Adaptive Changes in *Mycobacterium avium* Gene Expression Profile Following Infection of Genetically Susceptible and Resistant Mice

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ABSTRACT **We performed a comparative analysis of *Mycobacterium avium* transcriptomes (strain 724R) in infected mice of two different strains- resistant and susceptible to infection. Sets of mycobacterial genes transcribed in lung tissue were defined, and differentially transcribed genes were revealed. Our results indicate that *M. avium* genes coding for enzymes of the Krebs cycle, oxidative phosphorylation, NO reduction, fatty acid biosynthesis, replication, translation, and genome modification are expressed at high levels in the lungs of genetically susceptible mice. The expression of genes responsible for cell wall properties, anaerobic nitrate respiration, fatty acid degradation, synthesis of polycyclic fatty acid derivatives, and biosynthesis of mycobactin and other polyketides is increased in the resistant mice. In the resistant host environment, *Mycobacterium avium* apparently transitions to a latent state caused by the deficiency in divalent cations and characterised by anaerobic respiration, degradation of fatty acids, and modification of cell wall properties.**
KEYWORDS *Mycobacterium avium*, **transcriptome analysis** *in vivo*, **coincidence cloning, RNA-seq.**

INTRODUCTION

Infectious diseases caused by intracellular pathogenic bacteria represent a significant challenge in health care. The course of the infection depends not only on the protective mechanisms (native and acquired immune response, and mucous barriers), but also on the specific expression of bacterial genes. Altered expression as a response to the immune reaction of the host organism is critical for the survival and functioning of pathogenic bacteria. An analysis of these changes is important for understanding how infectious diseases proceed and developing effective approaches towards their treatment.

Mycobacterium avium are widespread mycobacteria that become intracellular pathogens in humans in the absence of normal T-cell-mediated immunity [1, 2]. These bacteria are found in approximately 70% of incurable AIDS patients and are believed to be the main cause of death in such patients [3]. In patients with weakened immunity (older people and children), *M. avium* may cause chronic lung diseases [4–6]. Experiments modelling the infection in mice of the C57BL/6 (B6) and derivative strains with knockout mutations in genes essential for immunity showed that T-cell-mediated immune response to *M. avium* had both defensive, as well as pathogenetic functions. In such an infection, the balance between the immune response and pathogenic processes in lung tissue is very similar to that of tuberculosis [7–9]; therefore, we can assume that the diseases caused by these mycobacteria are similar not only in their immune system mechanisms, but also in the mechanisms employed by the pathogens to overcome this defence.

It has been shown that mice of the I/St (I/StSnEgY-Cit) and B6 strains differ in their ability to resist an *M. avium*-induced infection [10]. Respiratory infection in B6 mice leads to a prolonged infiltration of lung tissue by macrophages and neutrophils, leading to the formation of necrotic lung granulomas and death. In contrast, in the I/St mice the infection is controllable, produces moderate infiltration of lung tissue, leading to small and medium granulomas without a necrotic centre, and the animals survive. The susceptibility of B6 mice to the *M. avium*-induced infection was shown to result from the presence of the nonfunctional allele of the *Nramp1* (natural resistance-associated macrophage protein-1) gene in their genome. The protein coded for by that gene consists of 12 transmembrane domains and is expressed at the membranes of late lysosomes and phagosomes. Nramp1 functions by removing divalent cations

Table 1. Oligonucleotides and primers used for coincidence cloning.

	Name	5'–3' structure
Suppressive adapter 1A (resulted from anneal of equimolar mixture of 1A long and 1A short)	1A long	GTAATACGACTCACTATAGGGCAGCGTGGTCGCGGCCGAGAG
	1A short	CTCTCGGCCG
Suppressive adapter 1B	1B long	GTAATACGACTCACTATAGGGCAGGGCGTGGTGCGGAGGGCGGC
	1B short	GCCGCCCTCC
Suppressive adapter 2A	2A long	GTAATACGACTCACTATAGGGCAGGCAGGCGGTGGTGGGCAGGC
	2A short	GCCTGCCCAC
Suppressive adapter 2B	2B long	GTAATACGACTCACTATAGGGCAGCGGAGGCGGTAGGAGGCGGA
	2B short	TCCGCCTCCT
External primer	T7	GTAATACGACTCACTATAGGGC
Internal primers	pr 1A	AGCGTGGTCGCGGCCGAGAG
	pr 1B	AGGGCGTGGTGCGGAGGGCGGC
	pr 2A	AGGCAGGCGGTGGTGGGCAGGC
	pr 2B	AGCGGAGGCGGTAGGAGGCGGA

(Fe^{2+}, Mn^{2+}, etc.) from phagosomes, thus depriving the mycobacteria of important metabolites [10].

