Usefulness of *acr* Expression for Monitoring Latent *Mycobacterium tuberculosis* Bacilli in 'In Vitro' and 'In Vivo' Experimental Models

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Abstract

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Real-time RT-PCR was used to quantify the expression of genes possibly involved in *Mycobacterium tuberculosis* latency in *in vitro* and murine models. Exponential and stationary phase (EP and SP) bacilli were exposed to decreasing pH levels (from 6.5 to 4.5) in an unstirred culture, and mRNA levels for 16S rRNA, sigma factors *sigA,B,E,F,G,H* and *M*, Rv0834c, *icl*, *nirA*, *narG*, *fpbB*, *acr*, *rpoA*, *recA* and *cysH* were quantified. The expression of *acr* was the one that best correlated with the CFU decrease observed in SP bacilli. In the murine model, the expressions of *icl*, *acr* and *sigF* tended to decrease when bacillary counts increased and *vice versa*. Values from immunodepressed mice (e.g. α/β T cells, TNF, IFN-γ and iNOs knock out strains), with accelerated bacillary growth rate, confirmed this fact. Finally, the expression of *acr* was maintained in mice following long-term treatment with antibiotics. The quantification of *acr* expression could be useful for monitoring the presence of latent bacilli in some murine models of tuberculosis.

Introduction

Latent *Mycobacterium tuberculosis* bacilli are probably the main problem in the eradication of tuberculosis. Their presence in infected tissues makes the treatment of both infected and tuberculosis patients difficult, forcing them to follow long chemotherapy schedules. However, as the diagnosis of tuberculosis infection is essentially immunologically based [1], it is not known for sure whether latent bacilli are present in infected patients, although other methods have also been used in the study of latency. In experimental models and in HIV-positive humans, induction of immunodeficiency often results in reactivation of disease implying that bacilli must have been there. In addition, *M. tuberculosis* DNA can often be demonstrated in lung tissue by PCR during latency in humans [2]. This fact and the progressive knowledge on the genetics and proteomics of *M. tuberculosis* accumulated over the last 10 years [3, 4] will help us to better understand the nature of latent bacilli and to obtain better tools to detect them.

Classical works by Wayne and Sohaskey [5] on the origin of latent *M. tuberculosis* focused on a hypothesis concerning the histopathology of human lesions that suggested that the metabolism of latent bacilli may be adapted to low oxygen pressure in order to fit the conditions developed in granulomas, i.e. the presence of compact necrosis

(*caseum*) and their surrounding fibrosis. As a result, many works studied bacilli in '*in vitro*' models in order to evaluate their expression. In this sense, overexpression of the enzyme isocitrate lyase (*icl*) from these bacilli was one of the first to explain a metabolic change needed for survival in these conditions [6]. On the other hand, classical works by Balasubramanian *et al.* [7] and Opie and Aronson [8] show clearly that *M. tuberculosis* also occurs in tissue outside of granulomas, thus questioning this approach.

An increased production of 16 kDa α crystalline protein (*acr*) was also observed after the exposure of *M. tuberculosis* to nitric oxide donors, thus resembling the conditions induced by the immune response [9]. Interestingly, the presence of this protein was linked to an increase in cell wall thickness, which is characteristic of bacteria in stressful conditions [10], and to other specific stressful conditions for *M. tuberculosis*, such as treatment with antibiotics (e.g. rifampicin or streptomycin) [11]. This increased production was also related to a higher expression of the sigma factor F (*sigF*), a σ subunit of the RNA polymerase that is stimulated under stress. The study of sigma factors added another powerful link in order to try to understand the mechanisms associated with the metabolic changes that may lead to the induction of latent bacilli [12].

In some murine models of tuberculosis, where granulomas usually have no necrosis and do not trigger a powerful fibrotic reaction [13] that would justify the hypothesis that latent bacilli must adapt to anaerobic conditions, the presence of latent bacilli was well documented a long time ago (i.e. when studies conducted at Cornell University gave name to the homonymous experimental model). Bacilli were detected in this model after a long resting time despite a prolonged antibiotic treatment that resulted in a long period in which no viable counts were detected in the tissues [14]. Despite some criticism on the relation of this model to the induction of latent bacilli, which associated it with antibiotic tolerance [15], this model questions the adaptation of bacilli to low oxygen pressure, as granulomatous lesions are uncommon at the end of this prolonged antibiotic treatment. More recent studies with transgenic *M. tuberculosis* lacking specific genes revealed new issues, such as the adaptation of bacilli to obtain nutrients, where *icl* becomes a crucial gene for obtaining energy from the host fatty acids [16].

Another interesting fact concerning the murine tuberculosis murine model is that these animals may control infection after acquiring specific immunity. However, this reaction only allows them to reduce the bacillary concentration by 1 $\log_{10}$ [13]. The question on how the remaining bacilli resist the immune response is another good point that is worth assessing when studying the nature of latent bacilli. In fact, a long time ago, Wallace [17] demonstrated that bacilli obtained from chronically infected mice lungs were more resistant to heat stress than those obtained from lungs in the acute phase of infection. This author also demonstrated that bacilli from old cultures had a greater resistance to heat than those from young cultures. The interpretation of this finding suggested that chronically infected lungs had bacilli with a less active metabolism, thus increasing their ability to resist stressful conditions.

