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Functional and Genetic Characterization of the Tap Efflux Pump in *Mycobacterium bovis* BCG

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Efflux pumps extrude a wide variety of chemically unrelated compounds conferring multidrug resistance and participating in numerous physiological processes. *Mycobacterium tuberculosis* possesses many efflux pumps, and their roles in drug resistance and physiology are actively investigated. In this work we found that *tap* mutant cells showed changes in morphology and a progressive loss of viability upon subcultivation in liquid medium. Transcriptome analysis in *Mycobacterium bovis* BCG revealed that disruption of the *Rv1258c* gene, encoding the Tap efflux pump, led to an extensive change in gene expression patterns during stationary phase, with no changes during exponential growth. In stationary phase, Tap inactivation triggered a general stress response and led to a general repression of genes involved in cell wall biosynthesis, in particular the formation of the peptidoglycan; this suggested the accumulation of an unknown Tap substrate that reaches toxic concentrations during stationary phase. We also found that both disruption and overexpression of *tap* altered susceptibility to many clinically approved antibiotics in *M. bovis* BCG. Acriflavine and tetracycline accumulation assays and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) potentiation experiments demonstrated that this phenotype was due to an active efflux mechanism. These findings emphasize the important role of the Tap efflux pump in bacterial physiology and intrinsic drug resistance.

Bacterial efflux pumps are energy-dependent membrane proteins capable of exporting a wide variety of compounds from the cytoplasm. Substrates of efflux pumps include antimicrobials, synthetic compounds, lipids, toxic metabolites, host-derived antimicrobial agents, and virulence factors, among others. Such a heterogeneous substrate profile allows bacterial efflux pumps to play diverse roles in bacterial cell physiology, drug resistance, detoxification, and virulence (41). In fact, the ability of many bacterial pathogens such as *Salmonella enterica* serovar Typhimurium, *Pseudomonas aeruginosa*, *Campylobacter jejuni*, or *Neisseria gonorrhoeae* to cause disease relies on the activities of efflux pumps (42, 47). In addition, efflux-mediated drug resistance has become clinically relevant in some bacterial pathogens, such as *P. aeruginosa*, and attempts have been made to develop efflux pump inhibitors as a strategy to overcome drug resistance (27). Efflux pumps usually confer low levels of drug resistance but can also play critical roles in the evolution to high levels of resistance. The activities of drug transporters can facilitate the progressive acquisition of chromosomal mutations conferring higher levels of resistance to a particular antibiotic (46). In addition to their roles in drug resistance and virulence, efflux pumps have also been associated with cell division (11, 25) and have physiological roles supporting pH homeostasis and alkali tolerance (24). Interestingly, the physiological functions of these pumps may foster their persistence as potential resistance determinants (24).

In *Mycobacterium*, the genus including the important human pathogen *Mycobacterium tuberculosis*, clinically relevant drug resistance is mainly due to chromosomal mutations in genes encoding the drug target or prodrug-activating enzymes (29). As in other bacterial pathogens, drug efflux pumps could contribute to the acquisition of such mutations in *M. tuberculosis* and explain why mutations in the target genes were not found in many low-level resistant strains (8). In this regard, rifampin resistance in *M. tuberculosis* has been traditionally correlated with specific mutations in the gene encoding the β-subunit of the RNA polymerase (*rpoB*). It has recently been proposed that the level of rifampin resistance in these mutant strains is defined by efflux (28); moreover, it was shown that activation of efflux pump genes by rifampin led to cross-resistance, i.e., a decrease in susceptibility to ofloxacin. The loss of particular efflux pumps also results in *M. tuberculosis* strains having a decreased virulence phenotype in an animal model of infection (6), probably due to their inabilities to properly secrete and locate essential cell envelope components (5). In addition, efflux pumps in mycobacteria also play fundamental roles in intrinsic drug resistance, oxidative stress responses, cell wall assembly, and growth (8, 15, 36, 53). These findings highlight the relevance of efflux pumps for establishing latency, in which a subpopulation of mycobacteria that are slowly dividing, metabolically active, and drug tolerant is able to persist in tuberculosis (TB) patients. The persistent state of mycobacterium has some similarities to cultures in stationary growth phase (23).

We have previously characterized the Tap efflux pumps from

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TABLE 1 Strains used in this study

Strain	Description	Reference or source
M. bovis BCG Pasteur 1173	Wild type	Laboratory collection
M. bovis SUM36	Derivative of wild type containing vector pSUM36	This study
M. bovis KOTap	Derivative of wild type with the *tap* gene disrupted; tap_{TB}::Ω*hyg*	This study
M. bovis PAZ11	Derivative of wild type expressing the tap_{TB} gene under its own promoter in the multicopy pSUM36 plasmid	This study
M. bovis AC48	Derivative of wild type expressing the tap_{FR} gene under its own promoter in the multicopy pSUM36 plasmid	This study
M. bovis KOP55	Derivative of wild type with the *p55* gene disrupted; *p55*::Ω*hyg*	53
M. bovis KO2333	Derivative of wild type with the *stp* gene disrupted; *stp*::Ω*hyg*	52

Mycobacterium fortuitum and *M. tuberculosis*, focusing on their contributions to drug resistance. These genes were expressed in the fast-growing nonpathogenic species *Mycobacterium smegmatis*. Here we extend these studies, further documenting the role of Tap efflux pumps in providing intrinsic drug resistance as well as its essential roles in physiology, growth, and cell morphology in *Mycobacterium bovis* BCG Pasteur, a slow-growing, more closely related model system for *M. tuberculosis*.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and chemicals. Strains used in this study are listed in Table 1. Sequences of oligonucleotides and plasmids used are available upon request. *M. bovis* BCG was cultivated at 37°C and 5% CO_2 in Middlebrook 7H9 broth (Difco) supplemented with 10% Middlebrook ADC (Difco) and 0.05% (vol/vol) Tween 80 or on Middlebrook 7H10 agar plates (Difco) supplemented with 10% (vol/vol) oleic acid-albumin-dextrose-catalase (OADC; Difco). *Escherichia coli* was grown at 37°C in LB broth or on LB 1.5% agar plates. For the selection of vectors in mycobacteria, hygromycin or kanamycin was added to cultures at final concentrations of 10 mg/liter or 20 mg/liter, respectively. Plasmids were maintained in *E. coli* with appropriate antibiotics for selection (ampicillin 100 mg/liter, kanamycin 20 mg/liter).

DNA manipulations. DNA manipulations were carried out according to standard techniques (54). Mycobacterial genomic DNA isolation was performed as previously described (43). Southern blotting was done using the ECL direct nucleic acid labeling and detection system (Amersham Biosciences) according to the manufacturer's instructions. Both *E. coli* and mycobacteria were transformed by electroporation with a gene pulser (Bio-Rad Laboratories Inc., Richmond, CA) (43).

