showed mutation at *inhA* regulatory region. Thirty six (16.5%) of them had a C to T mutation at −15 in *inhA* regulatory region (*inhA* C −15 T). Among these strains, 21 had additional mutation at *katG* 315. One each had additional concurrent mutations at *katG* Ser315Thr/*inhA* T −8 A, *katG* Ser315Thr/*inhA* T −8 C and *katG* Ser315Thr/*inhA* C −34 T. Twelve (5.5%) had a G to A at position −47 of the *inh*A regulatory region in association with the *katG* Ser315Thr substitution. And twelve (5.5%) phenotypic MDR strains were without mutation in both *katG* gene and *inhA* regulatory regions.

4. Discussion

Recent reports indicate that MDR-TB is increasing in Bangladesh[5,6] but there is scarcity of data on molecular mechanism of drug resistance. Studies on the RIF resistance in several countries demonstrated that majority of resistant strains had mutation at *rpoB* gene.[7,12,13] And the mutation percentage varied from 78 to 100% worldwide.[1,14] In this study, *rpoB* mutation was detected in 95% (207 out of 218) of MDR strains from Bangladesh (Table 2), which is consistent with the data obtained in majority of countries studied. The most frequently observed amino acid substitution occured in Bangladesh strains at codon 531 of the *rpoB* gene (57.4%) and was similar to those reported from Nepal (57.8%),[22] Morocco (59.6%),[23] Singapore (54.9%),[24] Thailand (58.5%)[25] and Brazil (56.1%).[26] However, significantly lower percentage of *rpoB* mutation at codon 531 was reported from Vietnam (39.2%),[27] Hungary (34.5%)[28] and Poland (32.4%).[29] Surprisingly low percentage was detected in strains isolated from Eastern China (3.6%).[30] On the contrary, high percentage of mutation at codon 531 was reported from Germany (75.7%),[31] Kazakhstan (82.7%)[24] and Spain (72.3%).[32] Second highest mutation at codon 526 (22.9%) was observed in Bangladesh as has been reported from many countries. There are some clinical strains of *M. tuberculosis*, which are phenotypically resistant to RIF but lack of any detectable mutation at *rpoB* gene. Eleven strains (5%) from Bangladesh had this characteristic. These types of discrepancy may happen as a result of the mixture of wild type and resistant strains (10), mutation outside the *rpoB* core region,[33,34] altered permeability of cell wall and metabolism of antibiotics.[7,35]

Unlike RIF, NIH resistance is associated with mutations in several genes such as *katG*, *inhA*, *kasA*, *ahpC* and *oxyR*.[7] Besides, mutations in genes like *furA*, *iniA*, *iniB* and *iniC* are responsible for INH resistance in a very limited number of strains of *M. tuberculosis*.[17] Majority of INH resistant strains carried mutations at codon 315 of the *katG* gene and 20–35% strains contained mutation at the *inhA* regulatory region.[7,14] In the present study, point mutations at *katG* gene and *inhA* regulatory region were 83.9 and 16.5%, respectively (Table 3). The amino acid substitution at *katG* Ser315Thr has been reported to be favorable for the bacteria because this change damages the activation process of INH with retaining 30–40% of the catalase–peroxidase activity essential for virulence.[36] Mutations at codon 315 of the *katG* gene similar to our finding was reported in other studies from Nepal (82.2%),[22] Southern Turkey (76.7%)[37] and Ghana (80.1%).[38] In contrast, higher percentage of mutation was reported from Russia (90.9%)[39] and Kazakhstan (98.4%),[40] and lower percentage was reported from United States (32.1%),[41] Canada (45.5%)[42] and Spain (60.8%).[32] In general, a higher percentage of this type of substitution mutation was observed in high TB burden country like Bangladesh, compared to intermediate or low TB prevalent countries.

Up-regulation mutations in the regulatory region of *inhA* (preceding the *mabA-inhA* operon) results in the over expression of *InhA*, thereby contributes to the acquisition of INH resistance via a titration mechanism.[7,14] Several studies have demonstrated about 10–34% of INH-resistant cases with mutations in the *inhA* regulatory region16, 43. In our study, 17.9% (39 out of 218) of INH resistant strains carried mutations in *inhA* regulatory region with the majority (16.5%) had mutation at position −15 (36 out of 218). Percentage of mutation at −15 in *inhA* regulatory region in this study is comparable with studies from Southern Turkey (16.7%),[37] Russia (13.9%)[39] and Ghana (13.3%).[38] High percentage of *inhA* mutation was reported from United States (46.4%),[41] Tunisia (36.1%)[44] and Canada (26.0%).[42] In contrast, none or low percentage was reported from Spain (0%),[32] Myanmar (0%)[45] and Germany (1.94%).[31]

It was noteworthy that one and twelve isolates carried novel mutations T to C at position −34 and G to A at position −47 of the *inh*A regulatory region, respectively, in association with the *katG* Ser315Thr amino acid substitution. The contribution of these novel mutations on INH resistance is not clear and necessary to be elucidated in future studies.

Substantial numbers of isolates with dual mutations in the genes associated with RIF or INH resistance in the isolates, particularly from inpatients were found (Tables 2 and 3). We have carefully reanalyzed the raw data to find that all isolates with dual mutation in *rpoB* and *katG* except for that with Asp516Tyr and Ser531Leu amino acid substitution in *rpoB* showed clear single peaks at the mutational positions indicating dual mutations. In contrast, sequence peaks at the both mutational sites in the isolate with Asp516Tyr and Ser531Leu in *rpoB* showed overlapping feature indicating the mixed population. These results indicated the low prevalence of mixed population with two different lineages in these isolates. Constant treatment at the hospital might select the strains with high-level resistance to both RIF and IHN or those with low fitness cost through acquiring secondary mutations.

We, then, compared the mutation rate in both RIF and INH resistant strains from inpatients and outpatient to correlate with nosocomial infection and specific mutations. Isolates without any mutation in RRDR was higher in outpatients than in inpatients (12.3% vs 2.5%). In contrast, no significant difference was observed between strains from inpatients and outpatients with respect to INH resistance. Mutations outside RRDR or other mechanism giving RIF resistance might contribute to higher transmission between outpatients. The precise comparison of mutations between strains isolated from inpatients and outpatients did not give any clear difference. However, more than 10% (n = 6) of strains from outpatients had His to Arg substitution at codon 526 in *rpoB* of RIF resistant strains compared to none in inpatient strains. Strains with this mutation might have high means of transmission.