Transition from acute to chronic infection is related to the development of acquired cell-mediated immunity, mediated by the activation of antigen-specific T cells and macrophages, which in turn results in a 'static' equilibrium where bacterial replication is dramatically reduced [18, 19]. Recent works have demonstrated that the induction of this Th1-mediated response in a tuberculosis murine model following infection induces changes in *M. tuberculosis* transcription in the lungs of immunocompetent mice for up to week 8 postinoculation, measured by real-time RT-PCR. It transiently increases the expression of genes *acr* and Rv262c (with an unknown function) and decreases that of genes Rv2623 (increased *in vitro* with low oxygen levels), *sodA* and *C* (superoxide dismutases that tend to decrease with low oxygen levels), *fbpbA*, *B* and *C* (fibronectin-binding proteins that encode for antigen mycolyl transferases required for cell envelope biogenesis 85A, B and C), *pstS1* (encoding for 38 kDa antigen). Finally, the expression of *esat-6* slightly decreased [20, 21]. The expression of all these genes increased in IFN-γ knock out mice. Interestingly, data from *fbpB* contradicted the results of a previous work with immunocompetent mice that showed hyperexpression during stress [22].

At this point, which are the mechanisms used by macrophages activated against *M. tuberculosis* to induce the latter's slower metabolism? Essentially two: the decreasing the pH inside the phagosome, then promoting their binding to lysosomes [23–25], and also the production of reactive nitrogen intermediates (RNI) [9].

The aims of our work were to study the expression of latency-related genes with a real-time RT-PCR system in an 'in vitro' unstirred culture, allowing a progressive limited O_2 concentration; to study the induction of stressed bacilli by decreasing pH and using two types of bacilli (i.e. from cultures in an exponential and a stationary phase); to establish a parallelism between bacilli in the acute and chronic phases in a murine model of tuberculosis. The analysis of latency-related genes in this model allowed us to choose the most representative genes and study their expression in different murine models (including the chemotherapy and the Cornell models) in order to find a useful marker for latent bacilli. We concluded that the expression of *acr* is a solid marker for the presence of these bacilli in infected tissues.

Materials and methods

Bacteria. Mycobacterium tuberculosis standard strain NC007416 (H37Rv) was used and grown in Proskauer Beck medium containing 0.01% Tween 80 to mid-log phase and stored at $-70\,°C$ in 2 ml aliquots. Next, these aliquots were grown in Middlebrook 7H9 broth until mid-log phase ($OD_{595} = 0.4–0.6$) to be inoculated in other tubes to obtain the exponential phase (EP) or stationary phase (SP) cultures. Growth of mycobacteria was studied in Middlebrook 7H9 broth modified to pH 4.5, 5, 5.5, 6 and 6.5. All the cultures were unstirred and incubated at $37\,°C$. Evolution of CFU/ml was followed against time by plating serial dilutions on nutrient Middlebrook 7H11 agar (Biomedics s.l., Madrid, Spain) and counting bacterial colony formation after 21 days incubation at $37\,°C$. BACTEC® 13A mycobacteria-enriched culture vials (Becton Dickinson Diagnostic Instrument Systems, Sparks, MD, USA) were used for qualitative recovery of bacilli from those cultures were viable counts were not obtained. Briefly, when *M. tuberculosis* growth $^{14}CO_2$ is produced and liberated into the vial head space. After being inoculated with 1 ml of the sample, the culture vial is periodically inserted into the BACTEC® grand 460 TB instrument (Becton Dickinson) for testing, which consists of aspiration of the supernatant gas and assay or its radioactive content. A positive reading indicates the presence of viable micro-organisms in the vial.

Media and reagents. Middlebrook 7H9 broth was prepared in sterile conditions and supplemented with oleic acid dextrose complex, 0.2% glycerol and 0.05% Tween 80 to avoid clumping (Becton Dickinson). The 7H9 broth was acidified to pH 4.5, 5, 5.5, 6, 6.5 with the addition of 2N HCl filter sterilized. One millilitre aliquots of the modified 7H9 broth were added to sterile, capped glass tubes 10×100 mm, with a total fluid capacity of 9 ml.

Growth assay under several pH levels. Mycobacterium tuberculosis H37Rv was grown in Middlebrook 7H9 broth without modifications from one of the aliquots of H37Rv in 7H9 broth until exponential and stationary phase. EP was determined by measuring the $OD_{595} = 0.3–0.5$, and was reached after 5 days of incubation, whereas SP was determined at $OD_{595} = 0.9–1$, at about 15 days of incubation. Similar CFU values (up to 8 $\log_{10}$) were obtained. For each assay, the EP and SP cultures were inoculated in capped glass tubes previously acidified in triplicate and studied the survival and mRNA expression taking as time-point the day 0, 1, 4, 7, 10 and 15. Approximately, 10^7 bacilli in a 50 µl were added to each tube. The cultures were incubated at 37 °C without agitation, and serial dilutions were plated onto Middlebrook 7H11 agar at the indicated days after infection and counted as described above.

Estimation of oxygen consumption. An additional fourth capped glass tube was used in each experiment with a sterile solution of methylene blue (500 µg/ml) to obtain a dye concentration of 1.5 µg/ml. Reduction and decolourization of this dye served as a visual indication of oxygen depletion as described elsewhere [5].