Strain construction. The nucleotide sequence of the *M. tuberculosis* *Rv1258c* gene (http://genolist.pasteur.fr/TubercuList/) is identical to that of *BCG1316c* from *M. bovis* BCG Pasteur 1173 P2 (http://genolist.pasteur.fr/BCGList/). In this study, both *Rv1258c* and *BCG1316c* will be referred to as tap_{TB} and the *tap* gene from *M. fortuitum* as tap_{FR}.

(i) Overexpression. The tap_{TB} and tap_{FR} genes expressed under the control of their respective promoters were cloned previously into the pSUM36 vector, yielding pPAZ11 (9) and pAC48 (2). Plasmids pPAZ11 and pAC48 were introduced to *M. bovis* BCG, resulting in *M. bovis* BCG PAZ11 and *M. bovis* BCG AC48, respectively.

(ii) Disruption. A suicide delivery plasmid was constructed containing the tap_{TB} gene interrupted by the insertion of a hygromycin resistance cassette (Ω*hyg*) with flanking termination sequences (49). Briefly, a 2.3-kb PCR product from *M. tuberculosis* H37Rv genomic DNA containing tap_{TB} was cloned into pUC19. The tap_{TB} gene was then interrupted by the insertion of the Ω*hyg* cassette. The *tap*::Ω*hyg* fragment was isolated by PstI digestion and cloned into the PstI-digested p2NIL vector (44), yielding pVZ16. A cassette containing *lacZ* and *sacB* genes from pGOAL17 (44) was then cloned into the single PacI site of pVZ16 to generate the suicide delivery vector pVZ17. pVZ17 was used to transform *M. bovis* BCG. Single- and double-crossover (DXO) transformants were selected as described elsewhere (44). Candidates for DXO were analyzed by PCR with primers for the tap_{TB} gene flanking the Ω*hyg* insertion point. The mutant DNA generated a large PCR fragment compared to the wild-type fragment, because it included the inserted hygromycin cassette (data not shown). Candidates with the expected PCR patterns were streaked onto plates with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and either kanamycin or hygromycin for phenotypic analysis. Finally, Southern blotting was done to confirm DXO (Fig. 1). The *M. bovis* BCG strain with the disrupted tap_{TB} gene was named *M. bovis* BCG KOTap (Table 1).

Drug susceptibility assays. MICs were determined using 2-fold serial dilutions of compounds. For determination in liquid medium, the resazurin assay was carried out essentially as previously described (51), except plates were incubated for 8 days at 37°C and for two additional days after the addition of the redox indicator (resazurin). For agar-based determinations, exponential-phase cultures were diluted to 10^5 cells/ml, and 10 μl was spotted onto 25-ml 7H10 agar plates containing serial 2-fold antibiotic dilutions. Additionally, 10-fold serial dilutions of a 10^7-cell/ml expo-

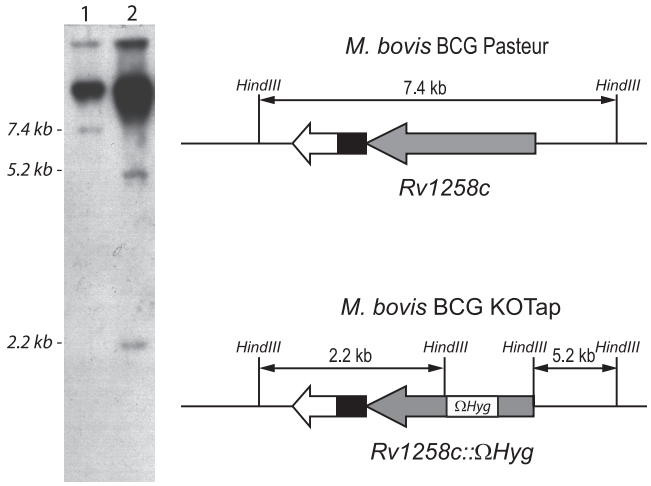

FIG 1 Southern blot analysis for *tap* gene inactivation in *M. bovis* BCG. Genomic DNA isolated from *M. bovis* BCG wild type (lane 1) and the *M. bovis* BCG double-crossover (DXO) strain (lane 2) was digested with HindIII and hybridized to a probe corresponding to a 0.86-kb tap_{TB} internal region flanking the Ω*hyg* insertion point. HindIII digestion released the Ω*hyg* cassette, yielding two fragments of 5.2 kb and 2.2 kb for the DXO strain, while a single 7.4-kb fragment was observed in the wild-type strain. Loading wells and undigested DNA can be also seen on the upper part of the image. The diagram showing the expected HindIII digestion fragment is not shown to scale. In *M. bovis* BCG Pasteur, the RD13 deletion (4) comprises the region containing the *Rv1255c*, *Rv1256c*, and *Rv1257c* genes, compared to the *M. tuberculosis* H37Rv genome. The chimeric gene after the fusion of the *Rv1255c* and *Rv1257c* genes is shown in white and black.

nential-phase culture were inoculated on a fixed subinhibitory concentration of antibiotic using a Steer replicator. A conventional disc diffusion assay was used to test the redox compounds. Briefly, an exponential-phase culture dilution containing 10^6 cells/ml was spread on Middlebrook 7H10 agar plates, and discs containing the redox compounds were placed onto the lawn. Visible growth was scored after 21 days of incubation. Experiments were carried out in triplicate and repeated at least three times.

Accumulation experiments. (i) [^{3}H]tetracycline accumulation. Uptake experiments were essentially performed as previously described (10, 35). Exponential-phase cultures of *M. bovis* BCG wild-type and KOTap strains growing in 7H9 broth were harvested by centrifugation at room temperature, washed twice with 0.1 M potassium phosphate (pH 7.0), and resuspended in prewarmed assay phosphate buffer (0.1 M potassium phosphate [pH 7.0], 1 mM $MgCl_2$). Aliquots of 1 ml were preincubated for 15 min at 37°C with vigorous aeration by shaking, and the assay was started by the addition of [^{3}H]tetracycline (0.76 Ci/mmol; New England Nuclear) to a final concentration of 5 μM. At various time intervals thereafter, 50 μl of the suspension was removed, diluted in 1 ml of ice-cold 0.1 M potassium phosphate (pH 7.0) buffer containing 0.1 M LiCl, and immediately filtered through a 0.45-μm-pore-size filter (Millipore). The filter was rapidly washed twice with 4 ml of the same buffer and dried. The radioactivity was then determined in a Beckman LS 7000 liquid scintillation counter by using Ecolume scintillation cocktail (ICN Biomedicals). Uptake experiments were performed at least three times.

