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Global transcriptional response to vancomycin in *Mycobacterium tuberculosis*

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In order to gain additional understanding of the physiological mechanisms used by bacteria to maintain surface homeostasis and to identify potential targets for new antibacterial drugs, we analysed the variation of the *Mycobacterium tuberculosis* transcriptional profile in response to inhibitory and subinhibitory concentrations of vancomycin. Our analysis identified 153 genes differentially regulated after exposing bacteria to a concentration of the drug ten times higher than the MIC, and 141 genes differentially expressed when bacteria were growing in a concentration of the drug eightfold lower than the MIC. Hierarchical clustering analysis indicated that the response to these different conditions is different, although with some overlap. This approach allowed us to identify several genes whose products could be involved in the protection from antibiotic stress targeting the envelope and help to confer the basal level of *M. tuberculosis* resistance to antibacterial drugs, such as Rv2623 (UspA-like), Rv0116c, PE20-PPE31, PspA and proteins related to toxin–antitoxin systems. Moreover, we also demonstrated that the alternative sigma factor σ^E confers basal resistance to vancomycin, once again underlining its importance in the physiology of the mycobacterial surface stress response.

INTRODUCTION

Mycobacterium tuberculosis remains one of the world's most prevalent and serious pathogens. It is estimated that every year 2 million people die as a direct result of tuberculosis and that there is a reservoir of 2 billion cryptically infected people (Dye *et al.*, 1999). Of these asymptomatic carriers, around 5% will develop active disease at some stage in their lives and in doing so will contribute to the ongoing transmission of infection (Raviglione, 2003). The recent emergence of multidrug-resistant (MDR) and extensively drug-resistant (XDR-TB) strains (Gandhi *et al.*, 2006) has raised the importance of searching for alternative targets to develop new anti-mycobacterial drugs.

Due to the importance of its physiological role and its difference from the eukaryotic cell surface, the bacterial cell wall is one of the best candidates in the search for new drug targets. A large number of antibacterial drugs currently in use are directed against surface components or metabolic pathways that are involved in their synthesis. For example, β-lactams, cephalosporins, glycopeptides, phosphomycin, bacitracin and cycloserine inhibit peptidoglycan biosynthesis, while polymyxin interferes with the cell-membrane structure. Moreover, compounds such as isoniazid, pyrazinamide, ethambutol and ethionamide target typical components of the mycobacterial cell surface. In spite of a good knowledge of the chemical composition of the mycobacterial surface, not much is known about its organization and physiology (Barry, 2001). The recent demonstration of the presence of an outer membrane will surely boost research in this field (Hoffmann *et al.*, 2008; Zuber *et al.*, 2008).

One strategy to study bacterial surface physiology is to characterize the variation of the bacterial transcriptional profile in response to exposure to compounds able to perturb surface homeostasis. This approach has been successfully used to study the global transcriptional response of *Bacillus subtilis* and *Staphylococcus aureus* in response to several inhibitors of peptidoglycan biosynthesis (Cao *et al.*, 2002; Mascher *et al.*, 2003; Utaida *et al.*, 2003), and in *M. tuberculosis* to study the transcriptional response to the

Abbreviations: HCL, hierarchical clustering; SAM, significance analysis of microarrays.

The data discussed in this publication have been deposited in the NCBI Gene Expression Omnibus under GEO Series accession number GSE12364.

Five supplementary tables are available with the online version of this paper.

detergent SDS (Manganelli *et al.*, 2001) and to the mycolic acid biosynthesis inhibitor isoniazid (Wilson *et al.*, 1999). These experiments contributed to the understanding of the physiological mechanisms used by bacteria to maintain surface homeostasis and to identify several potential targets for the development of new antibacterial drugs.

In this work we used this approach to characterize the variation of the *M. tuberculosis* global transcriptional profile in response to inhibitory and subinhibitory concentrations of vancomycin. Our analysis has identified a significant transcriptional response associated with bacteria exposed to a concentration of the drug ten times higher than the MIC (153 genes differentially regulated) as well as bacteria growing in a concentration of the drug eightfold lower than the MIC (141 genes differentially regulated).

METHODS

Bacterial strains and growth conditions. *M. tuberculosis* strains H37Rv, ST28 (*sigE* mutant) and ST29 (*sigE* complemented mutant) (Manganelli *et al.*, 2001) were grown in Middlebrook 7H9 (Difco) supplemented with 10 % albumin-dextrose-sodium chloride complex (ADN) (Jacobs *et al.*, 1991), 0.2 % glycerol and 0.05 % Tween 80 (Sigma) at 37 °C in rotating bottles (40 r.p.m.).

Cultures growing in subinhibitory concentrations of vancomycin were obtained by inoculating bacteria in 35 ml 7H9 (at an OD_{540} of 0.05) containing 5 μg ml^{-1} of the drug and following their growth until they reached an OD_{540} between 0.2 and 0.3. Cultures exposed to inhibitory concentrations of vancomycin were obtained by adding 400 μg ml^{-1} of the drug to 35 ml bacterial cultures grown in 7H9 to an OD_{540} of 0.2–0.3 and incubating at 37 °C for an additional 1 or 4 h.