RNA extraction and cDNA synthesis. For the *in vitro* study, 0.9 ml were snap-frozen in liquid nitrogen at indicated time-points and kept at −70 °C until its study. RNA extraction was performed using TRIzol (Gibco BRL, Grand Island, NY, USA) in combination with FastPrep products (Qbiogene Inc., Illkirch, France). Briefly, Lysing Matrix B tubes (Qbiogene Inc.) were filled with an adequate volume of TRIzol and sample, and homogenized in the cell fragmenter FastPrep FP120 for two cycles of 45 and 20 s at maximum speed chilling on ice between cycles. Next, RNA extraction was performed following manufacturer recommendations. Total RNA concentration was determined by spectrophotometry and, in addition, a denaturing agarose gel was used to check RNA stability. Total RNA was subjected to a DNase treatment with DNA-free kit (Ambion, Woodward Austin, TX, USA). Subsequently, 5 µg RNA was reverse transcribed using a Superscript RT kit (Gibco BRL) under the manufacturer recommendations using Random hexamer (Gibco BRL) to obtain cDNA.

For the murine model samples, the right middle lobes were snap frozen at −70 °C in liquid nitrogen and RNA extraction and DNase treatment were done exactly as described above. Next, two reverse transcriptase procedures were performed to obtain murine cDNA and bacterial cDNA in tissues. The murine cDNA was obtained as described before [25] but using OligodT (Gibco BRL) instead of Random Hexamer and the procedure to obtain bacterial cDNA in tissues was done according to *Transcriptor Reverse Transcriptase* (Roche Biochemicals, Idaho Falls, ID, USA) recommendations using specific primers strategy for each gene.

Quantitative analysis using real-time PCR. Regarding the *in vitro* study, quantitative analysis of 16 genes related to metabolism, cell wall biogenesis and sigma factors were studied in the LightCycler™ System (Roche Biochemicals) and are shown in Table 1.

We chose *sigA* and *rpoA* as markers of active growth; *sigB,E,F,G,H* and *M* as stress markers [12]; *recA* as it is included in the response of many bacteria against DNA damage (although recent studies have demonstrated that this gene has no role in *M. tuberculosis* [26]); *cysH* because it has been related to an antioxidant response in *M. tuberculosis* [27]; *icl* and *narG* as they have classically been related to the persistence of bacilli at low oxygen levels [5, 28–30], and *nirA*, for these same reasons, although it has been recently discovered that its nitrite

Table 1 Primer sequences for PCR

Gene	Sequence 5′–3′	Tª
16SrRNA	GCTTTAGCGGTGTGGGATGAGCC	68
(*rrs*)	CGCACGCTCACAGTTAAGCCGTG	
sigA	GAGATCGGCCAGGTCTACGGCGTG	60
(Rv2703)	CTGACATGGGGGCCCGCTACGTTG	
sigB	CGCCGCCGGTGAAGTCGAA	60
(Rv2710)	CAGGTCGCGTTTTCGGTTCT	
sigE	GTTGCCGACGGTGACGACTTG	65
(Rv1221)	CGCGGACCTGTTGGGGATGAG	
sigF	GCGGGTCGGGCTGGTCAAC	67
(Rv23286c)	CCTCGCCCATGATGGTAGGAAC	
sigG	CTGCGTAGGCTCATTGACGTG	65
(Rv0182c)	CGGTGTGGGCGGAGAAGTC	
sigH	CGCTGTTTCTTGCGATAG	60
(Rv3222c)	AGGACTTGCTCCAGGA	
sigM	ACCGCGCAGGTCGAGACC	60
(Rv3911)	GGGTGTCGGCGATCGAATAG	
PE_PGRS	GAGGACAGCAATGCCC	62
(Rv0384c)	GGCCTGATCTTTGCCC	
icl	GCGGAGCAGATCCAGCAGGT	64
(Rv0467)	GCGGCGGGCCAGCGTGTGCTC	
nirA	GAAGGAGAACCCCCAAT	62
(Rv2391)	CCTTCTTCAGCTCTTCGT	
narG	ACAAGCTGAAGCTCTCG	65
(Rv1161)	ACTGTACGTTGGAGTCG	
fbpB	GAGCGGTGGGAACAACTCA	60
(Rv1886c)	ACCGGCATGACTATCGACAGT	
acr	GAAGACGAGATGAAAGAGGGG	62
(Rv2031c)	GTAAGAATGCCCTTGTCGTAGG	
rpoA	AATTCTTGGTCTCCACG	62
(Rv3457c)	GCTGAACGATAAGGGCA	
recA	AGGCGCTGCGGAAAATGA	66
(Rv2737c)	CACCGACGCGTAGAACTT	
cysH	CAACATGGCTGATGCG	61
(Rv2392)	GCGAACAAGTCCTTGC	

reductase activity is inactive in *M. tuberculosis* [6, 29]. Finally, we also chose *acr* and *fbpB* because of their antigenic relevance in immunity against *M. tuberculosis*, and the close relation to latency demonstrated by the former [20, 21], although strong expression of *acr* has been also demonstrated in human active lung TB [31].

The cycle conditions and standards for every gene were performed as previously described [32].