Acriflavine accumulation. Exponential-phase cultures of *M. bovis* BCG SUM36 and PAZ11 cultures grown in 7H9 broth were washed twice and resuspended in 0.1 M potassium phosphate (pH 7.0). Cells were incubated at 37°C for 15 min to allow stabilization of the intrinsic fluorescence (F_0) of the cells. The experiment was initiated by the addition of acriflavine at a final concentration of 5 μM. At every time point, fluorescence was measured for 20 s, and the highest [$F_{1(max)}$] and lowest [$F_{1(min)}$] values were recorded. The mean fluorescence of the two values was then calculated (F_1). Acriflavine accumulation was expressed as an arbitrary unit (F_1/F_0). Acriflavine has an excitation wavelength (λ_{ex}) of 485 nm and an emission wavelength (λ_{ex}) of 501 nm. Fluorescence was determined using a Perkin Elmer LS-3 fluorimeter.

RNA extraction, cDNA labeling, microarray hybridization, and data analysis. Two independent cultures of *M. bovis* BCG wild-type and KOTap strains grown in 75-cm^2 tissue culture standing flasks were harvested during exponential growth phase (1 week; optical density at 600 nm [OD_{600}] = 0.2 to 0.3) or stationary growth phase (6 weeks; OD_{600} = 0.8 to 1.0). Cultures were pelleted at room temperature. After removing the supernatant, pellets were frozen on dry ice and stored at −80°C. RNA extraction was performed as previously described (30). Fluorescently labeled cDNA copies of total RNA were prepared by direct incorporation of fluorescent nucleotide analogues during a first-strand reverse transcription (RT) reaction as previously described (30). *M. tuberculosis* oligoarrays were obtained from the Center for Applied Genomics, International Center for Public Health (Newark, NJ). These microarrays consist of 4,295 70-mer oligonucleotides representing 3,924 open reading frames (ORFs) from *M. tuberculosis* strain H37Rv and 371 unique ORFs from strain CDC1551 that are not present in H37Rv. Each microarray was processed for hybridization as previously described (30). Hybridizations were performed using RNA extracted from two cultures, each started from different colonies. Each sample was hybridized twice through reverse labeling of their cDNAs. Fluorescence intensity data from each array were collected with an Affymetrix 428 scanner. Fluorescence intensities of Cy3 and Cy5 dyes at each spot were quantified using the ImaGene software 5.0 (BioDiscovery, Inc.), and data obtained from qualified spots on each chip were normalized using the print-tip Lowess implementation included in GEPAS v1.1 (62). The expression ratio for the wild-type and the mutant genes was determined from the normalized fluorescence intensity and was calculated as the average fold change. Significance analysis of microarrays (SAM) identified genes whose expression was affected by the disruption of *tap* (60), defined by a *q* value (percent chance that the gene is a false positive) of ≤1% and a minimum change in expression of 2.0- or 0.5-fold.

RT-qPCR. Reverse transcription was performed with random hexamer primers using murine leukoblastoma virus retrotranscriptase (Applied Biosystems), as previously described (30). Quantitative PCR (qPCR) was performed with SYBR green master mix (Applied Biosystems). After 10 min at 95°C to activate the enzyme, 40 amplification cycles were performed using an Applied Biosystems 7700 Prism spectrofluorometric thermal cycler (Perkin-Elmer) under the following conditions: 1 min denaturation at 95°C, 30 s annealing at 60°C, and 30 s extension at 72°C. Results were normalized to the amount of *sigA* mRNA, as previously described (31). RNA samples that had not been reverse transcribed were included in all experiments to exclude samples with significant DNA contamination. For each sample, melting curves were performed to confirm the purity of the products. Sequences of the primers for qRT-PCR are available upon request.

Auramine O stain. Samples were taken to an OD_{600} of 0.5 and fixed on a heating block for 1 h at 70 to 80°C. A fluorescent stain kit for mycobacteria (Fluka) was used. Glass slides were flooded with phenolic auramine O solution and stained for 30 min. Samples were decolorized for 1 min with an acid-alcohol solution. Finally, counterstaining was performed for 2 min using a potassium permanganate solution. Samples were air dried and visualized by fluorescence microscopy. Images were captured with a Hamamatsu Orca camera system coupled to a Nikon Eclipse TE2000-U microscope equipped with a 100× objective and a 10× ocular. Micrograph analyses were performed using ImageJ.

Microarray data accession number. Microarray data have been deposited in the Gene Expression Omnibus public database (http://www.ncbi.nlm.nih.gov/geo) under the accession number GSE32249.

RESULTS AND DISCUSSION

KOTap cells had a progressive growth defect and altered morphology. In the course of this study, we found that KOTap cells (*M. bovis* BCG with a disrupted homolog of the tap_{TB} gene) grew less rapidly and arrested growth prematurely after cycles of subcultivation in liquid cultures (Fig. 2). This progressive growth defect could be reversed by cultivation on solid medium; when these new single colonies were reinoculated into liquid medium, growth kinetics were similar to those of the original transformants. Interestingly, after subcultivation in liquid, the mutant bacilli became more elongated (Fig. 3). The progressive growth defect and altered morphology phenotypes were specific for the KOTap strain; other efflux pump mutants (*p55* [53] and *stp* [52]) were included for comparison and did not display the progressive growth defect (Fig. 2A) or elongated bacillus morphology (data not shown). Surprisingly, KOTap cells resembled the morphology observed after overexpression of *ftsZ* (12) or an *E. coli* AcrEF-TolC efflux pump mutant (25). The *ftsZ* gene determines molecular events involved in cell division in mycobacteria. Dziadek et al. (12) showed that overproduction of FtsZ generated filamentous cells that lacked visible septa and were not viable. Finally, while the *p55* colonies were smaller than those of its parental strain (53), this phenotype was not observed in KOTap or KO2333 (data not shown), suggesting different physiological roles for these efflux pumps.

Inactivation of the Tap efflux pump in *M. bovis* BCG greatly alters global gene expression in stationary phase but not during exponential growth. Efflux pump studies have traditionally focused on their roles in conferring intrinsic drug resistance in bacteria (26), a function having potential clinical implications (46). However, other documented roles in physiological homeostasis and virulence processes (47) suggest that antibiotic resistance