RNA extraction and cDNA labelling. Bacterial cultures were centrifuged for 5 min at 2500 ***g***. After removing the supernatant, pellets were frozen on dry ice and stored at −80 °C. The RNA from the frozen pellets was extracted following standard protocols as previously described (Maciag *et al.*, 2007). Fluorescently labelled cDNAs were produced as previously described (Provvedi *et al.*, 2008) by reverse transcription (RT) of total RNA with Superscript II (Invitrogen Life Technologies) in the presence of Cy3-dUTP or Cy5-dUTP (Amersham Pharmacia) by using random hexamers to prime cDNA synthesis (Invitrogen Life Technologies). Fluorescent probes were purified with the CyScribe GFX purification kit (GE Healthcare) and then concentrated to 7.5 μl by Microcon YM-30 centrifugal filter devices (Millipore) according to the procedure indicated by the manufacturers.

Microarrays and hybridizations. *M. tuberculosis* oligoarrays were obtained from the Center for Applied Genomics, International Center for Public Health (Newark, NJ, USA). These microarrays consist of 4295 70-mer oligonucleotides representing 3924 open reading frames (ORFs) from *M. tuberculosis* strain H37Rv and 371 unique ORFs from strain CDC 1551 that are not present in H37Rv.

Each microarray was incubated in prehybridization solution (2.8 % BSA, 0.1 % SDS) at 42 °C for 1 h. The slides were first rinsed in water for 2 min and then in propan-2-ol for an additional 2 min before air drying. Competitive hybridizations with equal amounts of purified Cy3/Cy5-labelled cDNA (approx. 60 pmol) were performed in duplicate with both dye arrangements, as previously described (Provvedi *et al.*, 2008).

Fluorescence intensity data from each array were collected with an Affymetrix 428 scanner; the scanned images were finally analysed with ImaGene 4.1 to calculate the median spot intensities.

Data processing and statistical and cluster analysis. Data were normalized with a web-based tool for Diagnosis and Normalization of spotted cDNA MicroArrayData (DNMAD) available at http://dnmad.bioinfo.cnio.es (Vaquerizas *et al.*, 2005) using the print-tip lowess method after background subtraction. Significance analysis of microarrays (SAM) was performed to identify those genes whose differential expression was considered significant (Tusher *et al.*, 2001). Differentially expressed genes were defined by a *q*-value (percentage chance that the gene is a false positive) <5 % and a minimum fold difference of ±1.5 between control and drug-treated samples. Cluster analysis was carried out by hierarchical clustering (HCL) using a complete linkage agglomeration method applied to a Pearson correlation similarity matrix. Both SAM and HCL were part of the TigerMultiExperiment Viewer package version 3.1 (TMeV) available at http://www.tm4.org/mev.html (Provvedi *et al.*, 2008).

RT-PCR. Reverse transcription was performed with random primers using murine leukoblastoma virus retrotranscriptase (MULV-RT) (Applied Biosystems). Briefly, 500 ng RNA was denatured at 98 °C for 2 min in the presence of the appropriate volume of water and then chilled on ice. The RNA sample was used to prepare 25 μl annealing mixture [5.5 mM $MgCl_2$, 0.55 mM (each) dNTPs, 0.25 mmol random hexamers; 32 U MULV, 10 U RNase inhibitor and 1× reaction buffer (Applied Biosystems)]. Samples were then incubated at 25 °C for 10 min, at 45 °C for 50 min, and finally at 95 °C for 5 min to allow the annealing of the random hexamers. Quantitative PCR was performed with SYBR green master mix (Applied Biosystems) under the following conditions: 1 min denaturation at 95 °C, 30 s annealing at 62 °C, and 30 s extension at 72 °C. Results were normalized to the amount of *sigA* mRNA, as previously described (Manganelli *et al.*, 1999). RNA samples that had not been reverse transcribed were included in all experiments to exclude significant DNA contamination. For each sample, melting curves were performed to confirm the purity of the products. Sequences of the primers for quantitative RT-PCR are available upon request.

Determination of growth inhibition by disc diffusion assay. *M. tuberculosis* strains were grown to early exponential phase and 100 μl culture containing 3×10^7 c.f.u. was spread on 20 ml 7H10 plates. Paper discs containing 10 μl vancomycin at a concentration of 10 mg ml^{-1} were placed on top of the agar. The diameter of the inhibition zone was measured after 20 days incubation at 37 °C.

RESULTS AND DISCUSSION

Whole-genome microarrays were employed to profile gene expression in *M. tuberculosis* H37Rv in response to inhibitory or subinhibitory concentration of vancomycin. Inhibitory conditions were achieved by exposing bacteria to 400 μg vancomycin ml^{-1}, a concentration ten times the MIC (10X-MIC). This concentration was chosen in order to determine a rapid arrest of bacterial cell growth (Fig. 1a). A 10X-MIC of vancomycin was also used by Cao *et al.* (2002) in *B. subtilis* to identify an inducible defensive response against antibiotics coordinated by alternative sigma factors. Subinhibitory conditions were determined by growing bacteria in the presence of decreasing vancomycin concentrations (twofold serial dilutions) starting from the MIC (40 μg ml^{-1}) (data not shown). The concentration of 5 μg ml^{-1} (1/8-MIC) was chosen since it allowed bacteria to grow with the same profile as untreated cells, although at a reduced rate (Fig. 1b).