As regards the *in vivo* study, quantitative analysis for IFN-γ, TNF-α, IL-4, IL-10, iNOS and RANTES, on postinfection weeks 0, 2, 3, 4, 5, 6, 9, 18 and 22 was performed using a LightCycler™ System. In addition, quantification of 16S rRNA, *icl*, *acr*, *sigF* and *sigH* expression at the same time-points was performed in tissues of infected mice.

Primer design and normalization to a housekeeping gene. All the primers were designed using the software LightCycler Probe Design™ (Roche Biochemicals).

For the *in vitro* study, all the primers are described in Table 1. 16S rRNA expression was analysed for every target sample in order to normalize for efficiencies in cDNA synthesis and RNA input amounts. A ratio was obtained according to 16S rRNA expression of every sample.

For the *in vivo* study, primers for the cytokines and housekeeping gene hypoxanthine phosphoribosyltransferase (HPRT) were used as previously described [32]. Specific primers for reverse transcriptase procedure to obtain bacterial cDNA are shown in Table 2. Normalization of efficiencies in cDNA synthesis and RNA input amounts was obtained according to HPRT expression of every sample. Moreover, the normalization of bacterial expression in tissues was done according to 16S rRNA expression that showed a good correlation with CFU/ml in the model of aerosol infection, $r^2 = 0.9425$ (Fig. 3B) and a constant profile. Normalization was not possible in the Cornell Model as after 8 weeks of chemotherapy no expression of 16S rRNA could be found.

Bacteria and infection. Mycobacterium tuberculosis standard strain H37Rv Pasteur was grown in Proskauer Beck medium as described above. Mice were aerogenically infected as previously described [32]. Briefly, mice were placed in the exposure chamber of an airborne infection apparatus (Glas-col Inc., Terre Haute, IN, USA). The nebulizer compartment was filled with 7 ml of a *M. tuberculosis* suspension at a previously calculated concentration to provide an approximate uptake of 20 viable bacilli within the lungs. Four mice were used for every time-point in every experimental group. The number of viable bacteria in the left lung was measured at weeks 0, 2, 3, 4, 5, 7, 9, 18 and 22 by plating serial dilutions on nutrient Middlebrook 7H11 agar (Biomedics s.l.) and counting bacterial colony formation after 21 days of incubation at 37 °C. Lungs were immediately extracted after euthanasia by means of a halothane overdose (Zeneca Farma, Pontevedra, Spain).

Mice. Our study was performed using specific pathogen-free (*spf*) C57BL/6 female mice, 6–8 weeks old, which had been obtained from Charles River (Bagneux Cedex, France). Knock out (KO) mice strains for IFN-γ (IFN-γ$^{-/-}$), chain TCR-α (TCR-α/β$^{-/-}$) and TNF (TNF-R1) were supplied by The Jackson Laboratory (Bar Harbor, ME, USA). Mice iNOS2 KO (NOS2$^{-/-}$) were kindly donated by Dr Irene García-Gabay from the Department of Pathology, University of Geneva, Switzerland. Mice were shipped in suitable travel conditions, with the corresponding certificate of health and origin. All the animals were kept under controlled conditions in a P3 High Security Facility with sterile food and water ad libitum. Chemotherapy was administered p.o. 5 days a week with isoniazid (25 mg/kg) and rifampicin (10 mg/kg).

Animal health. Mice were weighed once a week. They were supervised every day under a protocol paying attention to weight loss, apparent good health (bristled hair and wounded skin) and behaviour (signs of aggressiveness or isolation). Animals were euthanized with halothane (Fluothane, Zeneca Farma) overdose so as to avoid suffering. Sentinel animals were used to check *spf* conditions in the facility. Tests for 25 known mouse pathogens were all negative. All experimental proceedings were approved and supervised by the Animal Care Committee of 'Germans Trias i Pujol' University Hospital in agreement with the European Union Laws for protection of experimental animals.

Statistical analysis. SIGMA STAT (Jandel Scientific Software, San Rafael, CA, USA) was used to compare values. One-way ANOVA Dunnet's test was used to compare the values from the different pH conditions with the more physiological values (pH 6.5), shown in Figs 1 and 2. All Pairwise Multiple Comparison Procedure (Student–Newman–Keuls Method) were used to compare CFU and expression values shown in Figs 3–5. Differences were significant when marked with * for $P < 0.05$.
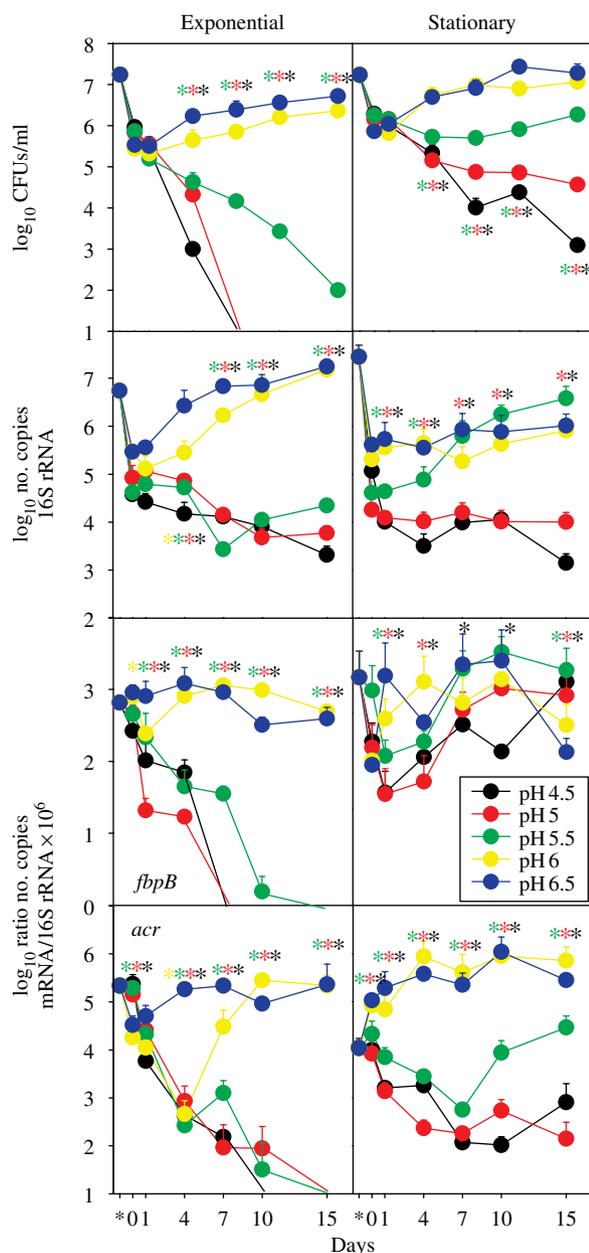
Table 2 Primer sequences for specific RT

