^5$
STM1	$3.6 \pm 0.4 \times 10^5$	$1.1 \pm 0.3 \times 10^5$	$1.5 \pm 0.2 \times 10^5$
STM2	$2.7 \pm 0.3 \times 10^5$	$9.0 \pm 0.2 \times 10^4$	$8.6 \pm 0.3 \times 10^4$
STM3	$5.1 \pm 0.5 \times 10^5$	$3.0 \pm 0.2 \times 10^5$	$2.6 \pm 0.4 \times 10^5$
STM4	$2.3 \pm 0.4 \times 10^5$	$2.0 \pm 0.5 \times 10^5$	$1.8 \pm 0.3 \times 10^5$
STM5	$3.0 \pm 0.5 \times 10^5$	$8.2 \pm 0.4 \times 10^4$	$2.0 \pm 0.5 \times 10^5$†
STM6	$3.9 \pm 0.2 \times 10^5$	$1.9 \pm 0.3 \times 10^5$	$1.6 \pm 0.4 \times 10^5$
STM8	$3.6 \pm 0.5 \times 10^5$	$8.1 \pm 0.2 \times 10^4$	$7.6 \pm 0.4 \times 10^4$
STM10	$3.7 \pm 0.3 \times 10^5$	$8.5 \pm 0.4 \times 10^4$	$1.0 \pm 0.3 \times 10^5$†
STM11	$4.7 \pm 0.3 \times 10^5$	$8.1 \pm 0.6 \times 10^4$	$8.5 \pm 0.2 \times 10^4$

*RAW 246.7 macrophage monolayers were infected with *M. avium* for 1 h and extracellular bacteria were removed by washing. Some wells were incubated with 2 mg SOD ml^{-1}, as reported in Methods. Monolayers were lysed at 24 h and viable intracellular bacteria quantified. The experiment was repeated three times.
†$P<0.05$ compared with the number of c.f.u. (ml macrophage lysate)$^{-1}$ at 24 h without adding SOD.

Table 4. Susceptibility of *M. avium* clones to the effect of nitric oxide

M. avium strain	No. of c.f.u. (ml macrophage lysate)$^{-1}$*		
	1 h	24 h	
		−N-MA	+N-MA
WT (MAC 104)	$6.4 \pm 0.3 \times 10^5$	$8.3 \pm 0.3 \times 10^5$	$8.7 \pm 0.5 \times 10^5$
STM1	$2.0 \pm 0.4 \times 10^5$	$8.1 \pm 0.3 \times 10^4$	$8.5 \pm 0.6 \times 10^4$
STM2	$4.4 \pm 0.2 \times 10^5$	$7.6 \pm 0.3 \times 10^4$	$8.1 \pm 0.2 \times 10^4$
STM3	$5.2 \pm 0.5 \times 10^5$	$4.1 \pm 0.3 \times 10^5$	$3.8 \pm 0.4 \times 10^5$
STM4	$2.6 \pm 0.2 \times 10^5$	$1.0 \pm 0.3 \times 10^5$	$9.8 \pm 0.2 \times 10^5$
STM5	$3.4 \pm 0.2 \times 10^5$	$9.8 \pm 0.3 \times 10^4$	$1.0 \pm 0.4 \times 10^5$
STM6	$3.9 \pm 0.5 \times 10^5$	$2.8 \pm 0.4 \times 10^5$	$2.7 \pm 0.6 \times 10^5$
STM8	$3.8 \pm 0.2 \times 10^5$	$8.3 \pm 0.3 \times 10^4$	$7.6 \pm 0.5 \times 10^4$
STM10	$4.6 \pm 0.3 \times 10^5$	$4.1 \pm 0.5 \times 10^4$	$1.6 \pm 0.3 \times 10^5$†
STM11	$2.5 \pm 0.5 \times 10^5$	$7.8 \pm 0.4 \times 10^4$	$1.2 \pm 0.6 \times 10^5$†

*RAW 246.7 macrophage monolayers were infected for 1 h with *M. avium*, washed, and all of the wells were treated with IFN-γ and with 100 μM *N*-methylarginine. The monolayers were lysed after 24 h and the intracellular bacteria quantified. The experiments were repeated three times.
†$P<0.05$ compared with the number of bacteria at 24 h without *N*-methylarginine.