In order to choose the time points at which to perform our analysis, we ran a preliminary microarray experiment in

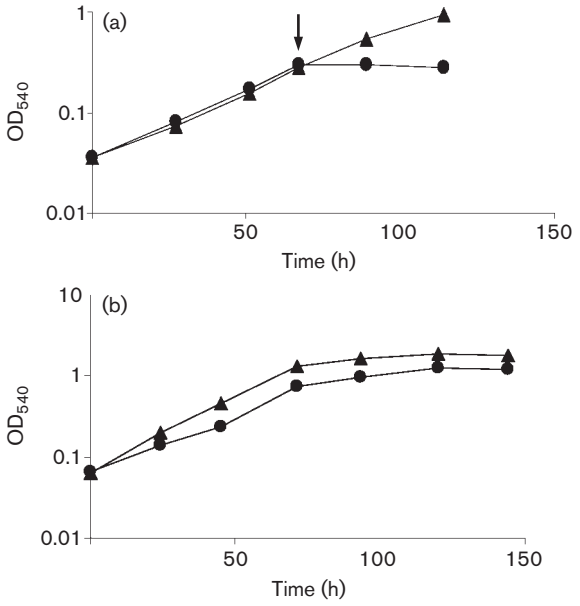

Fig. 1. Growth curve of *M. tuberculosis* in the presence (●) or absence (▲) of vancomycin. The vertical arrow in (a) indicates the time at which the drug (400 μg ml^{-1}) was added to one of the two cultures. Cultures in (b) were grown without or with 5 μg vancomycin ml^{-1}. Cultures for RNA extraction were harvested at OD_{540} of 0.2–0.3.

which bacterial culture at an OD_{540} of 0.2 was added to vancomycin at a concentration 10X-MIC, incubated for 30 min, 1 h, 2 h or 4 h, and then analysed to identify which genes were differentially expressed (data not shown). Under these conditions bacterial replication arrested rapidly (Fig. 1a), while the viable counts remained constant ($4.1 \pm 0.4 \times 10^7$ and $3.7 \pm 0.2 \times 10^7$ at time 0 and 4 h, respectively). We discarded the first time point (30 min), since very few genes, if any, were shown to be differentially regulated; time point 2 h was discarded since its expression profile was quite similar to the previous one (1 h) (data not shown). The analysis was than repeated on different biological samples exposed to vancomycin for 1 h or 4 h; in these conditions the number of differentially expressed genes was 59 and 153, respectively (see Supplementary Tables S1 and S2, available with the online version of this paper).

When similar experiments were performed in *B. subtilis*, 10 min exposure was enough to obtain the induction of 129 genes (Cao *et al.*, 2002), suggesting that some time is required for vancomycin to cross the mycobacterial outer membrane and exert its action. Quantitative RT-PCR was used to validate the differential expression of selected genes (Supplementary Tables S2 and S3, available with the online version of this paper). An analysis of the DNA array datasets using HCL indicated that the gene expression profile obtained after exposing bacterial cells to 10X-MIC vancomycin for either 1 h or 4 h was not globally different, since these tested conditions grouped in the same cluster (Fig. 2a).

When bacterial cultures were grown in the presence of the subinhibitory concentration of the drug, 141 genes were found to be differentially expressed (Table S3). A comparison by HCL between this set of data and that obtained from cultures exposed to inhibitory concentration of vancomycin for 4 h highlighted the presence of two distinct clusters, indicating that their gene expression profile was globally different (Fig. 2b).

However, several genes were found to be commonly expressed under more than one condition. In particular, 20 genes were commonly regulated after exposure to vancomycin 10X-MIC for 1 h and 4 h, while 30 genes were regulated both in the presence of vancomycin 10X-MIC for 4 h and when cells were grown in a subinhibitory concentration of the drug. Finally, nine genes were commonly regulated under all tested conditions (Fig. 3). These genes (Table 1) were all induced and encoded well-known stress proteins, such as Hsp and HtpX, and proteins of unknown functions. Among them were Rv2623, belonging to the UspA (universal stress proteins) family (O'Toole & Williams, 2003); Rv0516c, predicted as a putative anti-anti-sigma factor; Rv1057, similar to an archaeal surface layer protein and containing a β-propeller domain that could be involved in cell–cell interaction; Rv2050, which is an orthologue of the RNA-polymerase-associated protein RbpA of *Streptomyces coelicolor* (Paget *et al.*, 2001b); and PE20.

Hsp (also referred to as Acr2) belongs to the α-crystallin family of molecular chaperones and is induced in several stress conditions such as heat shock, oxidative stress and uptake by macrophages (Schnappinger *et al.*, 2003; Stewart *et al.*, 2002). HtpX is a putative membrane-bound zinc metalloprotease that has been suggested to participate in the proteolytic quality control of membrane proteins (Rodrigue *et al.*, 2006).