Gene	Sequence 5′–3′	Tª RT
16SrRNA (*rrs*)	GCCCGCACGCTCACAGTTAAG	62
acr (Rv2031c)	GGCTTCCCTTCCGAAA	60
icl (Rv0467)	CGCACCTGCTGGACGGCCA	62
sigF (Rv23286c)	CGAGCTAACGAATACGC	60
sigH (Rv3222c)	GCCGTCTGAGGAGACA	63

Results

### EP bacilli display a lower resistance against acid stress than SP bacilli

Figure 1 shows the entire picture of the '*in vitro*' experiments. Essentially, the decrease in pH values induces a

Figure 1 Influence of decreasing pH values in exponential (EP) and stationary (SP) bacilli on CFU and mRNA expression for *sigA, sigB, sigE, sigF, sigG, sigH, sigM,* Rv0834c, *icl, nirA, narG, fbpB, acr, rpoA, recA and cysH.* Data show the average and the standard deviation of the values obtained from three samples independently run at the same time. The experiment was repeated twice. One-way ANOVA Dunnet's test was used to compare the values from the different pH conditions against the more physiological one (pH6.5) at every time point. Differences were significant when marked with * or & ($P < 0.05$) in the case of CFU and 16S rRNA, respectively. Differences were significant when marked with * ($P < 0.05$) and stained with their corresponding colour in the case of mRNA expression. ND = not detected.

Figure 2 Influence of decreasing pH values in stationary (SP) and exponential (EP) bacilli on CFU and 16S rRNA, *fbpB* and *acr* mRNA expression. Data show the average and the standard deviation of the values obtained from three samples independently run at the same time. The experiment was repeated twice. One-way ANOVA Dunnet's test was used to compare the values from the different pH conditions against the more physiological one (pH 6.5) at every time-point. Differences were significant when marked with * ($P < 0.05$) and stained with their corresponding colour.

drop in the CFU from pH 5.5, which is especially strong in EP bacilli, as they become undetectable at day 15 postinoculation (pH 5.5) or even at day 7 (with pH 5 or 4.5). Note that the limit of detection in this case was 1 $\log_{10}$, and culturing these samples with the BACTEC® system confirmed the presence of bacilli in all cases. In the case of
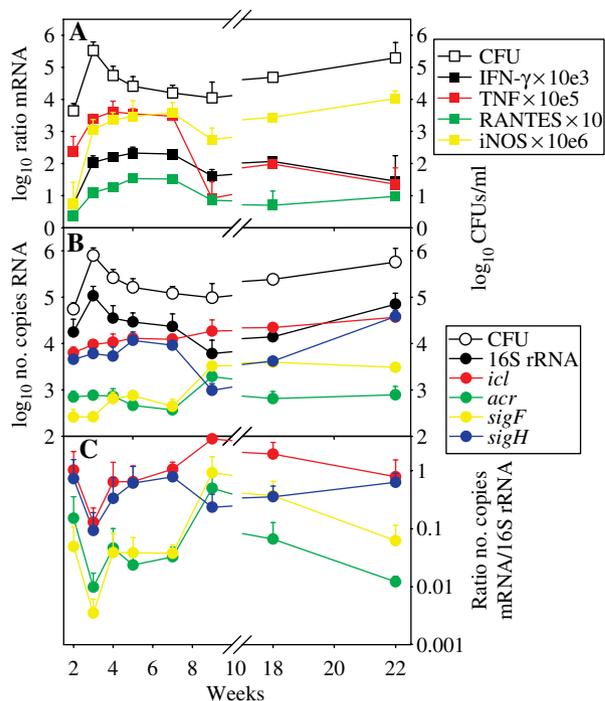

Figure 3 Evolution of immunity-related genes and latency-related genes in a murine model of infection followed by CFU values in the lung. Picture A shows the mRNA expression of IFN-γ, iNOS, TNF and RANTES, expressed as $\log_{10}$ of the ratio obtained after dividing every value by the expression of hypoxanthine phosphoribosyltransferase (HPRT) in each sample and multiplying it by a factor (ranging from 10 to 10^6). Local pulmonary expression of *icl*, *acr*, *sigF* and *sigH* mRNA (as well as 16S rRNA) are expressed as $\log_{10}$ of raw data in picture B and after normalizing for efficiencies by dividing every value by the 16S rRNA value of each sample in picture C. Data show the average and the standard deviation of the values obtained from four animals. The experiment was repeated twice.