The B6 immune response is characterized by an increased production of IFN-γ, TNF-α, and, especially, IL-12. We suppose that the differences in the immune response to *M. avium* infection are manifested in the differences in the pathogen expression in the lungs and lymphoid organs of mice of the susceptible strain versus those of the resistant strain, showing that the mechanisms essential for resistant host survival may not activate during infection of the susceptible host.

This work endeavoured to study the biochemical processes involved in the adaptation of *M. avium* to genetically different host organisms. We compared sequences transcribed in mice of the I/St and B6 strains in the 13th week of infection.

EXPERIMENTAL

Standard DNA and RNA procedures were carried out according to ref. [11]. Genomic DNA of the *M. avium* 724R strain was isolated according to the procedure described in ref. [12].

Infection

Mice of inbred strains I/StSnEgYCit (I/St) and C57BL/6YCit (B6) were bred and maintained under conventional, non-specific-pathogen-free conditions at the Animal Facilities of the Central Institute for Tuberculosis (Moscow, Russia) in accordance with guidelines from the Russian Ministry of Health (guideline 755) and the NIH Office of Laboratory Animal Welfare (assurance A5502-06). Female mice, 2.5–3.0 months old, were infected by the respiratory route with 1-2 × 10^3 viable CFU of *M. avium* 724R strain, characterized in ref. [13], using an inhalation exposure system (Glas-Col, USA) according to the procedure described in ref. [10].

RNA isolation and cDNA synthesis

RNA was isolated from the lungs of mice of both strains in the 13th week after infection, using the RNA Isolation System kit (Promega, USA). RNA samples were treated with DNase I (MBI Fermentas, Lithuania) to remove DNA traces. The first cDNA strand was constructed using oligonucleotide primers BR (5'-AAGC AGTGGTATCAACGCAGAGTAC$(N)_9$) and SMART

(5'-AAGCAGTGGTATCAACGCAGAGTACGCrGr GrG). Both primers (at 12 μM) were annealed with 2 μg of total RNA in 11 μl of solution. The mixture was incubated for 2 minutes at 70°C and then placed in ice for 10 minutes. cDNA was synthesised using reverse transcriptase PowerScript II (Clontech, USA). In parallel with reverse transcription (RT+), a reaction used as a control (RT-) without reverse transcriptase was performed. The RT+ and RT- mixtures were incubated at 37°C for 10 minutes, then for 40 minutes at 42°C. cDNA was synthesised in 30 PCR cycles (95°C for 20 sec, 64°C for 20 sec, and 72°C for 2 min) using 5S primers (5'-GTGGTATCAACGCAGAGT). Then, cDNA was purified using QIAquick PCR Purification kit (Qiagen, USA).

Coincidence cloning was carried out following the procedure described in ref. [14]. Genomic DNA of the *M. avium* 724R strain and total cDNA samples (synthesised using total RNA) were fragmented with restrictases RsaI and AluI. The obtained genomic DNA fragments were ligated with suppressive adaptors 1A for hybridisation with the I/St cDNA sample, and adapters 1B for hybridisation with the B6 cDNA sample (Table 1). Suppressive adapters 2A and 2B were ligated to cDNA fragments from the lung tissues of I/St and B6 mice, respectively. A mixture of 100 ng of the genomic DNA sample and 100 ng of one of the cDNA samples in 2 μl of the hybridisation buffer (50 mM HEPES, pH 8.3; 0.5 M NaCl; 0.02 mM EDTA, pH 8.0) was incubated at 99°C for 5 minutes (denaturation) and then at 68°C for 18 hours (renaturation). After this procedure, 100 μl of the hybridisation buffer at 68°C was added to the mixture, and 1 μl of the resulting solution was used as a template for PCR. The first PCR stage was performed in a 25 μl reaction volume containing 10 pmol of T7 primer. After incubation for 5 minutes at 72°C (filling-in sticky ends), 20 amplification cycles were carried out (94°C for 30 sec, 66°C for 30 sec, and 72°C for 90 sec). The second stage of amplification was performed with 10 pmole of internal primers pr1A/pr1B and pr2A/pr2B, and it consisted of 25 cycles (94°C for 30 sec, 68°C for 30 sec, and 72°C for 90 sec), using the PCR product of the first stage, diluted ten-fold. The amplification product was purified using the QIAquick PCR Purification kit (Qiagen) and then used for 454 sequencing.