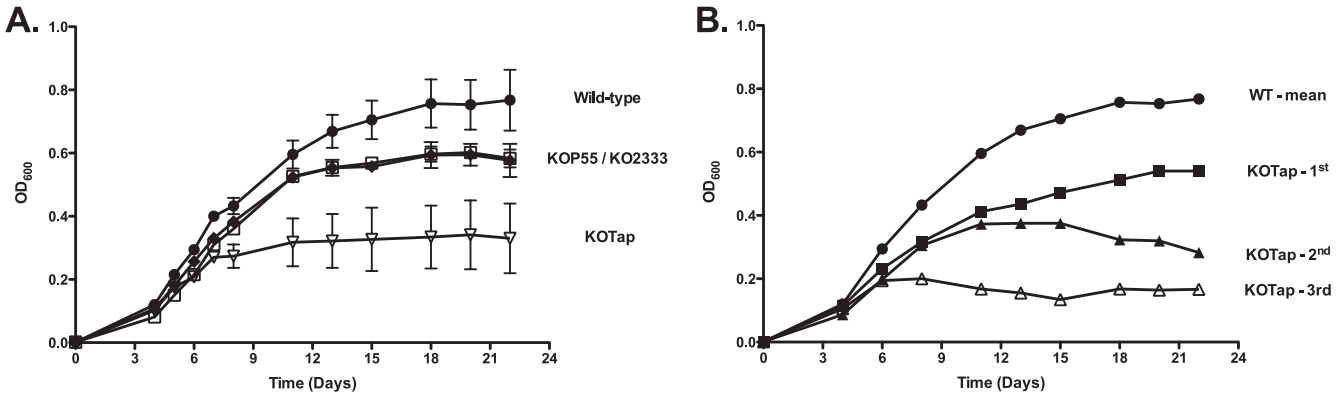

FIG 2 Growth kinetics of *M. bovis* BCG wild-type and efflux pump mutant strains. Single colonies were inoculated in 10 ml of broth as preinoculum and subcultivated at 37°C, 5% CO_2 in 100-ml cultures in 75-cm^2 tissue culture standing flasks with occasional shaking. After 1 week, strains were subcultivated in fresh media. Bacteria were subcultured on a weekly basis, i.e., the second subculture was inoculated from the first, and the third subculture from the second. (A) Wild type (circles), KOP55 (open squares), KO2333 (diamonds) and KOTap (inverted empty triangles). Growth curves are the means from the three subcultures and one representative of two experiments. Error bars represent standard errors of the mean (SEM). (B) Consecutive individual subcultivation growth curves are shown for KOTap (first subculture [squares], second subculture [triangles], and third subculture [open triangles]). The growth curve of the wild type is shown for comparison.

genes, particularly those encoding multidrug efflux transporters, could have evolved from existing genes with other physiological functions (24).

Because of the progressive growth defect observed in the KOTap strain, we used transcriptomic approaches to study the role of the Tap efflux pump during exponential and stationary growth and to define both *in vitro* growth dynamics and potential roles of Tap in TB latency. *M. bovis* BCG KOTap transformants were initially viable, indicating that tap_{TB} is not essential for growth *in vitro* during exponential growth. When gene expression

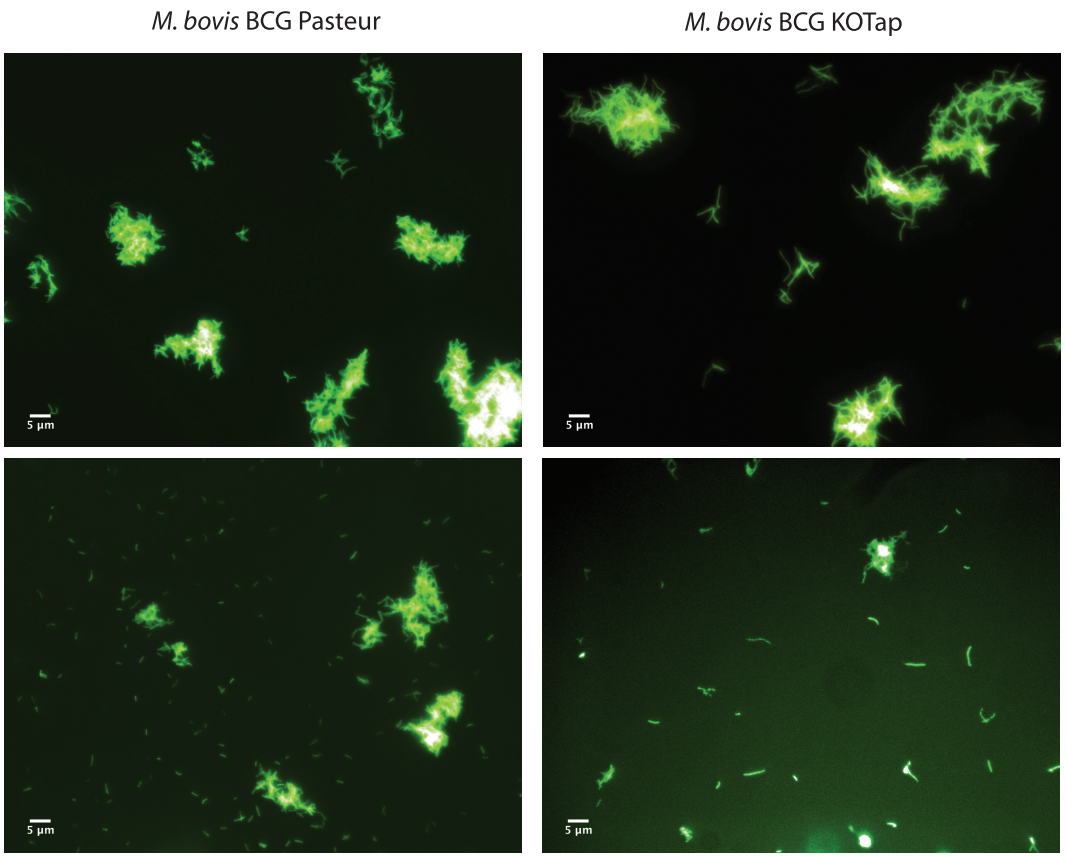

FIG 3 Morphology of *M. bovis* BCG wild-type and KOTap cells. Samples correspond to the second subcultivation at day 14. Two fields with aggregates and individual cells are shown for comparison. Bars, 5 μm.

TABLE 2 Total number of genes differentially expressed after Tap inactivation in *M. bovis* BCG

Growth phase[b]	No. of genes with altered expression[a]			
	Increased by:		Decreased by:	
	≥2	≥1.7	≤0.5	≤0.7
Exponential	0	7	0	0
Stationary	103	109	30	40

[a] Two cutoff values were used to define changes in gene expression (fold variation compared to wild-type levels of expression).
[b] Gene expression was analyzed during exponential growth phase (1 week: OD_{600} = 0.2 to 0.3) and stationary phase (6 weeks: OD_{600} = 0.8 to 1.0).

patterns of the *tap* mutant were compared to those of the wild-type strain during exponential growth (1 week; OD_{600} = 0.2 to 0.3), almost no differences were observed; only seven genes had slightly increased expression (Table 2; also see the supplemental material). In contrast, more than 100 genes had altered levels of expression in stationary-phase cultures; the induction or repression of a set of 18 relevant genes was validated by qPCR (Tables 2 and 3). A functional category analysis (http://tuberculist.epfl.ch/index.html) of the genes differentially expressed in stationary phase revealed a high proportion belonging to the virulence, de-

TABLE 3 Genes differentially regulated in *M. bovis* BCG during stationary phase due to inactivation of the Tap efflux pump