SP bacilli, CFU decrease progressively with the drop of pH, but a considerable CFU level is still detected in all cases (3 $\log_{10}$ minimum). Besides, the levels with pH 5.5 tend to recover and return to the ones obtained at pH 6.5 and 6. Interestingly, in EP bacilli, 16S rRNA can still be detected in all these cases, thus losing the correlation observed at pH values of 6.5 and 6. On the contrary, in SP bacilli, the levels of 16S rRNA tend to correlate better with CFU.

Table 3 summarizes the tendencies of mRNA values observed in Fig. 1. In general, the expression ratio of the genes tends to decrease and become undetectable with time and with the drop of pH, essentially from pH 5.5. Interestingly, there are some genes that stabilize in both EP and SP conditions. This is the case of *sigE* and *sigF*. Note that the expression of *sigG* and *sigH* tends to increase in EP conditions, whereas they decrease in SP conditions.

It is worth mentioning that the expression of Rv0834c, although not detected in the experiments with EP bacilli, can be detected with SP ones from days 1 to 4 when the pH drops from 5.5 to 4.5. Furthermore, the expression of
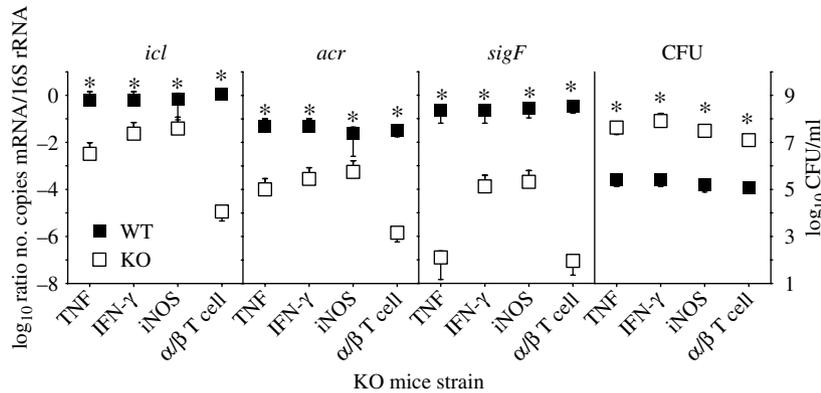
Figure 4 Pulmonary CFU values and expression of genes *icl*, *acr* and *sigF* in TNF, IFN-γ, iNOS and a/*b* T cell KO mice in the lung. Data were obtained at week 4 for TNF KO and IFN-γ, and at weeks 5 and 6 for the remaining genes. Data from C57BL/6 immunocompetent (WT) mice were obtained at these time points and drawn in parallel with the values obtained from each KO mice, depending on the week when they were obtained. Local pulmonary expression of *icl*, *acr* and *sigF* mRNA are expressed after normalizing for efficiencies by dividing every value by the 16S rRNA value of each sample. Differences between mice groups were significant when marked with * ($P < 0.05$).

fbpB is unique, as it seems not to be affected by the drop of CFU in SP bacilli, and remains stable at all pH values and at almost the same values as when the pH was 6.5. This is also the case of *icl* and *rpoA* expression, which decreased from a pH value of 5.5, but remained stable at pH levels of 5.5–4.5. The expression of *recA* can be included in this group, although it drops and becomes undetected at day 4. Finally, the expression of *acr* is the one that better correlates with CFU levels in SP bacilli.

Figure 2 shows the same values as Fig. 1 to better monitor the evolution of of 16S rRNA, *fbpB* and *acr* values with different pH values, and their relation with CFU.

The visual inspection of cultures supplemented with 1.5 mg of methylene blue/ml indicated a complete decolourization in all the conditions after 10 days of incubation.

The expression of *icl*, *acr*, *sigF* and *sigH* in the murine model inversely correlates with CFU

Figure 3A shows the expression of IFN-γ, TNF, RANTES and iNOS, reproducing the well-known tendency of the host Th1 immunity to control the CFU at the acute phase of the infection. As seen before [32], iNOS expression tends to increase in the later stages of chronic infection.

Figure 3C shows how the ratio between the number of mRNA copies of these genes divided by the 16S rRNA values inversely correlates with CFU. Significant differences have been found when comparing the CFU and the mRNA values using an All Pairwise Multiple Comparison Procedure (Student–Newman–Keuls Method), during the acute (weeks 2–4) and the chronic phases (weeks 7, 10 and 18) of infection, thus confirming such tendency in all the cases but *sigH*, where no difference was detected between weeks 10 and 18 (data not shown). Besides, *sigH* showed a tendency to increase between weeks 18 and 22 postinfection (although not significantly), whereas *sigF*, *acr* and *icl* decreased, although the difference in the latter was not significant (data not shown).