(Chan *et al.*, 1992; Gomes *et al.*, 1999). In fact, it seems that the inability of macrophages to produce nitric oxide leads to increased control of the infection (Appelberg & Orme, 1993; Bermudez, 1993; Gomes *et al.*, 1999). MAV4012 is a transcription regulator, and its inactivation was associated with increased susceptibility to ROIs and NO. Because MAV_4012 is a transcription regulator, one might suggest that the expression of this gene regulates the expression of a number of membrane-associated genes. When tested individually for susceptibility against NO, STM10, STM11 and, to a lesser extent, STM5 were susceptible. The reason for the discrepancy between the results obtained in macrophages and regarding STM5 susceptibility *in vitro* to NO is unknown at this point. MAV_4292 is a gene encoding a protein of unknown function. Of importance is the finding that other proteins, but not SOD or correlated protein, were identified associated with resistance to superoxide anion and reactive nitrogen intermediates. Future studies will address these specific questions.

Vacuole pH

We determined the pH of the vacuole of STM mutants and compared it to the WT pH. As shown in Table 5, while the *M. avium* 104 vacuole pH was 6.4 at 15 min and 6.9 at 24 h, STM1, STM5, STM10 and STM11 had significant acidification in the macrophage vacuole. The vacuole of STM6, although significantly more acidic than the WT bacterium, was less acidic than the vacuole of the other STM mutants.

The pH in the vacuole of the STM mutants was acidic, in contrast to the pH of the vacuole of the WT bacterium. Except for one strain, in which the gene inactivated is a regulator, all other mutants had significantly less decrease in the pH of the vacuole compared with *M. avium* 104. The interpretation of the findings suggests that MAV_4264 regulates a series of genes that participate in the bacterial inhibition of phagosome acidification.

Complementation of phenotype

Complementation of STM1, STM5, STM8, STM10 and STM11 mutants was accomplished and the complemented clones were then evaluated for attenuation in RAW 246.7 macrophages. The results, shown in Table 6, indicate that the complementation of STM1, STM5, STM8 and STM10 recovered the virulent phenotype, with similar results to WT, while the attempt to complement STM11 was only partially successful (Table 6).

Table 5. Vacuole pH of RAW 264.7 macrophages

Strain	pH	
	15 min	24 h
WT (MAC 104)	6.4	6.9
STM1	5.3*	5.6*
STM5	5.5*	5.7*
STM6	5.7*	6.5*
STM10	5.2*	5.1*
STM11	4.8*	4.5*

*$P<0.05$ compared with the WT MAC 104. Experiments were repeated three times.

Table 6. Ability of complemented strains STM1 comp, STM5 comp, STM8 comp, STM10 comp and STM11 comp to replicate in murine macrophages

Assays were repeated three times. The numbers represent means ± SD.

Strain	No. of c.f.u. (ml macrophage lysate)$^{-1}$	
	1 h	4 days
WT MAC 104	$2.3 \pm 0.3 \times 10^5$	$7.1 \pm 0.2 \times 10^5$
SMT1	$2.7 \pm 0.4 \times 10^5$	$7.0 \pm 0.4 \times 10^4$*
STM1 comp	$2.1 \pm 0.5 \times 10^5$	$6.7 \pm 0.4 \times 10^5$†
STM5	$3.8 \pm 0.4 \times 10^5$	$1.6 \pm 0.3 \times 10^4$*
STM5 comp	$2.5 \pm 0.6 \times 10^5$	$5.9 \pm 0.5 \times 10^5$†
STM8	$5.0 \pm 0.4 \times 10^5$	$6.7 \pm 0.3 \times 10^4$*
STM8 comp	$4.1 \pm 0.3 \times 10^5$	$6.9 \pm 0.4 \times 10^5$†
STM10	$2.8 \pm 0.2 \times 10^5$	$9.8 \pm 0.6 \times 10^3$*
STM10 comp	$3.4 \pm 0.4 \times 10^5$	$4.1 \pm 0.5 \times 10^5$†
STM11	$3.0 \pm 0.3 \times 10^5$	$6.1 \pm 0.3 \times 10^3$*
STM11 comp	$4.4 \pm 0.5 \times 10^5$	$9.5 \pm 0.4 \times 10^4$†

*$P<0.05$ compared with the WT MAC 104 at day 4.
†$P<0.05$ compared with the mutated strain at day 4.

Conclusions

In summary, by screening an STM library, we have identified a number of virulence genes in *M. avium* required for the survival of the bacteria in macrophages and in the experimental host. Several of the genes appear to be directly associated with the resistance to macrophage mechanisms of killing.

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