Bangladesh is surrounded by India and Myanmar in three and one borders, respectively. There are in and out migration of people among these countries. This situation favors transmission of pathogen among people of these three countries. Data on the

Table 4
Frequency of the mutations in rpoB RRDR in RIF-resistant clinical isolates in Myanmar, India and Bangladesh.

Mutated codon(s)	% Mutations in different geographic regions*			
	North India (reference 46; n = 93)	India† (reference 43; n = 149)	Myanmar (reference 45; n = 29)	This study (n = 218)
511	9.7	1.3	–	0.5
513	–	0.7	–	2.3
516	20.5	11.5	3.4	9.6
526	20.4	22	17.2	22.9
531	38.7	59	55.2	57.8
533	–	4	3.4	2.8
Others	23.7	3.3	20.6	6.8

* Including isolates having mutations at multiple codons.
† North India (n = 110) and South India (n = 39).

Table 5
Frequency of the mutations in KatG 315 or/and inhA promoter region −15 in INH-resistant clinical isolates in Myanmar, India and Bangladesh.

Locus	% Mutations in different geographic regions*			
	North India (reference 48; $n = 121$)	South India (reference 476; $n = 70$)	Myanmar (reference 40; $n = 96$)	This study ($n = 218$)
katG 315	55.4	65.7	63.5	83.9
InhA −15	25.7	11.4	–	16.5
Others†	27.3	23.9	36.5	14.2

* Including isolates having mutations at both loci.
† Including other mutations and no mutations.

mutation at codon 531 RIF resistant strains showed similar trend to India[43] and Myanmar[45] and distinct from North India[46] (Table 4). In contrast, the occurrence of *katG* codon 315 alterations among Bangladesh strains (83.9%) was higher than those reported in South India,[47] Myanmar[45] and North India[48] (Table 5). *M. tuberculosis* strains with similar type of mutations found suggested the possible transmission among the people of these three countries. Genotypes from clinical isolates consisting of drug-susceptible and resistant phenotypes have been published from these three countries. Spoligotype analysis of clinical isolates in urban Bangladesh[49] revealed 22.9, 33.3 and 16.7% of the isolates to be East African Indian (EAI), Beijing and Central Asian (CAS) type, respectively. In contrast, distribution of these three genotypes was 25.0, 15.5 and 7.1%, respectively in rural Bangladesh.[50] Distinct distributions of Spoligotypes were observed in Myanmar[51] (EAI: 41.8%, Beijing: 32.7%, CAS: 0%), northern India[52] (EAI: 10.5%, Beijing: 8.6%, CAS; 36.2%) and southern India[53] (EAI: 84.4%, Beijing: 1.9%, CAS; 3.9%). Difference in strain types in the five regions in three countries might reflect the differences in proportions of certain mutations associated with either RIF- or INH-resistance. Further analysis of genotypes focusing on MDR strains will confirm this.

In conclusion, this study provides valuable information of mutations at *rpo*B and *kat*G genes and *inh*A regulatory regions of clinical isolates of *M. tuberculosis* from Bangladesh. It expands our current knowledge and understanding of the molecular mechanisms of drug resistance and also assists in developing low cost tailor-based molecular tools for rapid susceptibility testing of *Mycobacterium* in Bangladesh. Such methods could be appropriate choice for early detection of resistance and to initiate early treatment to stop further transmission of TB.

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Molecular Characterization of Multidrug-Resistant *Mycobacterium tuberculosis* Isolated in Nepal

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Despite the fact that Nepal is one of the first countries globally to introduce multidrug-resistant tuberculosis (MDR-TB) case management, the number of MDR-TB cases is continuing to rise in Nepal. Rapid molecular tests applicable in this setting to identify resistant organisms would be an effective tool in reversing this trend. To develop such tools, information about the frequency and distribution of mutations that are associated with phenotypic drug resistance in *Mycobacterium tuberculosis* is required. In the present study, we investigated the prevalence of mutations in *rpoB* and *katG* genes and the *inhA* promoter region in 158 *M. tuberculosis* isolates (109 phenotypically MDR and 49 non-MDR isolates collected in Nepal) by DNA sequencing. Mutations affecting the 81-bp rifampin (RIF) resistance-determining region (RRDR) of *rpoB* were identified in 106 of 109 (97.3%) RIF-resistant isolates. Codons 531, 526, and 516 were the most commonly affected, at percentages of 58.7, 15.6, and 15.6%, respectively. Of 113 isoniazid (INH)-resistant isolates, 99 (87.6%) had mutations in the *katG* gene, with Ser315Thr being the most prevalent (81.4%) substitution. Mutations in the *inhA* promoter region were detected in 14 (12.4%) INH-resistant isolates. The results from this study provide an overview of the current situation of RIF and INH resistance in *M. tuberculosis* in Nepal and can serve as a basis for developing or improving rapid molecular tests to monitor drug-resistant strains in this country.

With an estimated 9 million new cases and 2 million deaths every year, tuberculosis (TB) represents one of the most serious infectious diseases worldwide (35). The increasing spread of multidrug-resistant TB (MDR-TB), which is resistant to at least two drugs, including isoniazid (INH) and rifampin (RIF), and the recent emergence of extensively drug-resistant TB (XDR-TB), with additional resistance to a fluoroquinolone (FQ) and at least one of the three injectable second-line drugs, pose a significant threat to tuberculosis control (19, 35). Lack of adequate treatment, often due to irregular drug supply, inappropriate regimens, or poor patient compliance, is associated with the emergence of drug-resistant *Mycobacterium tuberculosis* (9, 13). In 2008, approximately 440,000 cases of MDR-TB were estimated throughout the world, and 58 nations had reported to World Health Organization (WHO) at least one case of XDR-TB (19, 21, 35). Among the countries listed in the WHO report, India and China had the highest burden of MDR-TB, together accounting for almost half of the world's total cases (19, 35). In Nepal, the incidence of all forms of TB was estimated to be 173/100,000 population, while the incidence of new smear-positive cases was at 77/100,000 in 2008 (14, 35). According to the national drug resistance survey conducted in 2006, the prevalences of MDR-TB in Nepal among new and retreatment cases were 2.9 and 11.7%, respectively (14).