Figure 4 also shows the tendency of the ratios of *icl*, *acr* and *sigF* to decrease when there is active bacillary growth, as found in TNF, IFN-γ, iNOS and α/β T cell KO mice at week 4 postinfection in the first cases and at weeks 5 and 6 in the last cases.

The expression of *acr* is the one best maintained, both after chemotherapy and in the Cornell model

Figure 5 shows the evolution of the expression of the *acr* gene. We also carried out preliminary studies with *icl*, but its expression was undetectable in tissues with bacterial concentrations lower than 2 log10 (data not shown). A prolonged chemotherapy period (16 weeks) resulted in
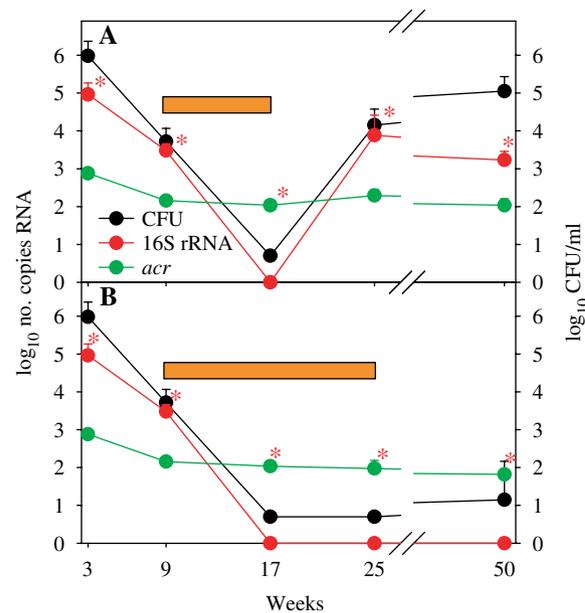
Figure 5 Evolution of 16S rRNA and *acr* expression in the murine model of tuberculosis following chemotherapy, expressed as log10 of raw data. Picture A shows the evolution of the infection after 8 weeks of treatment with isoniazid and rifampicin (25 and 10 mg/kg, respectively). Picture B shows the Cornell model obtained after 16 weeks of chemotherapy using the same drugs. Data show the average and the standard deviation of the values obtained from four animals save for week 50, when 10 animals were used. The experiment was repeated twice. Differences between 16S rRNA and *acr* values were significant when marked with * ($P < 0.05$).

Table 3 Tendency of the RNA expression in the 'in vitro' models

	Bacilli	
	EP	SP
CFU	Decrease to ND	Decrease
16S rRNA	Decrease and stabilize	Decrease and stabilize
mRNA		
sigA	Decrease to ND	Decrease to ND
sigB	Decrease to ND	Decrease
sigE	Stabilize	Stabilize
sigF	Stabilize	Stabilize
sigG	Increase	Decrease
sigH	Increase	Decrease
sigM	Decrease to ND	Decrease to ND
Rv0834c	ND	Increase to ND
icl	Decrease to ND	Decrease and stabilize
nirA	Decrease to ND	Decrease to ND
narG	Decrease to ND	Decrease to ND
fbpB	Decrease to ND	Stabilize
acr	Decrease to ND	Decrease and stabilize
rpoA	Decrease to ND	Decrease and stabilize
recA	Decrease to ND	Decrease and stabilize
cysH	Decrease to ND	Decrease to ND

ND = not detected.

disappearance of the expression of 16S rRNA but not that of *acr*. In this case, although viable counts were undetectable in most animals (i.e. in eight of 10 mice), the culture of samples in the BACTEC® qualitative system was positive for all of them.

Discussion

The aim of this study was to obtain information on the expression of different genes involved in the response to stress that may be crucial in the development of latency in *M. tuberculosis* to find a useful marker for latent bacilli. We would like to emphasize the inherent weakness of this type of experiments, because the conditions in the lungs of both animals and patients are poorly understood and are likely to be quite variable. Thus, we would like to stress that the conditions in the culture tubes and mice are only best-guess scenarios.

Regarding the design of the experiments, we initially carried out 'in vitro' experiments in unstirred cultures searching for those genes related to metabolism expressed after challenge in a stressful environment that resembles the conditions present in the phagosomes of activated macrophage (i.e. decreasing pH values with progressively low oxygen levels) [33, 34]. This information was later used to find latent bacilli in different murine models of aerosol-induced tuberculosis, including chemotherapy and the Cornell model.

Furthermore, it must be highlighted the limitation of this work, as it does not include the perception demonstrated by classical works [7, 8] and even a recent one [2] supporting the hypothesis that latent bacilli can also be

found outside of granulomas, and thus may not be under stressful conditions generated by the activated macrophages or the granuloma itself.

'In vitro' models have confirmed previous evidence for the ability to respond against stress, taking into account both the origin of bacilli and their metabolic stage: in EP or SP [17]. Bacillary concentrations in both populations decreased significantly at a pH of 5.5. The EP population was unable to overcome the situation and decreased to a point where no viable cells were detected. However, the SP population was able to recover. Hence, although viable counts may be detected when cultured in a richer medium, we considered that the EP population had a decreased ability to survive future acid shocks and thus did not represent a latent bacilli population in 'in vivo' models. Therefore, we did not consider the remaining expression of *sigE*, *sigF*, *sigH* and *sigG* experimented by EP bacilli as good markers for monitoring the presence of latent bacilli 'in vivo'. We considered *acr* the best potential marker for latent bacilli as its evolution was the one that better correlated with the SP bacilli CFU, among other genes (*icl*, *fbpB*, *rpoA* and *recA*) that also maintained their expression, even at the lower pH values but in a more stable manner.