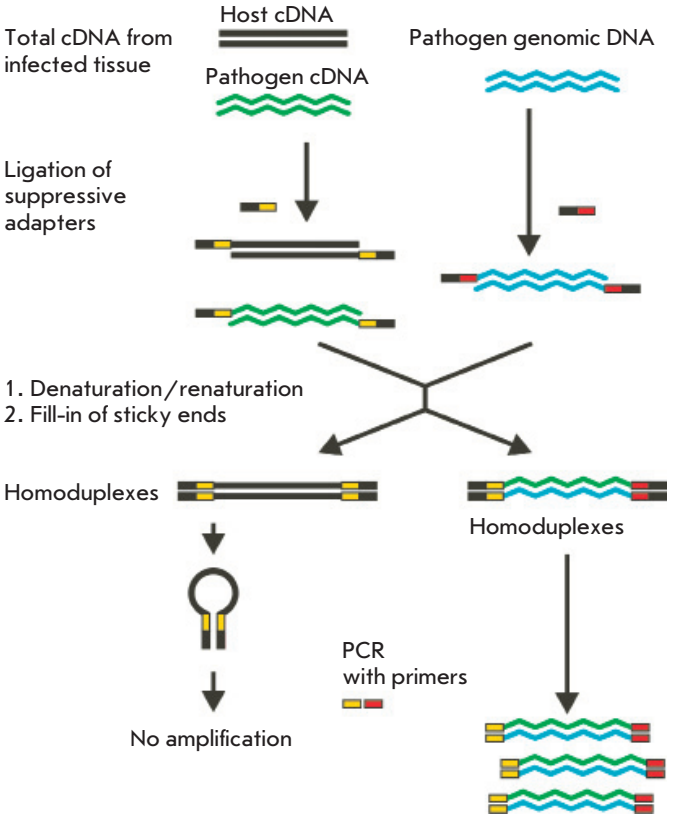

Fig. 1. Coincidence cloning. Suppressive oligonucleotide adapters are ligated to fragments of bacterial genomic DNA and total cDNA. The samples are mixed, denatured, and slowly renatured, which leads to the formation of two types of duplexes. Due to selective suppression of PCR, only heteroduplexes containing fragments of bacterial genomic DNA and bacterial cDNA are amplified.

454 SEQUENCING

Nucleotide sequences of cDNA libraries were determined by massive parallel pyrosequencing using the genetic analyser GS FLX (Roche, Germany) and a 20 x 75 cm picotitration plate. The sequences of 83,000 independent reactions were determined. The sequences were mapped to the genome sequence of the *M. avium* strain 104, since the *M. avium* 724R genome has not yet been sequenced. The number of cDNA fragments corresponding to each gene was determined using the BLASTn algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi). A sequence was considered to belong to a certain gene if a fragment of that sequence had more than 95% homology with the gene segment longer than 40 nucleotides. The *M. avium* genes, the expression of which in samples I/St and B6 is significantly different, were determined following the procedure described in ref. [15].

RESULTS AND DISCUSSION

The course of the pathology and immune response to *M. avium* infection in mice from the susceptible (B6) and resistant (I/St) strains are discussed in detail in refs. [10] and [16]. The airborne infected susceptible B6 mice died after 7 months, while the resistant I/St mice survived for longer than 11 months. In the susceptible B6 mice, the lung pathology developed quickly,

Table 2. *M. avium* genes differentially transcribed in the lungs of infected I/St and B6 mice.