Nepal is a landlocked country in Southeast Asia, bounded to the north by China and to the south by India, sharing an open border with India. Every year, a large number of people of Nepal and India cross the border for various purposes, such as work, study, trade, pilgrimage, cultural visits, and so on. According to the 2001 census of Nepal, 762,181 people were abroad, with 78% in India. The census recorded 116,571 foreign citizens residing in Nepal, 88% of whom were Indians (20). However, this information does not adequately cover the short-term and short-distance mobility that could significantly contribute TB epidemics in Nepal. Since drug resistance rates on one side of the border impact the other side of the border (33), a high proportion of MDR-TB in Nepal may reflect the possible dissemination of infection from surrounding two countries, mainly from India.

Rapid determination of the antimicrobial susceptibility pattern in clinical isolates of *M. tuberculosis* is important for the early administration of appropriate therapeutic agents for the prevention of additional resistance development (21). In this context, molecular characterization of drug resistance by identifying mutations in associated genes will be applicable for developing a potential rapid molecular drug susceptibility test as an alternative to conventional methods (16, 23).

The collection of data from different countries has indicated that resistance to RIF in >90% of cases is due to mutations resulting in an amino acid substitution within the 81-bp core region of the RNA polymerase β-subunit gene (*rpoB*), called the RIF resistance-determining region (RRDR) (8, 24, 26, 30). In contrast, INH resistance is mediated by mutations in several genes, most frequently within the *katG* gene, encoding a catalase-peroxidase which transforms INH into its active form (6, 11, 24), and in the promoter region of *inhA*, encoding a putative enzyme involved in mycolic acid biosynthesis. An upregulation mutation in the *inhA* promoter region results in the overexpression of InhA and develops INH resistance via a titration mechanism (24).

In the present study, we sought to determine the prevalence of resistance-associated mutations in three specific genes (*rpoB*, *katG*, and the *inhA* promoter region) of *M. tuberculosis* isolates in

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TABLE 1 Primers used for PCR amplification and sequencing of drug-resistance-associated genes in *M. tuberculosis*

Locus	Primer	Nucleotide sequence (5′–3′)	Target region (position)	Product size (bp)
rpoB[a]	TB *rpoB* S	CAGGACGTGGAGGCGATCAC	1519–1599[a]	278
	TB *rpoB* AS	GAGCCGATCAGACCGATGTTGG		
katG	TB *katG* S	ATGGCCATGAACGACGTCGAAAC	823–1140	392
	TB *katG* AS	CGCAGCGAGAGGTCAGTGGCCAG		
inhA	TB *inhA* S	TCACACCGACAAACGTCACGAGC	−50 to −1	231
	TB *inhA* AS	AGCCAGCCGCTGTGCGATCGCCA		

[a] Corresponding *E. coli* numbering was used for *rpoB*.

Nepal and to compare the frequency of different mutations with those in isolates circulating in the surrounding countries.

MATERIALS AND METHODS

Isolates. In total, 109 and 49 samples were randomly selected from MDR and non-MDR clinical isolates, respectively, in isolates bank at the German Nepal Tuberculosis Project (GENETUP) over a 3-year period from 2007 and 2010. The isolates were recovered from 158 patients living in nine different cities of Nepal, six of which have an open border with northern India. Of 109 MDR isolates, the numbers of isolated from each city were as follows: Kathmandu ($n = 70$), Biratnagar ($n = 8$), Bhairahawa ($n = 8$), Pokhara ($n = 7$), Birgunj ($n = 4$), Nepalgunj ($n = 4$), Dhangadi ($n = 4$), Butwal ($n = 3$), and Sarlahi ($n = 1$). Of the non-MDR isolates, 48 were obtained from patients in Kathmandu, and 1 was obtained from Biratnagar. Histories of previous TB treatment were available in 94.5% of the MDR and 42.9% of the non-MDR patients. A drug susceptibility test was performed using Löwenstein-Jensen medium by a conventional proportional method with the following critical drug concentrations of INH, RIF, streptomycin (STR), and ethambutol (EMB): 0.2, 40, 4, and 2 μg/ml, respectively (2).

DNA extraction. DNAs were prepared for PCR by mechanical disruption, as described previously (29). Briefly, the colonies were suspended in TE buffer consisting of 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA in a 2-ml screw-cap vial, one-fourth of which was filled with 0.5-g glass beads (0.1 mm; BioSpec Products, Inc., OK). Mycobacterial cells were disrupted by shaking with 0.5 ml of chloroform on a cell disrupter (Micro Smash; Tomy Seiko Co., Ltd., Tokyo, Japan) for 1 min. After centrifugation, the DNAs in the upper layer were concentrated by ethanol precipitation and dissolved in 100 μl of TE buffer.

Species differentiation multiplex PCR. *M. tuberculosis* species were identified on the isolates by a multiplex PCR with primer pairs designed to amplify three genetic regions (*cfp32*, RD9, and RD12), as described previously (18).

Sequencing of the *rpoB* and *katG* encoding regions and the *inhA* promoter region. PCRs were performed in a 20-μl mixture containing 0.25 mM (each) deoxynucleoside triphosphates, 0.5 M betaine, 0.5 μM concentrations of each primer (Table 1), 1 U of GoTaq DNA polymerase (Promega, WI), GoTaq buffer, and 1 μl of DNA template. The reaction was carried out in a thermal cycler (Bio-Rad Laboratories, CA) under the following conditions: denaturation at 96°C for 60 s, followed by 35 cycles of amplification at 96°C for 10 s, 55°C for 10 s, and 72°C for 30 s, with a final extension at 72°C for 5 min. The presence of PCR products was confirmed by agarose gel electrophoresis. PCR products were sequenced according to the manufacturer's protocol with the primers TB *rpoB* S, TB *katG* S, and TB *inhA* S for *rpoB*, *katG*, and *inhA*, respectively, and the BigDye Terminator v3.1 cycle sequencing kit (Life Technologies Corp., CA) using an ABI Prism 3130*xl* Genetic Analyzer (Life Technologies Corp.). The resulting sequences were compared to wild-type sequences of *M. tuberculosis* H37Rv using Bio-Edit software (version 7.0.9) (5).