Proteins of the UspA family are encoded in the genomes of bacteria, archaea, fungi, protozoa and plants, but their biological and biochemical function is still not fully understood. They are characterized by the presence of a typical domain that occurs either in isolation or fused to

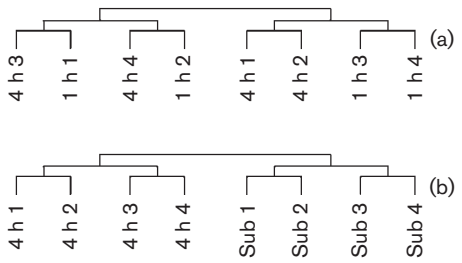

Fig. 2. Hierarchical clustering. (a) Clustering of gene profiles from *M. tuberculosis* cultures treated with 400 μg vancomycin ml^{-1} for 1 or 4 h. (b) Clustering of gene profiles from *M. tuberculosis* cultures treated for 4 h with 400 μg vancomycin ml^{-1} or grown in the presence of 5 μg vancomycin ml^{-1} (Sub).

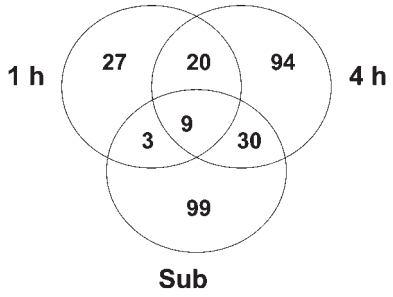

Fig. 3. Common and unique expression patterns of *M. tuberculosis* exposed to 400 μg vancomycin ml^{-1} for 1 h or 4 h, or exposed to 5 μg vancomycin ml^{-1} (Sub). Venn diagram showing the number of *M. tuberculosis* genes differentially expressed in the different conditions.

other domains. In *E. coli* this family of proteins is represented by at least five paralogues that are induced by overlapping, but non-identical stresses. Interestingly, members of this family are involved in the regulation of bacterial growth, as they are important for recovery from starvation and their inactivation causes an extended lag when stationary-phase cells are transferred to fresh media (Kvint *et al.*, 2003). Moreover, UspA production is strongly induced under conditions that cause growth arrest. The *M. tuberculosis* genome encodes at least eight proteins of this family (O'Toole & Williams, 2003). Rv2623 contains two UspA typical domains, and it was previously shown to be part of the dormancy regulon and to be upregulated in mouse lungs at the terminal stage of pathology (Florczyk *et al.*, 2001; Shi *et al.*, 2003). Its induction by inhibitory and subinhibitory concentrations of vancomycin suggests its involvement in *M. tuberculosis* growth control following exposure to stress.

Rv0516c encodes a soluble protein with a typical anti-anti-sigma factor domain. It was one of the genes with the largest reduction in transcription during early stationary-phase growth of a *M. tuberculosis* mutant harbouring a deletion of the gene encoding σ^F (Geiman *et al.*, 2004), and it has been shown to interact physically with this sigma factor in a two-hybrid system (Parida *et al.*, 2005). Its co-induction with σ^E opens the interesting hypothesis that this protein could be involved in the regulation of the activity of this sigma factor. Several attempts to express this protein in *M. tuberculosis* were unsuccessful, suggesting its toxicity (data not shown).

Rv2050 is an orthologue of RbpA, an RNA-polymerase-associated protein described in *S. coelicolor*, whose structural gene is induced by SigR in response to disulfide stress (Paget *et al.*, 2001a). Orthologues of this protein are only found in actinomycetes. Recently, it was shown that this gene is also induced by rifamicin, and that RbpA is able to stimulate the expression of the rRNA operon even in the presence of rifamicin, conferring a basal level of resistance to this drug (Newell *et al.*, 2006). In *M. tuberculosis*, Rv2050 was shown to be under the transcriptional control of SigH (SigR orthologue) and upregulated after heat shock and disulfide stress (Manganelli *et al.*, 2002). The finding that the expression of this gene is also induced by vancomycin suggests that Rv2050 might contribute to the basal level of drug resistance in mycobacteria as well, opening the possibility to consider it as a novel drug-target candidate.

The PE20 structural gene (Rv1806) displayed a very strong induction in all tested conditions. Moreover, this gene (together with its downstream gene encoding PPE31) was previously shown to be induced after exposure to SDS (Manganelli *et al.*, 2001), suggesting that these two proteins may have a specific role in surface stress response. Rv1807 (encoding PPE31) was induced after both 1 and 4 h of

Table 1. Genes induced in all conditions tested

Rv1807 was not included in this table since microarray data did not select it as an induced gene in cultures exposed to subinhibitory concentrations of vancomycin. However, RT-PCR data later showed that this was due to a technical problem (see text).

Rv number	Gene*	Fold induction (1 h)†	Fold induction (4 h)†	Fold induction (S)†	Gene product/function
Rv0251c	*hsp*	2.4 (0)	8.3 (0)	6.3 (0)	Molecular chaperone
RV0516c		1.8 (0)	4.0 (0) [9.5]	1.6 (0)	Putative anti-anti-sigma
Rv0563	*htpX*	1.6 (0)	4.5 (0)	2.9 (0)	Membrane-bound zinc metalloprotease
Rv1057		1.6 (0)	2.9 (0)	1.7 (0)	Unknown function
Rv1073		1.7 (0)	3.1 (0)	1.8 (0)	Unknown function
Rv1806	*pe20*	21.6 (4)	6.9 (0) [56.3]	191.9 (0) [83.5]	Unknown function
Rv2050		1.6 (0)	2.4 (0)	1.9 (0)	Putative RNApol associated protein
Rv2623		1.9 (0)	2.0 (0.2) [2.2]	16.0 (0) [2.7]	Member of UspA family
Rv2745c		1.9 (0)	2.1 (0)	3.0 (0)	Putative transcriptional regulator

*Genes are annotated as described by the Pasteur Institute on TUBERCULIST (http://genolist.pasteur.fr/TubercuList/).