Gene	Coded protein
Increased expression in the lungs of the I/St mice	
MAV_2015	MbtG; mycobactin lysine-N-oxygenase
MAV_1696	Glutamate dehydrogenase
MAV_1304	NarH; nitrate reductase, β-subunit
MAV_2379	MetH; vitamin B12-dependent methionine synthase
MAV_2385	Mce protein
MAV_2063	Mce protein
MAV_2386	Mce protein
MAV_0118	PPE protein
MAV_3109	RifB; polyketide synthase 7
MAV_0880	3-Ketosteroid-δ-1-dehydrogenase
MAV_3000	Acyl-CoA dehydrogenase
MAV_4019	Assumed acyl-CoA dehydrogenase
MAV_4679	Cyclopropane fatty acid synthase
Increased expression in the lungs of the B6 mice	
MAV_2514	PPE protein
MAV_2924	PPE protein
MAV_2926	PPE protein
MAV_2244	GlnA; glutamine synthetase
MAV_4011	NO-reductase, β-subunit
MAV_1074	SucC; succinyl-CoA-synthase, β-subunit
MAV_3303	AcnA; aconitate hydratase
MAV_1130	NADH-dehydrogenase I, H-subunit
MAV_4040	NADH-dehydrogenase I, H-subunit
MAV_1524	ATP-synthase F_1F_O, δ-subunit
MAV_5034	Transposase
MAV_1059	Transposase

accompanied by enhanced infiltration of lung tissue by immune system cells and increased production of pro-inflammatory cytokines IFN-γ, TNF-α, IL-6, and IL-12. Two parameters showing the susceptibility to infection correlated well: in the susceptible B6 mice, *M. avium* grew faster in lungs, and the lung pathology was deeper than in the resistant I/St mice.

We studied transcribed sequences *in vivo* using the coincidence cloning method we had developed previously [14, 17]. From the lungs of infected mice, we isolated total RNA, a mixture of the mice and bacterial RNA, with the amount of bacterial RNA being very small (less than 0.1–0.2% according to [18]). Using the total RNA from the I/St and B6 mice, total cDNA was synthesized. In the coincidence cloning method (Fig. 1), total cDNA and *M. avium* genomic DNA were denatured and renatured in one mixture. After a two-step selective PCR amplification, a set of fragments enriched with the bacterial cDNA fragments was obtained.

Qualitative (determination of nucleotide sequences of specifically expressed genes) and quantitative (the level of their expression) analyses of the sets were performed using parallel pyrosequencing.

The sequencing produced two libraries of *M. avium* cDNA sequences expressed in the lung tissue of infected I/St and B6 mice. We selected a series of genome loci, the expression of which was higher in sample I/St than in sample B6, and a series of loci, the expression of which was higher in sample B6 than in sample I/St (Table. 2). Locus annotation was performed using the KEGG (www.genome.jp/kegg) database. We anticipated that differential gene expression in the samples could be a manifestation of the microorganism's environmental adaptation; therefore, the products of the genes we found could be potential virulence factors.

We found the differential expression of the PPE gene family (*MAV_0118*, *MAV_2514*, *MAV_2924*, and *MAV_2926*). These proteins play an important role in the course of the mycobacterial infection because of both their antigen and immune functions. These acidic proteins, rich in glycine, are identified by the specific Pro-Pro-Glu (PPE family) and Pro-Glu (PE family) domains; they often contain polymorphic GC-rich sequences (PGRSs) and multiple copies of basic polymorphic tandem repeats. It is believed that these proteins are expressed on the cell's surface and are responsible for antigen variability, inducing different immune responses depending on the type of PE/PPE proteins expressed on the cell's surface [19]. Thus, the *MAV_0118* gene is expressed in the resistant mice, while the *MAV_2514*, *MAV_2926*, and *MAV_2924* genes are expressed in the susceptible mice. Since the mechanism of PPE protein action remains unknown, the above-mentioned observation is hard to explain; however, it

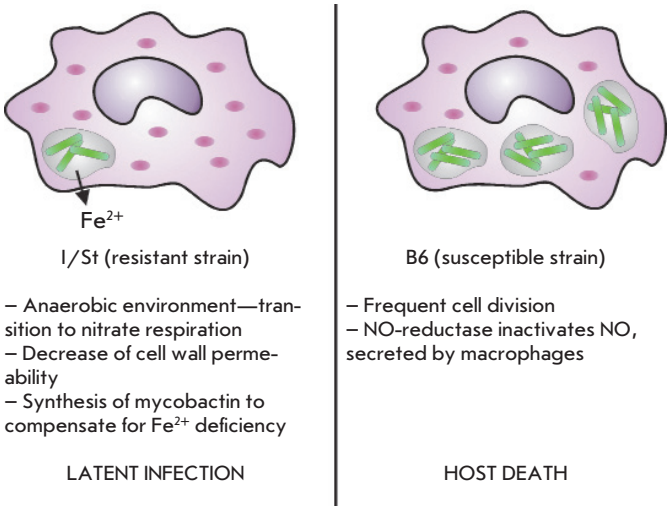

Fig. 2. *M. avium* metabolic state in the lungs of the I/St and B6 mice

is possible that the differential PPE expression is due to the differences in the immune responses.