RESULTS

Drug susceptibility patterns. Of the 109 MDR isolates, 102 were resistant to three or more first-line anti-TB drugs (Table 2). Forty-nine non-MDR isolates consisted of 41 fully susceptible and 2, 3, and 1 isolates with monoresistance against INH, STR, and EMB, respectively. Two isolates were resistant to both INH and STR.

Species identification. All 158 isolates showed three amplified bands corresponding to *cfp32*, RD9, and RD12 by multiplex PCR and were classified as *M. tuberculosis* (data not shown).

Mutations in the *rpoB* gene. Mutations in the RRDR of the *rpoB* gene were identified in 106 of 109 RIF-resistant (RIF^r) isolates (Table 3). A single nucleotide alteration in codon 531, resulting in the amino acid substitution of Ser to Leu, was most prevalent and observed in 62 isolates (56.9%). The second most affected codons were 516 and 526, which were found in 17 (15.6%) isolates each, and had 3 and 6 types of amino acid substitutions, respectively. Five (4.6%) isolates had a mutation in codon 513, and three (2.8%) had a mutation in codon 533. An insertion of Phe between codons 514 and 515 was observed in two (1.8%) isolates, one of which had an additional point mutation affecting codon 531. Two isolates carried double mutations in two separate codons, i.e., codons 513 and 526 and codons 516 and 533, respectively. No mutations were detected in the remaining 3 (2.8%) RIF^r and 49 RIF-susceptible (RIF^s) isolates.

Mutations in *katG* encoding region and *inhA* promoter region. Of 113 phenotypically INH^r isolates, 99 (87.6%) had *katG* mutations, the vast majority of which was the commonly described substitution *katG*(*Ser315Thr*) (Table 4). Only one isolate had a Ser-to-Asn substitution at *katG* position 315 (*katG* 315). *katG*(*Gly299Ser*) and *katG*(*Asp329Ala*) mutations were detected in two INH^r isolates. One isolate showed double mutations in two

TABLE 2 Drug susceptibility profile of 109 multidrug-resistant *M. tuberculosis* isolates

Characteristic	Resistance pattern[a]	No. of isolates
MDR	INH + RIF	7
	INH + RIF + EMB	6
	INH + RIF + STR	17
	INH + RIF + EMB + STR	79
Non-MDR	None	41
	INH	2
	STR	3
	EMB	1
	INH + STR	2

[a] INH, isoniazid; RIF, rifampin; STR, streptomycin; EMB, ethambutol.

TABLE 3 Distribution of mutations in the *rpoB* RRDR of 109 rifampin-resistant and 49 rifampin-susceptible *M. tuberculosis* isolates from Nepal

Mutated codon(s)	Amino acid change(s)[a]	Nucleotide change(s)	No. (%) of isolates	
			RIF^r (n = 109)	RIF^s (n = 49)
511	Leu→Pro	CTG→CCG	1 (0.9)	0 (0.0)
513	Gln→Leu	CAA→CTA	2 (1.8)	0 (0.0)
	Gln→Lys	CAA→AAA	2 (1.8)	0 (0.0)
514	Phe (ins)	TTC→TTCTTC	1 (0.9)	0 (0.0)
516	Asp→Val	GAC→GTC	13 (11.9)	0 (0.0)
	Asp→Phe	GAC→TTC	2 (1.8)	0 (0.0)
	Asp→Tyr	GAC→TAC	1 (0.9)	0 (0.0)
526	His→Tyr	CAC→TAC	5 (4.6)	0 (0.0)
	His→Arg	CAC→CGC	4 (3.7)	0 (0.0)
	His→Asp	CAC→GAC	3 (2.8)	0 (0.0)
	His→Cys	CAC→TGC	2 (1.8)	0 (0.0)
	His→Gly	CAC→GGC	1 (0.9)	0 (0.0)
	His→Leu	CAC→CTC	1 (0.9)	0 (0.0)
531	Ser→Leu	TCG→TTG	61 (56.0)	0 (0.0)
	Ser→Gln	TCG→CAG	1 (0.9)	0 (0.0)
	Ser→Val	TCG→GTG	1 (0.9)	0 (0.0)
533	Leu→Pro	CTG→CCG	2 (1.8)	0 (0.0)
531 and 514	Ser→Leu and Phe (ins)	TCG→TTG and TTC→TTCTTC	1 (0.9)	0 (0.0)
513 and 526	Gln→Lys and His→Asp	CAA→AAA and CAC→GAC	1 (0.9)	0 (0.0)
516 and 533	Asp→Ala and Leu→Pro	GAC→GCC and CTG→CCG	1 (0.9)	0 (0.0)
Wild type[b]	None	None	3 (2.8)	49 (100.0)

[a] Phe (ins), Phe insertion.
[b] That is, there were no mutations in the sequenced region.

separate *katG* codons: Thr275Ala and Ser315Thr. Mutations in the *inhA* promoter region were observed in 14 (12.4%) INH^r isolates; 12 of which had a mutation at −15 in the *inhA* promoter. Among the isolates with mutation in *inhA* promoter, three had additional mutation in *katG* 315, and one each had additional mutations in *katG* 285, *katG* 289, and *katG* 289 plus *katG* 296. No mutations in either region were identified in 7 (6.2%) INH^r and 45 INH^s isolates.

DISCUSSION

Antituberculosis drug resistance poses a significant threat to human health, which usually develops due to the alteration of drug targets by mutations in *M. tuberculosis* chromosomal genes (24, 26). Although a large number of mutations in several genes that confer resistance to *M. tuberculosis* have been reported from different countries, no study until now has managed to reveal the range of mutation in clinical samples from Nepal, one of the countries with the highest TB prevalence. Hence, in the present study, we attempted to identify the molecular basis of the drug resistance of *M. tuberculosis* circulating in Nepal.