Gene[a]	Rv no.[b]	*q* value[c]	Fold change in expression[d]	qPCR[e]	Functional category[f]	Gene product
Upregulated						
acr2	Rv0251c	0	12.03	18.14 ± 6.17	VDA	Heat shock protein
dnaK	Rv0350	0	8.52	14.21 ± 0.98	VDA	Chaperone
grpE	Rv0351	0	10.84		VDA	Hsp-70 cofactor
dnaJ	Rv0352	NA	NA	3.91 ± 0.82	VDA	Chaperone
hspR	Rv0353	0	8.12		VDA	Heat shock protein
clpB	Rv0384c	0	6.32		VDA	Heat shock protein
groEL2	Rv0440	0	9.68	20.43 ± 13.50	VDA	Chaperone
cysK2	Rv0848	0	4.52	10.60 ± 9.23	IMR	Cysteine synthase
trxB1	Rv1471	0	2.56	4.92 ± 1.16	IMR	Thioredoxin
nadA	Rv1594	0	1.97		IMR	Quinolinate synthetase
nadB	Rv1595	0	4.39		IMR	L-Aspartate oxidase
	Rv1812c	0	5.78		IMR	NADH dehydrogenase
	Rv1813c	0	8.31		CH	
katG	Rv1908c	0	2.54	3.0 ± 1.3	VDA	Catalase-peroxidase
furA	Rv1909c	0	4.94		RP	Ferric uptake regulation
	Rv2466c	0	5.78	5.16 ± 2.52	CH	DsbA-like
	Rv2912c	0	6.81		RP	Transcriptional regulator
	Rv2913c	0	24.75		IMR	D-Amino acid aminohydrolase
groEL1	Rv3417c	0	7.51	17.55 ± 11.64	VDA	Chaperone
groES	Rv3418c	0	6.35		VDA	Chaperone
bfrB	Rv3841	0	3.14		IMR	Bacterioferritin
whiB6	Rv3862c	0	6.00	4.39 ± 0.93	RP	Transcriptional regulator
trxC	Rv3914	0	3.31		IMR	Thioredoxin
Downregulated						
galE2	Rv0501	0	0.44	0.50 ± 0.17	IMR	UDP-glucose-4-epimerase
esxJ	Rv1038c	0.95	0.45		CWCP	ESAT-6-like
sigE	Rv1221	0	0.37		IP	Sigma factor
$eccB_5$	Rv1782	NA	NA	0.43 ± 1.38	CWCP	Esx conserved component
$eccCb_5$	Rv1784	0	0.44	0.36 ± 0.27	CWCP	Esx conserved component
PPE26	Rv1789	NA	NA	0.31 ± 0.11	PE/PPE	PPE family
esxM	Rv1792	0.51	0.44	0.13 ± 0.01	CWCP	ESAT-6-like
esxN	Rv1793	NA	NA	0.28 ± 0.02	CWCP	ESAT-6-like
eccA5	Rv1798	NA	NA	0.39 ± 0.12	CWCP	Esx conserved component
mpt70	Rv2875	0	0.36		CWCP	Secreted immunogenic
sugI	Rv3331	0	0.12	0.01 ± 0.00	CWCP	Sugar transporter
nagA	Rv3332	0.38	0.38		CWCP	GlcNAc6p deacetylase
esxU	Rv3445c	0	0.30		CWCP	ESAT-6-like
Asd	Rv3708c	0.55	0.41		IMR	Aspartate-semialdehyde dehydrogenase

[a] Genes were included in the table if their *q* value was ≤1 and the fold increase or decrease in gene expression was ≥2.0 or ≤0.5, respectively. Some genes with no expression data available (NA) were also included, provided they belonged to an induced or repressed gene cluster.
[b] Genes are annotated as described by the Pasteur Institute on TubercuList (http://genolist.pasteur.fr/TubercuList/).
[c] *q* value indicates the false discovery rate (FDR), i.e., the probability that the gene was falsely called (calculated by SAM). NA, not available.
[d] Values of change in gene expression by microarray experiments.
[e] Data represent mean values from two biologically independent experiments performed in duplicate. Standard deviation is shown.
[f] Functional categories as per TubercuList (http://genolist.pasteur.fr/TubercuList/). CWCP, cell wall and cell processes; CH, conserved hypotheticals; IP, information pathways; ISP, Insertion seqs and phages; IMR, intermediary metabolism and respiration; LP, lipid metabolism; PE/PPE, PE/PPE; RP, regulatory proteins; SR, stable RNA; VDA, virulence, detoxification, adaptation.

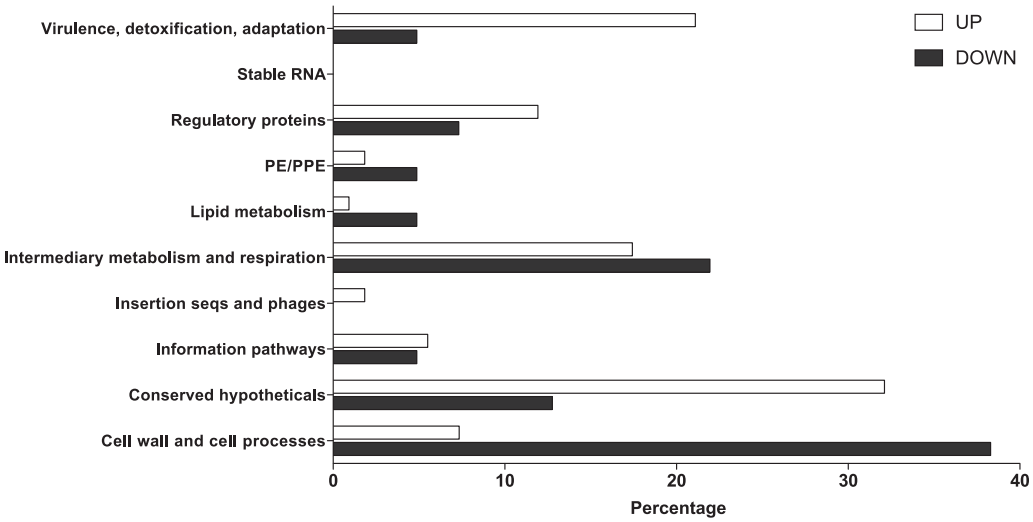

FIG 4 Functional categories of genes with increased (UP; cutoff value, 1.7) or decreased (DOWN; cutoff value, 0.7) expression in stationary phase in *M. bovis* BCG KOTap compared to wild-type strain. The total number of genes, either up- or downregulated (as shown in the supplemental material), is taken as 100%. The number of genes, as a percentage of the total, is depicted.