The expression of the *MAV_2244* locus is increased in the B6 sample. This gene is an ortholog of the *glnA1 M. tuberculosis* gene; it codes for glutamine synthetase, a key enzyme for nitrogen assimilation. It has been shown that this enzyme is important for *M. tuberculosis* persistence in macrophages. It is possible that in infected B6 mice *M. avium* enters into an environment auxotrophic for *L*-glutamine [20]. The *MAV_4011* locus that codes for the cytochrome b-containing subunit of NO-reductase is also worth mentioning. This enzyme reduces NO to N_2O, and it participates in the denitrification process in some soil microorganisms. However, no denitrification is observed in the *M. avium* from the susceptible B6 mice. It is speculated that *M. avium* use NO-reductase to get rid of the NO released by the macrophage into the endosomes and thus avoid the harmful effects of NO [21]. This could be the reason why *M. avium* are resistant to NO [22]. The expression of NO-reductase in *M. avium* from the lungs of the susceptible mice could be a result of the stronger immune response and increased NO production by macrophages.

In sample B6, we observed a more active and diverse expression of genes coding for the Krebs cycle enzymes: *MAV_1074* and *MAV_3303* coding for succinyl-CoA–synthesase and aconitate hydratase, respectively; and of genes coding for the proteins important for oxidative phosphorylation, as well as the respiratory electron-transport chain proteins: *MAV_1130*, *MAV_4040*, and *MAV_1524*. It is likely that in the susceptible mice, respiration is increased during persistent infection in order to supply the pathogen. The *MAV_4040* locus codes for one of the NADH-dehydrogenase I subunits, which is typical for the *M. tuberculosis* virulent form, during exponential proliferation of the pathogen [23].

The expression of the *MAV_5034* and *MAV_1059* genes coding for transposases in sample B6 indicates an enhanced level of gene rearrangements. Also, in sample B6, we detected an increased expression of the *MAV_5024* and *MAV_5027* genes coding for type II restriction-modification enzymes that protect cells from foreign DNA.

The increased expression of the *MAV_0382* (subunits of DNA-polymerase III) and *MAV_4450* (ribosomal protein) genes in sample B6 indicates an increase in the DNA replication level due to a more frequent mitosis, as well as a higher translation level.

In sample I/St, there was a very high level of expression of the *MAV_2015* gene that codes for mycobactin lysine-N-oxygenase (MbtG). This enzyme is responsible for one of the last stages of mycobactin synthesis; it is an iron-chelating agent that supplies the microorganism with iron from the environment [24]. It has been shown for *M. tuberculosis* that the activation of the *mbt B-H* gene cluster involved in mycobactin synthesis occurs either when the environment is depleted in iron [25] or in an anaerobic environment [26]. The expression of this gene is high in *M. avium* from the resistant I/St mice, but it is very low in the B6 mice. As mentioned above, these two mice strains differ in the *Nramp1* gene allele that codes an ionic pump which is assumed to pump out divalent cations from the endosomal region, where *M. avium* is located [16]. There is the functional allele of this gene in the I/St mice, as opposed to a nonfunctional one in the B6 mice. Apparently, the *M. avium* endosomes from the resistant mice are iron-deficient, and the microorganism synthesizes vast amounts of mycobactin in order to compensate for the deficiency.

In *M. avium* from the I/St mice, an increased expression of the *MAV_1696* gene coding for NAD^+-dependent glutamate dehydrogenase is observed. It is believed that, in contrast to $NADP^+$-dependent glutamate dehydrogenase, which is responsible for nitrogen assimilation, in microorganisms the former enzyme takes part in the glutamate catabolism, and this gene expression is independent of the NH_4^+ concentration. On the other hand, it has been shown recently that in *M. smegmatis* the expression of the *msmeg_4699* gene, an ortholog of *MAV_1696*, increases in response to NH_4^+ deficiency [27]. In addition, there is no gene coding for $NADP^+$-dependent glutamate dehydrogenase in the *M. avium* genome [28]. Some researchers speculate that, in mycobacteria, nitrogen assimilation involving NAD^+-dependent glutamate dehydrogenase may be more energy-efficient than via the GS/GOGAT pathway; this

being important, for example, when the pathogen is in a latent state [27].