RIF resistance is often considered as a surrogate marker for checking MDR-TB (7, 24). This hypothesis is supported by the finding in the present study that 100% of the RIF^r isolates were MDR. Consistent with previous studies that ca. 95% of RIF-resis-

TABLE 4 Distribution of mutations in *katG* gene and the *inhA* promoter region of 113 INH^r and 45 INH^s *M. tuberculosis* isolates from Nepal

Locus	Amino acid change(s)	Nucleotide change(s)	No. (%) of isolates	
			INH^r (n = 113)	INH^s (n = 45)
katG 315	Ser→Thr	AGC→ACC	86 (76.1)	0 (0.0)
	Ser→Thr	AGC→ACT	1 (0.9)	0 (0.0)
	Ser→Asn	AGC→AAC	1 (0.9)	0 (0.0)
katG 299	Gly→Ser	GGC→AGC	1 (0.9)	0 (0.0)
katG 329	Asp→Ala	GAC→GCC	1 (0.9)	0 (0.0)
katG 341	Trp→Gly	TGG→GGG	1 (0.9)	0 (0.0)
katG 275 and *katG* 315	Thr→Ala and Ser→Thr	ACC→GCC and AGC→ACC	1 (0.9)	0 (0.0)
inhA −15	NA[b]	C→T	6 (5.3)	0 (0.0)
inhA −8	NA	T→C	1 (0.9)	0 (0.0)
katG 285 and *inhA* −15	Gly→Asp and NA	GGC→GAC and C→T	1 (0.9)	0 (0.0)
katG 289 and *inhA* −15	Glu→Ala and NA	GAG→GCG and C→T	1 (0.9)	0 (0.0)
katG 289, *katG* 296, and *inhA* −15	Glu→Ala, Met→Val, and NA	GAG→GCG, ATG→GTG, and C→T	1 (0.9)	0 (0.0)
katG 315 and *inhA* −12	Ser→Thr and NA	AGC→ACC and T→A	1 (0.9)	0 (0.0)
katG 315 and *inhA* −15	Ser→Thr and NA	AGC→ACC and C→T	3 (2.7)	0 (0.0)
Wild type[a]	None	None	7 (6.2)	45 (100)

[a] That is, there were no mutations in sequenced regions of *katG* and *inhA* promoter.
[b] NA, not applicable.

TABLE 5 Frequency of the mutations in *rpoB* RRDR in RIF^r *M. tuberculosis* isolates in India and China reported by seven groups

Mutated codon	% Mutations in different geographic regions[a]							
	Northern India (n = 93)	India 1[b] (n = 149)	India 2[c] (n = 44)	Southern China (n = 60)	Eastern China 1[d] (n = 242)	China[e] (n = 72)	Eastern China 2 (n = 53)	This study (n = 109)
511	9.7	1.3	6.0	3.3	3.3	1.4		0.9
513		0.7	2.0	2.6	2.9	1.4		4.6
516	20.5	11.5	4.0	5.0	7.4	4.2	7.5	15.6
518	7.5		2.0			1.4		
522	5.4			2.6	1.7	2.8		
526	20.4	22.0	19.0	11.6	19.4	36.1	30.2	15.6
531	38.7	59.0	53.0	58.3	61.2	37.5	58.5	58.7
533		4.0	2.0	5.0	5.0	1.4		2.8
Others	10.8	1.3	13.7		2.1	4.2		1.8
None		2.0	2.0	10.0	3.7	9.7	7.5	2.8

[a] The values include isolates with mutations at multiple codons. Source references for the various regions were as follows: northern India (27), India 1 (28), India 2 (15), southern China (4), eastern China 1 (12), China (36), and eastern China 2 (10).
[b] Includes northern India (n = 110) and southern India (n = 39).
[c] Includes southern India (n = 35), northern India (n = 6), and western India (n = 3).
[d] Collected only in Shanghai (n = 242).
[e] Includes southern China (n = 26), northern China (n = 16), and eastern China (n = 30).

tant *M. tuberculosis* isolates worldwide have mutations within the 81-bp core region of the *rpoB* gene, we found mutations in this region in 97.3% of RIF^r isolates. The most frequently mutated codon in our study was codon 531 (58.7%), which was similar to those reported in clinical isolates from India (15, 27, 28), China (4, 10, 12, 36), and other geographical regions (3, 31) (Table 5). Although low frequencies of mutations in codon 516 in clinical isolates have been reported from various parts of China (4, 12, 36), we found a higher frequency of this mutation (15.6%), which was comparable to that of northern India (20.5%) (27).

Phenotypically RIF^r isolates with no *rpoB* mutations in our study were 2.8%, similar to those reported previously (3, 10, 12, 26, 28). Therefore, this finding suggested that majority of RIF^r isolates in Nepal could be rapidly detected by screening for the most common genetic alterations in RRDR of the *rpoB* gene, although the prevalence of isolates lacking mutations also needs to be considered.

Previous studies indicated that INH resistance was mediated by mutations in several genes, most commonly *katG*, particularly in codon 315, and the promoter region of *inhA* (6, 11, 16, 24). Accordingly, we found that 87.6 and 12.4% of phenotypically INH^r clinical isolates had point mutations in *katG* and in the *inhA* promoter region, respectively, and the frequencies were similar to those reported by other researchers (1, 3, 8). However, no deletion or insertion in *katG* was detected in any isolates in the present study. This result confirmed previous reports from different geographic regions of the rarity of this event in causing INH resistance (4, 8, 10, 11, 12, 16, 22). The seven (6.2%) INH^r *M. tuberculosis* isolates had no resistance-associated alterations in the two targets analyzed, indicating that resistance in these isolates could be due to mutations present outside of the sequenced area or in other genes (e.g., *kasA* and *ndh*) (6, 8, 26).

It has been postulated that the amino acid substitution *katG*(*Ser315Thr*) is favored by the bacteria because this alteration was elucidated to spoil INH activation and, on the other hand, to retain 30 to 40% of the catalase-peroxidase activity necessary for virulence (25); however, the prevalence of the *katG*(*Ser315Thr*) substitution in *M. tuberculosis* isolates around the world varies, especially with regard to the prevalence of TB. In general, a higher prevalence of this substitution has been observed in high TB burden regions, often with the predominance of Beijing and MDR *M. tuberculosis* strains, compared to regions where the prevalence of TB is intermediate or low (10, 17). The present study documented the prevalence of the KatG Ser315Thr substitution in 81.4% of INH^r isolates, which was not as high as those reported in INH^r isolates in northeastern Russia (93.6%) (17) but was comparable to those in Lithuania and Germany (85.7 and 88.4%, respectively) (1, 26). The occurrence of the KatG Ser315Thr alteration among Nepalese isolates was higher than that reported in India (16, 22) and in China (4, 10, 12) (Table 6).