toxification, adaptation (VDA), intermediary metabolism and respiration (IMR), conserved hypotheticals (CH), and cell wall and cell processing (CWCP) groups, suggesting a major adaptation to a stress generated by the disruption of tap_{TB} (Fig. 4). A comparative analysis of the genes up- or downregulated revealed a greater proportion of the upregulated genes in the VDA category, while downregulated genes were more abundant in the CWCP and lipid metabolism (LM) categories. Interestingly, Stewart et al. (58) reported a similar response of *M. tuberculosis* after heat shock, "with a bias toward adaptation/detoxification and regulatory genes, and away from cell wall-associated genes." These results clearly indicated that Tap was needed to maintain balanced physiological functions during late stationary phase and pointed toward a potential role of the Tap efflux pump in latency. As bacterial growth is arrested during stationary phase, toxic waste products accumulate inside the cell and must be cleared from the cytoplasm. We suggest that normal physiological substrates of Tap are toxic by-products which accumulate during stationary phase when nutrients are limited. The inactivation of Tap would trigger a shift in cell metabolism to counteract the physiological stress produced by the accumulation of its endogenous physiological substrates. Detailed analyses of the genes and operons differentially expressed in the KOTap strain described in the following sections support this hypothesis (Table 3) (a complete list of differentially regulated genes is provided in the supplemental material).

Genes upregulated during stationary phase upon tap_{TB} disruption. In general, the expression levels of stress response genes, including members of the HspR regulon (*acr2*, *dnak*, *grpE*, *dnaJ*, *clpB*, *bfrB*, and *hspR*), were highly upregulated. This set of genes responds to heat shock (58) and antibacterial agents produced by the immune system (57, 64). Increased expression of heat shock proteins is also promoted by the accumulation of unfolded polypeptides (39); in agreement with this, we observed that chaperone genes *groEL1* and *groEL2* were also highly upregulated (Table 3).

Genes involved in oxidative stress responses were also upregulated. *Rv2466c* is predicted to contain thioredoxin and disulfide oxidoreductase domains; it is a SigH-dependent gene induced under anaerobic cultures (38) that is associated with oxidative stress responses in *M. tuberculosis* (50). Other upregulated genes were involved in the biosynthesis of reducing equivalents, including *nadA*, *nadB*, and *Rv1812c* (encoding a putative NADH dehydrogenase); thioredoxins (*trxB1* and *trxC*); or genes responding to stress conditions (*katG* [19] and *furA* [13]) (Table 3).

The gene *Rv2913c*, annotated as a D-amino acid aminohydrolase, displayed the highest increase in expression (25-fold) in the tap_{TB} mutant. D-Amino acids are abundant components of peptidoglycan (PG) present in the peptide that cross-links the linear glycan chains in the cell wall (61). As discussed below, Tap could be involved in PG-recycling pathways that are growth rate dependent. In the *tap* mutant, toxic intermediates of cell wall assembly may accumulate during stationary phase. The increased expression of D-amino acid aminohydrolases might serve as an alternative detoxifying system.

The expression of *whiB6* also increased; it is one of seven *whiB*-like genes in *M. tuberculosis*, which are thought to play roles in transcriptional regulation (56). Interestingly, *whiB6* appears to be nonessential for the *in vitro* transition from exponential to stationary phase (63). However, in a study of the seven WhiB paralogs in *M. tuberculosis*, Geiman et al. (17) showed that *whiB6* was the most highly induced by diverse stresses, including heat shock, oxidative stress, and treatments that affected cell wall integrity. Accordingly, WhiB6 might specifically respond to the stress cause by the inactivation of the Tap efflux pump. Interestingly, WhiB7 is thought to control the expression of tap_{TB} (37), suggesting a regulatory link between these stress responsive WhiB proteins.

Genes downregulated during stationary phase upon tap_{TB} disruption. More than one-third of the genes downregulated in the KOTap strain belonged to the CWCP category (Fig. 4), suggesting that the stress conditions caused by tap_{TB} disruption resulted in the slowdown of cell wall-related processes. Among these genes, *sugI*, encoding an efflux pump of the major facilitator superfamily (MFS) thought to be involved in sugar transport, was the most downregulated (Table 3). It forms an operon with the

TABLE 4 Sensitivity of *M. bovis* BCG KOTap to redox compounds

Redox compound[a]	Disc load (μmol)	Inhibition zone[b] (mm) for *M. bovis* BCG strains	
		Wild type	KOTap[c]
Hydrogen peroxide	80	15	18
	40	13	14
Plumbagin	0.01	26	30
Diamide	20	0	18

[a] Dimethyl sulfoxide, which was used as the solvent for some of these compounds, had no effect on the growth of either strain (data not shown).
[b] Zones of inhibition were recorded after 21 days at 37°C.
[c] KOTap is *M. bovis* BCG with the tap_{TB} gene inactivated.

TABLE 5 Antimicrobial susceptibility of *M. bovis* BCG Tap-derivative strains

Compound	MIC for *M. bovis* BCG strains (mg/liter)[a]			
	Wild type	KOTap[b]	PAZ11[c]	AC48[d]
2′-*N*-Ethylnetilmicin	1	0.5	ND	ND
6′-*N*-Ethylnetilmycin	2	1	ND	ND
Acriflavine	1	1–0.5	4	2
Chloramphenicol	4	4	8	8
Clarithromycin	0.03	0.03–0.015	0.03	0.06–0.25
Ethambutol	4	4	8	4
Gentamicin	1	0.5	2–4	8
Isoniazid	0.2	0.2	0.4	0.4
p-Aminosalicylate	0.125	0.03	1	0.25
Rifampin	0.012	0.012	0.012	0.025
Streptomycin	0.2	0.2	0.4	1.6
Spectinomycin	1	0.25	4	4
Tetracycline	1	0.25	4	4
Tetracycline + CCCP[e]	ND	ND	1	1
Triclosan	8	4	32	8–16
Vancomycin	10	5	20–40	40

[a] MICs were assayed over a range of 2-fold dilutions of antibiotics. No difference in sensitivity between wild-type and Tap-derivative strains was observed for the following compounds: amikacin (MIC_{WT} = 0.125 mg/liter), carbonyl cyanide *m*-chlorophenylhydrazone (MIC_{WT} = 5 mg/liter), ciprofloxacin (MIC_{WT} = 0.125 mg/liter), clofazimine (MIC_{WT} = 0.05 to 0.1 mg/liter), chlorpromazine (MIC_{WT} = 4 mg/liter), econazole (MIC_{WT} = 6.4 mg/liter), ethionamide (MIC_{WT} = 4 mg/liter), fluconazole (MIC_{WT}, >64 mg/liter), gatifloxacin (MIC_{WT} = 0.03 mg/liter), moxifloxacin (MIC_{WT} = 0.03 to 0.06 mg/liter), and ofloxacin (MIC_{WT} = 0.25 mg/liter). ND, not determined.
[b] KOTap, *M. bovis* BCG with the tap_{TB} gene inactivated.
[c] PAZ11, *M. bovis* BCG strain with the pPAZ11 plasmid (tap_{TB} cloned into pSUM36).
[d] AC48, *M. bovis* BCG containing pAC48 plasmid (tap_{FR} cloned in pSUM36).
[e] CCCP was present at a concentration of 2 mg/liter.