†Values in parentheses represent false discovery rate (FDR), the probability that the gene was falsely called (calculated by SAM); values in square brackets represent the fold induction obtained by qRT-PCR. S, subinhibitory concentration.

exposure to vancomycin. Although the microarray data did not indicate it as an induced gene in cultures exposed to subinhibitory concentrations of vancomycin, quantitative RT-PCR revealed that it was induced as well (tenfold) under this condition (Table S3). Interestingly, in a recent report the PE20-PPE31 couple was found to be a vaccine candidate able to confer a significant level of protection in a guinea pig aerosol infection model (Vipond *et al.*, 2006). PE and PPE are two families of surface-associated proteins of unknown function typical of mycobacteria, characterized by conserved N-terminal domains (Gey van Pittius *et al.*, 2006). Several PPE-encoding genes are in tandem with a sequence encoding a PE, and in one case the PE and the PPE proteins were shown to form a dimer (Strong *et al.*, 2006).

Genes induced after exposure to 10X-MIC vancomycin

A total of 153 genes were differentially expressed during exposure to 10X-MIC vancomycin. Of these, 110 genes were upregulated and 43 were downregulated (Table S2). As indicated in Fig. 4(a), the most represented functional categories of induced genes include genes encoding conserved hypothetical proteins with unknown function (36 %) and genes involved in intermediary metabolism (13.5 %).

Genes involved in metabolism. Several genes involved in defence against xenobiotics and oxidative stress, as well as genes typically induced during growth arrest, were upregulated: *cysD* and *cysNC* encode an ATP sulfurylase, which catalyses the synthesis of activated sulfate (APS), which is then phosphorylated at the 3′ hydroxyl to form PAPS. This last compound can transfer sulfuryl groups to various metabolic recipients and is widely used by cells to regulate metabolism. Alternatively, the sulfuryl moiety of PAPS can be reduced to sulfide and incorporated into cysteine and, from there, into other reduced-sulfur metabolites. It has been shown previously that the *cysDNC* operon is induced upon oxidative stress, in stationary phase and in the intra-phagosomal milieu, suggesting regulation of sulfur assimilation in response to toxic oxidants (Hampshire *et al.*, 2004; Pinto *et al.*, 2004). Interestingly sulfolipids, important mycobacterial surface components able to inhibit phagosome–lysosome fusion, are sulfated by PAPS (Pinto *et al.*, 2004), suggesting a role of the *cysDNC* operon also in determining surface composition.

Other metabolic genes involved in oxidative stress response induced in these conditions are *trxB1*, encoding a thioredoxin; *bfrB*, encoding a bacterioferritin, whose role could be required to remove the ferrous ions that can react with metabolically generated hydrogen peroxide to yield very toxic hydroxyl radicals, and a group including *icl*, *pdhA*, *scoA*, *ndh* and *narU*. The proteins encoded by these genes use and regenerate reducing equivalents from fatty acids in the absence of external electron acceptors (*icl*, *pdhA* and *scoA*) (Boshoff & Barry, 2005), or during the microaerophilic respiration that is typical of cultures subjected to severe nutrient starvation or slow growth (*ndh*, *narU*) (Boshoff & Barry, 2005; Clegg *et al.*, 2006).