Van Soolingen et al. (32) reported that strains with amino acid substitutions in *katG* 315 are more likely to develop resistance to

TABLE 6 Frequency of the mutations in *katG* 315 and/or the *inhA* promoter region −15 in INH^r isolates in India and China reported by five groups

Locus	% Mutations in different geographic regions[a]					
	Northern India (n = 121)	Southern India (n = 70)	Southern China (n = 50)	Eastern China 1 (n = 131)	Eastern China 2[c] (n = 242)	This study (n = 113)
katG 315	55.4	64.3	60.0	61.8	72.7	82.3
inhA −15	25.6	11.4	8.0	21.4	8.3	10.6
Others[b]	27.3	28.6	36.0	18.3	21.5	10.8

[a] Values include isolates with mutations at both loci. Source references for the various regions were as follows: northern India (16), southern India (22), southern China (4), eastern China 1 (10), and eastern China 2 (12).
[b] Includes both other mutations and no mutations.
[c] Collected only in Shanghai (n = 242).

other drugs. In this respect, we found a correlation between this alteration and resistance to other drugs: 100% of the isolates with a *katG* 315 substitution showed resistance to RIF. Meanwhile, this mutation was found among 92 in 109 (84.4%) of MDR and none in four non-MDR INH^r isolates. This is consistent with the finding of previous studies in which substitutions in codon 315 of KatG are more common in MDR isolates (6, 26, 31). Several studies from different countries have shown that ca. 10 to 34% of INH^r cases have mutations in the *inhA* promoter region (11, 34). In contrast, we identified mutations in only 12.4% of INH^r isolates, the majority of which was a C-to-T mutation at position −15.

Since Nepal shares an open border with northern India, there is a large amount of population movement between these countries (20). Patients from northern India usually come to Nepal because of cheaper TB treatment facilities in Nepal; thus, we postulated the frequent air-born transmission of TB between these points (33). By comparing data with neighboring countries, we observed a similarity between Nepalese and northern Indian RIF^r isolates in the occurrence of mutations in codons 531, 526, and 516 of the *rpoB* gene (Table 5). In contrast, the frequency of *katG*(*Ser315Thr*) substitution and C-to-T mutations at position −15 in the *inhA* promoter between Nepalese and northern Indian INH^r isolates showed a significant difference (Table 6). This discrepancy might not suggest transport but the possible emergence of MDR-TB in Nepal. For confirmation, molecular typing of strains circulating in Nepal and northern India seems to be necessary.

In conclusion, we provide here valuable information on mutations occurring at *rpoB*, the *katG* gene, and the promoter region of *inhA* in Nepalese clinical isolates of *M. tuberculosis*. These findings expand our current knowledge of the molecular mechanisms of drug resistance and also assist in improving current molecular techniques for the diagnosis of MDR-TB in Nepal. Such methods promise rapid detection rates compared to those achieved by methods based solely on culture of the isolates.

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Antitubercular Activity of Disulfiram, an Antialcoholism Drug, against Multidrug- and Extensively Drug-Resistant *Mycobacterium tuberculosis* Isolates

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The antimycobacterial activities of disulfiram (DSF) and diethyldithiocarbamate (DDC) against multidrug- and extensively drug-resistant tuberculosis (MDR/XDR-TB) clinical isolates were evaluated *in vitro*. Both DSF and DDC exhibited potent antitubercular activities against 42 clinical isolates of *M. tuberculosis*, including MDR/XDR-TB strains. Moreover, DSF showed remarkable bactericidal activity *ex vivo* and *in vivo*. Therefore, DSF might be a drug repurposed for the treatment of MDR/XDR-TB.

According to the updated guidelines of the World Health Organization, the medications effective against multidrug- and extensively drug-resistant tuberculosis (MDR/XDR-TB) are confined because only a limited selection of drugs is available; therefore, the development of novel or repurposed drugs with activity against MDR/XDR-TB is strongly desired (9). Disulfiram (DSF; tetraethyl thiuram disulfide) has been used orally in the clinical treatment of alcoholism since 1949 and has been proven to exert an inhibitory effect on aldehyde dehydrogenase *in vivo* with 80% bioavailability and established safety profiles (13, 33). Both DSF and its first metabolite, diethyldithiocarbamate (DDC), were reported to exhibit growth-inhibitory activity against bacteria, fungi, protozoa, and viruses (2, 18, 23, 26, 27). In the mid-1950s, the tuberculostatic effects of DSF and DDC were demonstrated *in vivo* using guinea pigs (17). Subsequently, it was reported that DDC enhances monocyte-induced antitubercular activity in both healthy volunteers and human immunodeficiency virus-infected patients *ex vivo* (16). Recently, the antitubercular activities of DDC and the nitric oxide synthase inhibitor pyrrolidine dithiocarbamate (PDTC) against nonreplicating *Mycobacterium tuberculosis* have been demonstrated (4). In addition, we reported the unique antimycobacterial activities of dithiocarbamates and also the potent antitubercular activities of compounds containing dithiocarbamate groups, such as dimethyldithiocarbamate (DMDC), DDC, and PDTC (14, 15). More recently, the mode of action of dithiocarbamates against *M. tuberculosis* has been reported to be through β-class carbonic anhydrases (β-CAs), which are considered possible drug targets (19). However, the mechanism of action of DSF remains unknown.

In the present study, we evaluated the antimycobacterial activities of DSF and its metabolites against *M. tuberculosis*, including MDR/XDR-TB clinical isolates, in more detail. Furthermore, the intracellular bactericidal activities of these compounds against a virulent strain, *M. tuberculosis* $H_{37}Rv$, within macrophages were examined *ex vivo*, and the bactericidal activity of DSF *in vivo* was determined using the mouse model of chronic TB. Finally, the mechanisms of action of these compounds were investigated by means of gene-overexpressing strains *in vitro*.