N-acetylglucosamine-6-phosphate (GlcNAc6p) deacetylase gene, *nagA*, also highly downregulated, which is involved in the pathway for recycling the amino sugar of the PG. It is estimated that 40% to 50% of the PG is degraded and reused each generation. In *E. coli*, NagA catalyzes the conversion of GlcNAc6p into glucosamine-6-phosphate (GlcN6P), which is then converted into UDP-GlcNAc, the main precursor of the PG biosynthetic pathway, by the GlmM and GlmU enzymes. Newton and coworkers (40) have suggested a role for SugI in the import of *myo*-inositol, a sugar needed for mycothiol biosynthesis and as a precursor to mycobacterial membrane and cell wall components. If so, why would inactivation of the Tap efflux pump have such a dramatic effect on the expression of the *sugI-nagA* operon during stationary phase but not during exponential growth? In addition to having a structural role, amino sugars are an important energy source for bacteria because they supply both carbon and nitrogen. This could be of particular importance in stationary phase, in which nutrients are limited. In this context, Tap could be involved in the maintenance of sugar phosphate homeostasis. The inactivation of Tap would lead to GlcN6P accumulation, causing toxicity (21, 48), which would explain the upregulation of genes involved in general stress responses. A model in which PG recycling is shut down to prevent the accumulation of sugar phosphates is supported by the observation that other genes involved in cell wall processes are also repressed (Table 3). These include the *galE2* gene encoding a possible UDP-glucose-4-epimerase, involved in the biosynthetic arabinogalactan-peptidoglycan complex (MAPc) pathway of *M. tuberculosis* (7), and *asd*, encoding an L-aspartic-β-semialdehyde dehydrogenase. The *asd* gene forms an operon with *ask*, an aspartokinase; both genes are essential for the biosynthetic pathway of diaminopimelate (DAP), which forms essential interpeptide PG cross-linking (45). These observations suggest a general repression of genes involved in cell wall biosynthesis, in particular the formation of the PG.

Most sigma factors are involved in the regulation of stress responses, nutrient adaptation, and cell differentiation. In our analysis, sigma E was the only sigma factor whose expression was altered in the KOTap mutant strain (Table 3). The *sigE* gene is essential for growth in macrophages and virulence in mice (32, 34). In addition, a *sigE* mutant is more sensitive to membrane-disrupting agents and vancomycin as well as heat shock and oxidative compounds such as hydrogen peroxide or plumbagin (33). Interestingly, in addition to vancomycin the KOTap strain was more sensitive to the oxidative compounds diamide, hydrogen peroxide, and plumbagin (Tables 4 and 5), suggesting a role (direct or indirect) in cell wall stability and in the maintenance of the oxidative balance within the cell, as previously described for another efflux pump in mycobacteria (53).

Finally, several genes ($eccB_5$, *eccCb5*, *eccA5*, *esxJ*, *esxM*, *esxN*, and *esxU*) encoding members of the immunologically active early secretory antigenic target-6 (ESAT-6) family of proteins, along with the major secreted immunogenic Mtp70 protein, were repressed in the KOTap strain (Table 3). The *esat-6* gene is found in the RD1 (region of difference 1; absent from all *M. bovis* BCG strains), and it has been correlated with virulence (18). We confirmed the repression of the genes contained in the ESAT-6 gene cluster region 5 of *M. tuberculosis* (Table 3), involved in antigen secretion (18). These results suggest a link between the Tap efflux pump and virulence.

Mycobacterial Tap efflux pumps contribute to intrinsic antibiotic resistance in *M. bovis* BCG. The mycobacterial Tap efflux pumps from *M. fortuitum* and *M. tuberculosis* were originally reported to confer tetracycline and aminoglycoside resistance in *M. smegmatis*, a fast-growing *Mycobacterium* (2). The levels of resistance conferred and the substrate specificities differed between the two efflux pumps; in general, Tap_{FR} provided higher levels of resistance to a larger number of drugs than Tap_{TB} (2). Tap_{FR} used the electrochemical gradient to extrude tetracycline from the cell, and this efflux activity could be inhibited by several compounds, such as the protonophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (51). In the present study, the contribution of these Tap efflux pumps to antimicrobial resistance was character-

ized in *M. bovis* BCG, a much closer relative to *M. tuberculosis* than *M. smegmatis*.

M. bovis BCG derivatives were constructed overexpressing tap_{TB} or tap_{FR} genes, or with the tap_{TB} gene interrupted by the insertion of a hygromycin resistance cassette (Table 1; Fig. 1). Antimicrobial susceptibility to a set of compounds with diverse cellular targets was determined for these strains (Table 5). In general, variations in sensitivity due to the interruption of the tap_{TB} gene were lower (2- to 4-fold) than those due to overexpressing either tap_{TB} or tap_{FR} (4- to 8-fold). The substrate specificity profile was also larger when either *tap* gene (tap_{TB} or tap_{FR}) was overexpressed; there are two possible explanations for these differences. First, the disruption of tap_{TB} could lead to a compensatory increase in the levels of expression of other efflux pump genes with similar substrate recognition profiles. Second, it is well recognized that efflux pumps nonspecifically extrude a wide variety of chemically and structurally unrelated compounds and that constitutive overexpression may provide for increased resistance, a phenotype that would not be detected in studies of efflux pump mutants (42); here, tap_{TB} and tap_{FR} were overexpressed on a multicopy plasmid (5 to 10 copies per cell). A >4-fold change in sensitivity was observed for acriflavine, gentamicin, *p*-aminosalicylic acid (PAS), streptomycin, spectinomycin, tetracycline, triclosan, and vancomycin (Table 5). The degree of drug sensitivity probably correlates with the specificity of Tap for a particular substrate. In this regard, PAS (a second-line oral anti-TB agent), spectinomycin, and tetracycline were identified as specific Tap substrates in *M. bovis* BCG since a ≥4-fold change in sensitivity was observed in mutant and overexpressing strains compared to the wild-type strain.