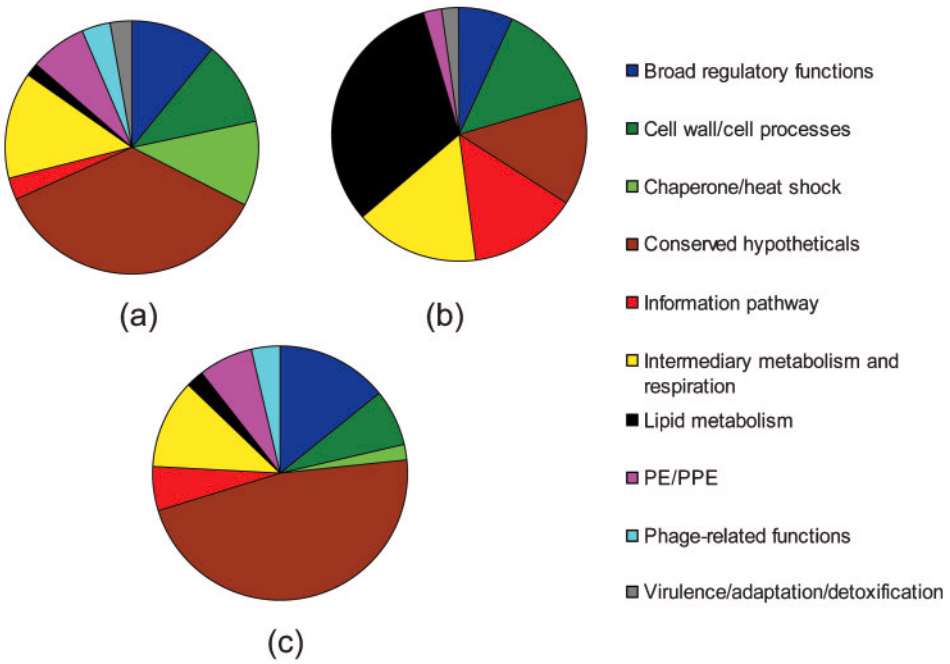

Fig. 4. Functional categories of genes upregulated (a) or downregulated (b) after exposure to 400 μg vancomycin ml^{-1} for 4 h; (c) functional categories of genes upregulated in *M. tuberculosis* cultures grown in the presence of 5 μg vancomycin ml^{-1}.

Cell wall and cell processes. Rv2743c, encoding a conserved transmembrane protein, was found to be induced together with the upstream genes Rv2745c and Rv2744c. This putative operon was upregulated in *M. tuberculosis* also after treatment with SDS and its transcription is under the control of the ECF sigma factor σ^E (Manganelli *et al.*, 2001); Rv2745c is predicted to be a transcriptional regulatory protein, whereas Rv2744c is highly homologous to the *E. coli* PspA (phage-shock protein A) (Rodrigue *et al.*, 2006). In *E. coli*, PspA is part of a cytoplasmic membrane protection system involved in suppressing proton leakage from damaged membranes (Kobayashi *et al.*, 2007). In the presence of a specific surface stress signal (a decrease of the proton-motive force due to cytoplasmic membrane permeabilization), a transmembrane protein activates PspA, which releases the transcriptional factor PspF and associates with the internal surface of the cytoplasmic membrane, causing its stabilization (Darwin, 2005). Although Rv2745c and Rv2743c do not share any similarity with the transcriptional regulator and the transmembrane protein, respectively, involved in the *E. coli* PspA system, it is tempting to speculate that they represent their functional analogues. Experiments to address this issue are currently under development in our laboratory. The gene Rv0116c encodes a surface protein of unknown function containing a peptidoglycan-binding domain and is induced also in cells exposed to subinhibitory concentration of the drug. Even though the function of this protein is unknown, it is possible to hypothesize that it is involved in the biogenesis or structural organization of the cell wall, making it an interesting candidate for future studies. *mmpL5-mmpS5* encode proteins resembling efflux pumps belonging to the resistance-nodulation-division (RND) family (Cole *et al.*, 1998). The genome sequence of *M. tuberculosis* contains 14 genes encoding putative transport proteins of the RND superfamily (Jain *et al.*, 2008). Interestingly, these two genes are also induced in response to SDS-mediated surface stress (Manganelli *et al.*, 2001), and recently their overexpression has been found to be responsible for azole resistance via an efflux mechanism (Milano *et al.*, 2009). Involvement of inner-membrane efflux pumps in response to drugs targeting peptidoglycan biosynthesis is commonly accepted even if difficult to explain, since these drugs do not need to enter inside the cell to exert their action (Danilchanka *et al.*, 2008).

Beyond the genes encoding the coupled PE20-PPE31, also the genes encoding the coupled PE35-PPE68 (part of the Esx-1 cluster), PPE32, PPE33, PE17 and PE_PGRS30 were induced in these conditions, underscoring the importance of these surface-associated protein families in the biology of the mycobacterial envelope.

Chaperones and protein degradation. Besides *hsp* and *htpX*, which have been mentioned before, several other chaperone-encoding genes were induced by 10X-MIC vancomycin, suggesting that high concentrations of this drug promote protein misfolding or aggregation, probably due to growth arrest. *hspX* (a paralogue of *hsp*, referred to as *acr1*) encodes a protein belonging to the α-crystallin family of molecular chaperones, which is associated with *in vivo* growth and persistence (Timm *et al.*, 2003) and with microaerophilic conditions (Desjardin *et al.*, 2001). *hspX* has been proposed to play an active role in slowing the growth of *M. tuberculosis in vivo* immediately following infection. Other chaperone-encoding induced genes were *dnaK*, *grpE*, *dnaJI*, *groEL1*, *groEL2*, *groES* and *clpB*, whose protein products have been shown to interact with each other in order to prevent protein aggregation and support the refolding of damaged proteins (Dougan *et al.*, 2002). Only one gene involved in protein degradation was induced: Rv2115c (*mpa*); this gene encodes an AAA ATPase forming ring-shaped complexes homologous to those found in proteasome caps of eukaryotes and was shown to have a role in oxidative stress response and in virulence (Darwin *et al.*, 2005).