MATERIALS AND METHODS

Bacterial strains. *M. tuberculosis* $H_{37}Rv$ ATCC 25618, *M. tuberculosis* $H_{37}Ra$ ATCC 25177, *Mycobacterium avium* ATCC 25291, and *M. avium* ATCC 35718 were purchased from the American Type Culture Collection (ATCC). *Mycobacterium smegmatis* JATA 64-01 was provided by M. Takahashi (Research Institute of Tuberculosis, Japan Anti-Tuberculosis Association, Japan). *Mycobacterium bovis* BCG strain Tokyo 172 (BCG Tokyo) was purchased from BCG Japan, Co. Ltd. *M. avium* 104 was provided by Deborah Geiman (John Hopkins University). Clinical isolates of *M. tuberculosis* were isolated at the Higashi Nagoya National Hospital (Japan). Mycobacterial strains were cultured in Middlebrook 7H9 broth (Difco) supplemented with 10% ADC (5% bovine serum albumin [fraction V], 2% dextrose, and 0.005% bovine liver catalase) including 0.05% Tween 80 or on Middlebrook 7H11 agar (Difco) supplemented with 10% OADC (5% bovine serum albumin [fraction V], 2% dextrose, 0.005% bovine liver catalase, and 0.05% alkalinized oleic acid) at pH 6.6. *Staphylococcus aureus* 209PJC-1, *S. aureus* RN4220, *S. aureus* MF490, *Enterococcus faecalis* ATCC 19433, *Enterococcus faecium* ATCC 19434, *Escherichia coli* JM109, *Klebsiella pneumoniae* ATCC BAA-1705, and *Pseudomonas aeruginosa* PAO1 were grown on Mueller-Hinton agar (Becton, Dickinson).

Drug and reagent preparation for *in vitro* and *in vivo* studies. Isoniazid (INH), rifampin (RIF), streptomycin (STR), ethambutol (EMB),

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TABLE 1 Antimycobacterial activities of DSF and its metabolites[a]

Strain	MIC (μg/ml)					
	DSF	DDC	S-Me-DDC	S-Me-DTC	S-Me-DTC sulfoxide	S-Me-DTC sulfone
M. tuberculosis $H_{37}Rv$	1.56	3.13	>100	>100	25	12.5
M. tuberculosis $H_{37}Ra$	1.56	3.13	>100	>100	25	12.5
M. bovis BCG strain Tokyo 172	3.13	6.25	>100	>100	50	25
M. avium subsp. *avium* ATCC 25291	25	>100	>100	>100	>100	>100
M. avium subsp. *avium* ATCC 35718	25	>100	>100	>100	>100	>100
M. avium subsp. *hominissuis* 104	25	>100	>100	>100	>100	>100
M. smegmatis JATA 64-01	25	>100	>100	>100	>100	>100

[a] The antimycobacterial activities of each agent were determined by broth dilution test using Middlebrook 7H9 broth containing albumin, dextrose, catalase, and Tween 80 at pH 6.6. Experiments were performed in duplicate. These results are representative of three separate experiments. DSF, disulfiram; DDC, diethyldithiocarbamate; S-Me-DDC, *S*-methyl *N,N*-diethyldithiocarbamate; S-Me-DTC, *S*-methyl *N,N*-diethylthiocarbamate.

ethionamide (ETH), *p*-aminosalicylic acid (PAS), ciprofloxacin (CIP), and bathocuproinedisulfonic acid disodium salt (BCPS) were purchased from Sigma-Aldrich Co. Thiuram, DSF, DMDC, DDC, PDTC, and amikacin disulfate salt (AMK) were purchased from Wako Pure Chemical Industries, Ltd., Japan. Kanamycin (KAN) was purchased from Meiji Seika Kaisha, Ltd., Japan. The bulk powder of disulfiram was provided by Mitsubishi Tanabe Pharma Corporation, Japan. *S*-Methyl *N,N*-diethyldithiocarbamate (S-Me-DDC), *S*-methyl *N,N*-diethylthiocarbamate (S-Me-DTC), S-Me-DTC sulfoxide, and S-Me-DTC sulfone were purchased from Toronto Research Chemicals Inc., Canada. Hydrophilic or hydrophobic agents were dissolved in distilled deionized water (DDW) and dimethyl sulfoxide, respectively. Before examination, the stock solutions were diluted with assay broth, i.e., 7H9 broth. For *in vivo* use, RIF and DSF were dissolved or suspended in 5% gum arabic solution.

BDT and agar dilution method. The broth dilution test (BDT) for the determination of MICs was performed as previously described (31). The starting drug concentration was 100 μg/ml. In the cases of INH and RFP, the concentrations were 10 and 1 μg/ml, respectively. The MIC_{90}s of test compounds against clinical isolates were defined as the antimicrobial concentrations that showed 90% growth inhibition of the strains. The agar dilution method for the determination of MICs using 7H11 agar at pH 6.6 was performed according to the *Manual of Clinical Microbiology* (22). The starting concentration of the drugs INH, RIF, and CIP was 3.2 μg/ml, and that of DSF, DDC, STR, EMB, KAN, and PAS was 0.125 μg/ml. The drug susceptibility test for clinical isolates was performed with the broth MIC MTB assay (Kyokuto Pharmaceutical Industrial Co., Ltd., Tokyo, Japan), and the assay was conducted according to the method of the provider (32). To determine the synergistic effects of DSF and DDC with metal ions, i.e., Zn^{2+}, Cu^{2+}, Co^{2+}, or Al^{3+}, we prepared Sauton broths with or without a metal salt, such as $ZnSO_4$, $CuSO_4$, $CoCl_2$, or $Al_2(SO_4)_3$. The 10-fold-concentrated bacterial culture was next compared to the one used for normal BDT. The MICs against several bacteria, except mycobacteria, were determined using the agar dilution method, as recommended by the Clinical and Laboratory Standards Institute (6).