The *MAV_2379* gene coding for B_{12}-dependent methionine synthase MetH is expressed at a high level in *M. avium* from the I/St mice. This protein is involved in the final stage of methionine synthesis. In the *M. avium* genome, this reaction is controlled by MetE-B_{12}-independent methionine synthase, which is not expressed in the presence of vitamin B_{12} [29]. The regulation of the *metH* gene expression has not been studied in detail, so the reason for the increase in its expression in the resistant mice is not quite clear.

The *MAV_1304* locus coding for the β-subunit of nitrate reductase is of particular interest. This gene is orthologous to the *narH* gene of *M. tuberculosis*. Its product is a subunit of anaerobic nitrate reductase NarGHJI, an enzyme enabling nitrate respiration in the absence of oxygen. Mutants lacking NarH cannot reduce nitrogen under anaerobic conditions [30]. When this gene was deleted in *M. bovis*, BCG bacteria demonstrated normal growth *in vitro* with sufficient oxygen supply; however, they appeared significantly less virulent when used for infecting mice [31]. The expression of the *MAV_1304* gene in *M. avium* from the lungs of the I/St mice might be an indication that, due to the harmful effects of the host's defence systems, the microorganism is subjected to anaerobic conditions and has to switch to nitrate respiration.

The *MAV_2063, MAV_2385*, and *MAV_2386* genes coding for proteins from the Mce family are expressed in the resistant mice. The function of the Mce proteins has not been clarified, although it is known that they enable invasiveness. These proteins supposedly represent a new group of ABC-transporters participating in the remodeling of the cell's membrane [32].

The *MAV_4679* locus coding for an enzyme involved in the synthesis of mycolic acids is expressed at a high level in *M. avium* from the I/St mice. An ortholog of this gene in *M. tuberculosis* is important for persistence in mice lungs. Mutants of this gene cannot cause lung infection in mice [33].

The *MAV_3109* locus codes for the RifB protein and is an ortholog of the *M. tuberculosis pks7* gene. An increased expression of the gene is observed in infected mice of the resistant strain. The protein product of this gene codes for an enzyme involved in the synthesis of phthiocerol dimycocerosate, one of the components of the mycobacterial cell wall, which ensures its impermeability [34].

The *MAV_0880* locus codes for 3-ketosteroid-δ-1-dehydrogenase, one of the enzymes involved in cholesterol catabolism. During the *M. tuberculosis*-induced infection, cholesterol provides the pathogen with energy for persistence in macrophages [35]. In *M. avium* from the I/St mice, we observed an increased expression of the *MAV_3000* and *MAV_4019* genes coding for enzymes degrading fatty acids: acyl-CoA-dehydrogenase and acyl-CoA-synthase. During persistence in macrophages, the catabolism of fatty acids is the primary energy source for *M. tuberculosis* [36].

CONCLUSION

This paper contains the first description of the *M. avium* transcriptome during infection *in vivo*. Until now, only a single publication on the *M. avium paratuberculosis* transcriptional response to various factors *in vitro* [37] has been available.

We employed the model of genetic control of susceptibility to *M. avium* infection and disease severity in mice in order to detect the sequences that are transcribed differently in infected mice from the genetically resistant and genetically susceptible strains, i.e. when the pathogen persists in genetically different microenvironments. We obtained data on the qualitative and quantitative differences in the transcription profiles of genes of bacteria persisting in the resistant and susceptible mice, which indicate some changes in the metabolism of *M. avium* (Fig. 2).

In the course of the infection in the genetically susceptible organism (B6 strain), we found an increased expression of several genes responsible for nitrogen assimilation, NO reduction, the Krebs cycle, and oxidative phosphorylation, as well as replication and translation. The infection proceeds with active division of the mycobacteria and death of the host organism.

In the course of infection in the genetically resistant organism (I/St strain), we found an increased expression of several genes responsible for the modification of the cell surface's properties, switching to anaerobic nitrate respiration, degradation of fatty acids, synthesis of polycyclic derivatives of fatty acids, and biosynthesis of mycobactin and other polyketides. In general, the changes in the *M. avium* metabolism are an indication that, in the resistant mice, the bacterial pathogen transitions to the latent state, because of the deficit in divalent metal ions. ●

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