Toxin–antitoxin systems. Four genes encoding two toxin–antitoxin (TA) protein pairs (Rv0550c-Rv0549c and Rv1991c-Rv1990c) and four genes encoding proteins related to toxic components of TA systems (Rv1720c, Rv2142c, Rv2602 and Rv2865) were found to be induced. TA systems are widespread in prokaryotes and are usually organized in operons composed of two genes in which the first ORF usually encodes an antitoxin that combines with and neutralizes a regulatory 'toxin' encoded by the second ORF. Typically, expression of the operon is negatively autoregulated by the antitoxin or by an antitoxin–toxin complex (Magnuson, 2007). TA systems were originally characterized as factors encoded by low-copy-number plasmids to eliminate plasmid-free cells emerging as a result of segregation or replication defects, ensuring plasmid dissemination among bacteria (Gerdes *et al.*, 1986; Jaffe *et al.*, 1985). TA modules are also widespread on bacterial chromosomes. These chromosomal TA cassettes (cTAs) are homologous to those identified on plasmids but apparently fulfil different functions. Experimental evidence suggests that cTAs are stress-response elements that help cells to survive adverse growth conditions by attenuation of protein synthesis and retardation of cell growth. In response to environmental stress, the antitoxin is degraded by host proteases, thereby causing an increased expression of the toxin, whose activity promotes slow growth (Hayes, 2003; Pandey & Gerdes, 2005). Interestingly, TA systems are over-represented in the genomes of slowly growing bacteria (Pandey & Gerdes, 2005). *M. tuberculosis* H37Rv contains 38 TA loci on its chromosome, suggesting that their biological functions are particularly beneficial to this micro-organism, which is able to arrest growth during intracellular phases of its life cycle (Arcus *et al.*, 2005). Indeed, TA pairs have been found to be part of the dormancy regulon and are induced by hypoxia and subinhibitory concentrations of nitric oxide (Voskuil *et al.*, 2004). The induction of TA genes after exposure to vancomycin suggests that these proteins play an important

role in regulating the growth rate of mycobacteria in response to diverse stress conditions, and also suggests that they could act as cell cycle arrest factors that induce cells to enter a dormant state as a protection against an adverse environment.

Regulatory proteins. As indicated in Table S2, we noted that several genes regulated by the surface stress-specific alternative ECF sigma factor σ^E were induced, suggesting a role of this regulator in the response to this drug. However, unexpectedly, *sigE* did not appear as one of the significantly upregulated genes. To determine whether this was an artefact due to the microarray technique, we measured *sigE* mRNA levels by quantitative real-time PCR. We found that *sigE* was indeed induced when cells were treated with both inhibitory and subinhibitory concentrations of vancomycin (Tables S2 and S3). Accordingly, *sigB*, Rv0465c and Rv0516c, encoding other regulators already shown to be downstream of σ^E, were induced as well in these conditions.

Finally, another interesting gene induced under these conditions is represented by *whiB6*, whose product belongs to a family of small transcriptional regulators typical of *Streptomyces* and other actinomycetes. This gene has been recently shown to be induced after exposure to surface stress (SDS, ethanol), oxidative stress (diamide, cumene peroxide) and heat shock (Geiman *et al.*, 2006). Members of this family have been shown to be involved in differentiation, spore formation and septation in *Streptomyces* (Soliveri *et al.*, 2000).

Genes repressed after exposure to 10X-MIC vancomycin

As shown in Fig. 4(b), the treatment with 10X-MIC vancomycin induced a strong repression of genes required for lipid metabolism (32 %). In particular, we found a strong repression of the *ppsABCDE* and *drrABC* operons, which are responsible for dimycocerosate biosynthesis and transport (Azad *et al.*, 1997); *kasAB* and *accD6*, involved in the biosynthesis of mycolic acids, were strongly repressed as well, together with other genes involved in lipid synthesis (*pks1*, *pks6*, *pks15*, *fadD22* and *desA2*). These data suggest that under conditions of growth arrest bacteria slow down the synthesis of energy-expensive cell-wall components. Additional genes involved in metabolic pathways were *nuoM* and *nuoN*, encoding two subunits of a NADH dehydrogenase typically expressed during rapid growth in aerobiosis. Their repression fits well with the induction of *ndh*, which encodes a protein with similar function, but typical of slow, microaerophilic growth (Boshoff *et al.*, 2004; Boshoff & Barry, 2005). *hemB*, *hemD* and *hemZ* encode enzymes responsible for the biosynthesis of haem, an oxygen-binding prosthetic group contained in several proteins involved in cytoplasmic metabolic pathways; *hemZ* was recently shown to be an essential gene in *M. tuberculosis* (Parish *et al.*, 2005). *rpfE* encodes a protein homologous to the resuscitation-promoting factor (*rpf*) gene of *Micrococcus luteus*, whose product is required to resuscitate the growth of dormant cultures of this bacterium. The *M. tuberculosis* genome contains five genes with significant homology to *rpf*. Even though null mutations in single *rpf* genes did not affect *M. tuberculosis* physiology, suggesting some redundancy of functions, multiple *rpf* mutations significantly attenuated *M. tuberculosis* virulence in a mouse infection model. These mutants were also unable to resuscitate spontaneously *in vitro*, demonstrating the importance of the Rpf-like proteins of *M. tuberculosis* in resuscitation from the non-culturable state (Downing *et al.*, 2005). Finally, several genes encoding ribosomal proteins were downregulated, suggesting a reduced rate of protein synthesis in these conditions.