The constant expression of *icl* in SP conditions is remarkable. Recent work has confirmed that expression of this gene is induced by acid, and has been suggested to be a marker of low pH levels [35]. Thus, its activity may be related to the acid stress. The strong stability of *fbpB* expression in SP bacilli, even with progressively lower pH values, is in accordance with previous data from 'in vitro' tests that used this expression as a marker for bacilli viability in drug susceptibility studies [36]. The expression of *rpoA* should be solely interpreted as a marker of RNA polymerase activity [37]. Moreover, the *recA* expression should be interpreted with caution, as nowadays it does not seem to be related to response against DNA damage.

Interestingly, in this model the expression of Rv0834c gene confirmed its relation with the response against acid stress [38], and it represents a significant difference between SP and EP populations. In addition, it would explain why EP bacilli are not able to survive against such adverse environment.

The interpretation of the kinetics of *narG*, *nirA* and *cysH* is difficult in the context of SP cultures; we may accept that *narG* is closely associated with growth under low oxygen levels, but this seems not to be the case with *nirA*, so far [5, 29]. The only possible interpretation suggests that, in our system, *narG* is only needed by cells that are trying to grow in order to face the progressive lack of O_2 (and growing cells are precisely the ones that are more susceptible to be killed because of low pH values). It seems that the cells that survive present another type of metabolism (which is even less active) to survive in a combined stress (i.e. low oxygen levels and low pH values). The behaviour of *cysH* may be explained as a decrease in

the production of new glycolipids in the cell wall caused by a decreased metabolism, as *cysH* is involved in the synthesis of sulpholipid I [39], although it could also be caused by the relative low amount of oxidants in the culture, which would render its activity not essential for survival [27].

A major criticism of this *in vitro* model is related to the nature and the maintenance of stress conditions. In fact, although the induced pH range fits in the physiological values obtained in phagosomes [24], the sudden induction of stress is not physiological. In an *ex vivo* model, a pH decrease to 4.5 takes at least 24 h, so it is more progressive [24] and thus bacilli might display a better adaptation to this situation. Another limiting point is the time during which these cells have been challenged to stress. Until now, there is no data on the duration of these conditions in the phagosome, as *ex vivo* experiments on the acidification of *M. tuberculosis*-infected phagosomes do not last for more than hours to 2 days [40, 41].

A major technical limitation in our work consists on the lower number of 16S rRNA copies obtained, as it tended to be 1 $\log_{10}$ lower in our system compared with CFU. This is a crucial point, as previous works had revealed that this concentration was usually 3 $\log_{10}$ higher than CFU [20, 21]. This difference may be explained by the methodology used: samples were previously treated to remove DNA and this probably made the technique more stringent as it may have lost an important percentage of RNA, thus reducing sensitivity. In previous studies [20, 21] DNA was not previously removed, and data were obtained by subtracting the values from samples not treated with RT to the RT-treated samples.

Another technical limitation is related to the *in vivo* model and the interference generated by the massive presence of eukaryotic mRNA did not allow us to evaluate the expression of the genes selected in the *in vitro* experiments, thus adding another limitation to our study. Even when a specific RT primer was used in all cases, efficacious results were only obtained from 16S rRNA, *sigF*, *sigH*, *icl* and *acr*. Interestingly, the expressions of *icl*, *acr*, *sigF* and *sigH* tended to decrease during the acute phase of infection in the murine model (between weeks 2 and 3 postinoculation) and increased at the beginning of the chronic phase (from week 7). Even in this latter phase, expression tended to decrease when average values of bacillary concentration increased save for *sigH*, which tended to be stable. Data obtained from mice lacking crucial genes for the control of bacilli (α/β T cells, TNF, IFN-γ and iNOS), and harbouring actively growing bacilli also support this view when compared with wild type mice. These data reinforce the hypothesis that the expression of these genes tends to be stimulated when the host controls actively growing bacilli and favours the presence of a latent population, although these data do not agree with previous works [20, 21].

In our experiments with chemotherapy and the Cornell model, we decided to further study the expression of *acr* expression, because *icl* values were undetectable in tissues with a low bacillary concentration. We did not study the expression of *sigF* or *sigH* because the *in vitro* tests with EP bacilli did not support further study as their physiological meaning might be misleading. Moreover, the expression of *acr* has been closely related to latency, and thus could be a valuable marker [11]. Interestingly, the expression of *acr* was maintained even when the levels of 16S rRNA were undetectable following a long period of chemotherapy. Therefore, this was the best value for detecting the expression of latent bacilli with the RT-PCR methodology used, and its technical limitations.

In conclusion, data from experimental *in vitro* stressing systems with progressive low oxygen levels and decreasing pH values have supported the search for a useful marker monitoring latent bacilli in a murine model of tuberculosis. In this sense, the expression of *acr* may be a new tool for monitoring this population and contributes to future works directed to its eradication.

Acknowledgments

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