Two complementary approaches were used to confirm that this phenotype was due to an efflux mechanism. First, tetracycline susceptibility assays in the presence of CCCP were carried out using *tap*-overexpressing strains (Table 5); in *M. bovis* BCG, CCCP reduced Tap activity by inhibiting the electrochemical gradient across the membrane, similar to what was previously observed in *M. smegmatis* (51). Second, tetracycline and acriflavine accumulation experiments were performed (Fig. 5). [^{3}H]tetracycline efflux experiments showed that KOTap cells accumulated approximately twice as much tetracycline as wild-type cells, achieving a steady-state level of accumulation within about 3 min (Fig. 5A). Similarly, acriflavine accumulation assays were performed with the *M. bovis* PAZ11 strain (tap_{TB} overexpression). When acriflavine is accumulated inside the cells, its fluorescence emission is quenched upon binding to the DNA. The tap_{TB}-overexpressing PAZ11 culture had higher levels of fluorescence (Fig. 5B) and a 4-fold increase in MIC to acriflavine (Table 5). Together, these data showed that Tap_{TB} provided tetracycline and acriflavine resistance in *M. bovis* BCG by active efflux.

The contribution of Tap to intrinsic resistance to first-line anti-TB drugs ethambutol, rifampin, and isoniazid was first suggested by the fact that small increases in resistance were reproducibly observed in overexpressing strains (Table 5). To validate these results, 10-μl portions of 10-fold serial dilutions of the culture (original inoculum, 10^7 cell/ml) were inoculated on agar plates at a fixed subinhibitory concentration of isoniazid (0.05 mg/liter). Under these conditions, the wild-type strain grew at a 10^{-2} dilution; the overexpressing strain (tap_{TB} or tap_{FR}) grew at a 10^{-4} dilution (data not shown), documenting its ability to better adapt to or tolerate isoniazid.

The levels of drug resistance conferred by efflux pumps are generally low compared to what is seen for mutations in drug target genes and, unlike other bacteria (46), no clinically relevant efflux pump mutations have yet been identified in *M. tuberculosis*. While some authors have reported increases in tap_{TB} expression after antibiotic treatment in clinical isolates (20, 55), a direct correlation between tap_{TB} levels of expression and its contribution to antibiotic resistance has not been demonstrated in these clinical isolates. In fact, increased expression of an efflux pump gene upon antibiotic exposure does not necessarily mean that the antibiotic is a substrate of the efflux pump. Antibiotics might have deleterious cellular effects beyond their molecular targets (22) and thus generate other metabolic signals. It is possible that the induction of efflux pump genes might be a response to counteract antibiotic downstream effects, including oxidative stress (16). In addition, increased expression of efflux pumps may permit bacteria to survive otherwise lethal concentrations of substrate antibiotics and thus allow selection for target mutations that increase drug resistance levels. This phenomenon is known as "drug target resistance masking" (14). Together, this underlines the role of efflux pumps in the development of high levels of multidrug resistance.

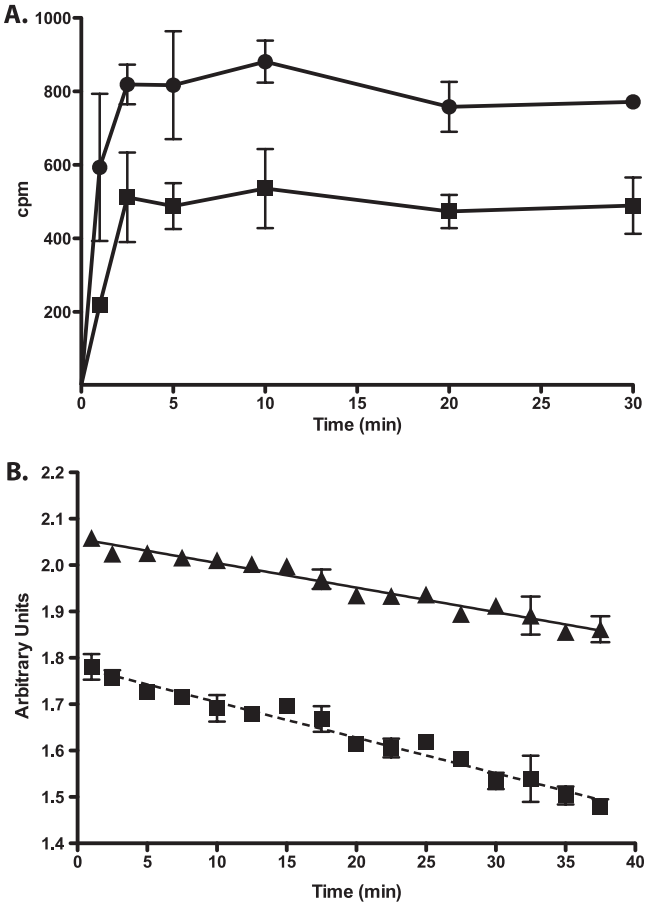

FIG 5 Tetracycline and acriflavine accumulation by intact cells of *M. bovis* Tap-derivative strains. (A) [^{3}H]tetracycline uptake by wild-type (squares) and KOTap mutant (circles) cells. (B) Acriflavine uptake by SUM36 (squares) and the tap_{TB}-overexpressing PAZ11 (triangles) cells. [^{3}H]tetracycline and acriflavine were added to the cells at time zero. Acriflavine fluorescence emission is quenched upon binding to the DNA; thus, in contrast to [^{3}H]tetracycline, a lower value reading indicates intracellular accumulation. The results are the average of three experiments, and error bars indicate standard deviations.

Importantly, in an era in which tuberculosis (TB) therapeutic options are limited and multidrug-resistant (MDR) and extensively drug-resistant (XDR) TB strains are on the rise (23), a Tap inhibitor could provide new strategies for TB therapy allowing shortened treatment, perhaps by targeting the drug-tolerant subpopulation of cells (1). One interesting approach to accelerate this process would be to identify drugs clinically approved for other therapeutic applications as Tap inhibitors, such as verapamil (1). This approach, known as "repurposing" (3), would allow the timely introduction of new TB therapies. A recent report validated this concept *in vivo*. Louw et al. (28) infected mice with an MDR-TB strain and showed improved efficacy of rifampin when administered in combination with verapamil. However, the use of verapamil for TB therapy generates concerns about the pharmacological effects on cardiac conduction and potential extrapyramidal disorders associated with calcium channel blockers (59).

Perspectives. In this study, we have characterized the important physiological role of Tap in stationary phase (probably through its contribution to the maintenance of the cell wall structure) and to intrinsic drug resistance. Our results build on the study by Adams et al. (1), suggesting the importance of Tap in drug tolerance. This subpopulation of *M. tuberculosis* cells tolerant to anti-TB drugs is one of the causes of prolonged TB therapy. Therefore, the development of Tap inhibitors could provide a valuable new weapon to combat TB. Such inhibitors would have important therapeutic applications. First, they would allow the introduction of already available although clinically non-TB-effective antibiotics such as spectinomycin or tetracycline and make second-line anti-TB drugs such as PAS and streptomycin more effective. Second, assuming that Tap has a primary role in the development of antibiotic tolerance, such an inhibitor would potentially reduce the duration of the current standard treatment. However, further research needs to be performed to validate these hypotheses, which could provide new avenues for TB therapy.

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