Taken together, these data suggest that in the presence of 10X-MIC vancomycin, *M. tuberculosis* undergoes a strong stress response involving σ^E-mediated membrane stability protection, and a protein refolding programme. Interestingly, several genes that we found induced are also induced in conditions of slow growth and low oxygen tension. Metabolism is slowed down, probably by the action of TA systems and Rv2623 (UspA-like), reducing the synthesis of proteins and energy-expensive surface lipids and shifting to a microaerophilic lifestyle. Of great interest is the induction in these conditions of the genes encoding two coupled PE-PPE proteins and four other non-linked PE/PPE genes of this family. Since the function of these families of surface-associated proteins is still unknown, vancomycin-treated cultures could represent a simple model to study their function in detail. Experiments to address this issue are in progress in our laboratory. The overall result of this transcriptional response could help the bacteria to partially avoid being killed by the bactericidal action of vancomycin.

sigE confers basal protection to vancomycin

sigE was induced by vancomycin; in order to determine its importance in the response to this drug-mediated stress, we tested the sensitivity of a *sigE* mutant to this drug in an agar diffusion assay. As shown in Table 2, the mutant was more sensitive to vancomycin as compared to the wild-type parental strain. This phenotype was totally complemented when the wild-type gene was reintroduced at an ectopic locus on the chromosome, showing that the transcriptional response due to this alternative sigma factor is indeed able to alleviate the toxic action produced by this drug.

Genes differentially expressed in cultures growing 1/8-MIC vancomycin

In the presence of subinhibitory concentrations of vancomycin, a total of 141 genes were differentially expressed. Functional categories of the upregulated genes are shown in Fig. 4(c). The most represented category was that of the conserved hypothetical proteins with unknown

function (46.8 %), followed by that of genes involved in broad regulatory functions (14.2 %), most of which are required for transcriptional regulation (Table S3). Only four repressed genes were identified.

While the pattern of gene expression after exposure to 10X-MIC vancomycin gave some clear hints of the physiological conditions of the bacteria, results obtained with cultures exposed to the 1/8-MIC of the drug were more difficult to interpret. Some of the stress-response genes induced by 10X-MIC vancomycin, such as *clpB*, *hsp*, *htpX*, were induced also in these conditions, suggesting that cells were indeed exposed to stress, even if still able to divide. The induction of Rv2623, encoding a UspA-like protein, is interesting, since its upregulation was previously associated only with conditions of growth arrest (Florczyk *et al.*, 2001; Shi *et al.*, 2003), while our data suggest that its activity is also required for active growth under certain stress conditions. Cultures exposed to subinhibitory vancomycin concentrations will represent a valuable model to study the function of this interesting gene. Other genes induced in these conditions include *ethR-ethA*, encoding a monooxygenase responsible for the activation of ethionamide and its transcriptional repressor. Interestingly, the induction of these two genes was also observed after SDS-mediated surface stress (Manganelli *et al.*, 2001). Other induced genes included *parB*, encoding a chromosome-partitioning protein; Rv0116c (induced also at high vancomycin concentration), encoding a protein with a peptidoglycan-binding domain; *lat*, encoding an L-lysine ε-aminotransferase; *rsbW*, and Rv3288c, which were reported as being strongly induced in conditions of long starvation and stationary phase (Bacon *et al.*, 2004; Betts *et al.*, 2002).

The transcriptional response to vancomycin was compared to those detected in response to acidic pH (Fisher *et al.*, 2002), the thiol-specific oxidating agent diamide (Manganelli *et al.*, 2002), SDS (Manganelli *et al.*, 2001), nitric oxide (Ohno *et al.*, 2003), heat shock (Stewart *et al.*, 2002) and non-replicative persistence (NRP) (Muttucumaru *et al.*, 2004). As shown in Table S4, 46 % of the genes induced by SDS and 41 % of those induced after heat shock were also induced by vancomycin in at least one of the conditions tested; the overlap with the genes induced in the other stress conditions was lower. Table S5 shows the genes induced in all three vancomycin conditions, and their induction by other stresses. Interestingly, also in this case the highest overlap was with SDS and heat shock, suggesting some common features between conditions experienced by the bacteria during exposure to stresses of this kind and those experienced during growth with vancomycin.

Table 2. Sensitivity to vancomycin in a disc diffusion assay

The paper discs contained 100 μg vancomycin. The experiment, performed in triplicate, was repeated twice with independent bacterial cultures.

Strain	Inhibition zone diameter (cm)*
H37Rv	3.5±0.1
sigE mutant	5.0±0.2
sigE mutant compl.	3.5±0.1

*Mean±SD.

Conclusions

By analysing the variation of the global transcription profile of *M. tuberculosis* cultures exposed to inhibitory or subinhibitory concentrations of a drug that perturbs surface homeostasis, we demonstrated that the responses to these different conditions are different, although with some overlap. This approach allowed us to identify several genes whose products could be involved in the protection from antibiotic stress targeting the envelope and help to confer the basal level of *M. tuberculosis* resistance to antibacterial drugs. Some of them, such as those encoding Rv2623 (UspA-like), Rv0116c, PE20-PPE31, PspA and the toxin–antitoxin systems, will be very promising subjects for future studies to better understand the physiology of the mycobacterial envelope and could represent new drug targets. Moreover, our findings demonstrate that σ^E confers basal resistance to vancomycin, once again underlining its importance in the physiology of the mycobacterial surface stress response (Raman *et al.*, 2008).

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