Serum bactericidal test. The serum bactericidal test was performed according to the method of Byrne et al. (4) and the NCCLS (24). DSF, DDC, INH, and RIF were administered orally at 80 mg/kg of body weight, 80 mg/kg, 25 mg/kg, and 10 mg/kg, respectively. INH and RIF were used as positive controls. The serum bactericidal activity of the drugs was compared to that of a vehicle control, 5% gum arabic. The serum samples were collected by cardiopuncture. For DSF and DDC, serum was collected at 0.083 and 2 h after administration, in accordance with the time to the maximum concentration of DDC (0.083 h), as previously determined in our laboratory. For INH and RFP, serum was collected at 1 h after administration as previously described (4). For vehicle, serum was collected before and 2 h after administration. DDC and INH were also administered intravenously, and serum was collected at 0.083 h after administration. The pooled serum was heated at 56°C for 40 min in order to inactivate complement. Then, serum samples were filtrated using a 0.45-μm-pore-size filter followed by a 0.2-μm-pore-size syringe filter. *M. tuberculosis* $H_{37}Rv$ was cultivated in 7H9 broth supplemented with 10% ADC including 0.05% Tween 80 and was grown to log phase. The final inoculum was confirmed to be 4×10^6 CFU/well (100 μl) by means of a colony assay using a 7H11 agar plate supplemented with 10% OADC.

Intracellular antitubercular activities of DSF and DDC in differentiated THP-1 cells. Cells of the human acute monocyte leukemia cell line THP-1 (ATCC TIB-202) were purchased from ATCC. THP-1 cells were maintained in RPMI 1640 medium supplemented with 5% heat-inactivated fetal bovine serum, including 100 units/ml penicillin G (Meiji Seika Kaisha, Ltd., Japan) and 100 μg/ml streptomycin (Meiji Seika Kaisha, Ltd., Japan) in a humidified 5% CO_2 atmosphere at 37°C. The intracellular antitubercular assay was performed according to the method previously described (20).

Therapeutic efficacy in an experimental mouse model of chronic TB. In order to examine the therapeutic efficacy of DSF and to determine the therapeutic dose range, an experimental mouse model of chronic TB was used as previously described (20). Mice obtained from the Institute of Cancer Research (ICR) ($n = 5$ per group) were inoculated intravenously with 1×10^6 CFU/ml of *M. tuberculosis* $H_{37}Rv$ through the caudal vein, and the infection was allowed to develop for 28 days. The test compounds were then administered orally once daily for 28 days (RIF, 5 to 20 mg/kg; DSF, 40 to 160 mg/kg [2-fold dilutions]). The extracted lungs and spleen were homogenized with 1 ml DDW. The bacterial burden (CFU/organs) in either organ was counted using 7H11 agar plates to determine the therapeutic efficacy. Statistical analysis was conducted using Microsoft Office Excel 2007 software. Statistical significance was set at *P* values of <0.05, <0.01, and <0.001 compared to the vehicle control group.

Sulfonation mechanisms of DSF and DDC. *ethA*- or *ethR*-overexpressing strains were prepared as described previously and were used for MIC determination (1, 7).

RESULTS AND DISCUSSION

In accordance with previous reports, DDC exhibited antitubercular activity, with MICs of 1.56 to 3.13 μg/ml. Despite the use of conditions similar to those in a previous study (4), DSF exhibited potent antitubercular activity, with an MIC of 1.56 μg/ml at pH 6.6 using Middlebrook 7H9 broth (Table 1). Importantly, the MIC of the bulk powder of DSF provided by Mitsubishi Tanabe Pharma, which is generally used in the clinical treatment of alcoholism in Japan, was comparable to that of chemical reagent DSF (data not shown). Additionally, the MIC of DSF against BCG Tokyo using 7H11 agar plates was also equivalent to the MICs of anti-TB drugs, i.e., EMB and KAN (MICs = 1 to 4 μg/ml) (see Table S1 in the supplemental material). Among mycobacterial species, although the antibacterial spectrum of DSF is broad, the antimycobacterial activity of DDC was highly specific to slow-

TABLE 2 Antitubercular activities of DSF and DDC against drug-susceptible and -resistant clinical isolates of *M. tuberculosis*[a]

Clinical isolate	No. of strains	Agent	MIC (μg/ml)		
			Range	50%	90%
DS-TB	20	DSF	0.78–1.56	0.78	1.56
		DDC	1.56–3.13	1.56	3.13
DR-TB[b]	22	DSF	0.78–1.56	1.56	1.56
		DDC	1.56–6.25	3.13	3.13

[a] Experiments were performed in duplicate. These results are representative of two separate experiments. DS-TB, drug-susceptible TB; DR-TB, drug-resistant TB; DSF, disulfiram; DDC, diethyldithiocarbamate.
[b] DR-TB includes multidrug-resistant TB (n = 13) and extensively drug-resistant TB (n = 5). Drug resistance profiles are depicted in Table S3 in the supplemental material.

growing strains, such as *M. tuberculosis* and BCG Tokyo, indicating that the antibacterial spectrum of DSF is quite distinct from that of DDC (Table 1). Meanwhile, the antibacterial spectrum of DSF against other Gram-positive bacteria, such as *S. aureus* (MIC = 16 μg/ml), *E. faecalis* (MIC = 32 μg/ml), and *E. faecium* (MIC = 32 μg/ml), was similar to that of DDC (see Table S2 in the supplemental material). As previously reported, unlike DSF, DDC exhibited antibacterial activity, with MICs of 128 μg/ml against Gram-negative bacteria, i.e., *E. coli*, *K. pneumoniae*, and *P. aeruginosa* (see Table S2 in the supplemental material) (28).

DSF is well-known to be a prodrug and is enzymatically metabolized to DDC in blood, followed by activation owing to certain reactions, such as *S*-methylation, oxidation, and sulfonation (1). The antimycobacterial activities of the metabolites of DSF, i.e., S-Me-DDC, S-Me-DTC, S-Me-DTC sulfoxide, and S-Me-DTC sulfone, were determined by BDT *in vitro*. The results revealed that *S*-methylation of the sulfhydryl group of DDC led to deactivation (MIC > 100 μg/ml), in spite of its oxidation, and the metabolites obtained by sulfonation had partially restored antimycobacterial activities (MICs = 12.5 to 25 μg/ml) (Table 1). Therefore, the results suggest that not only DSF but also its metabolites, namely, DDC, S-Me-DTC sulfoxide, and S-Me-DTC sulfone, are biologically active in the human body, which complicated the assessment of the antitubercular activity of DSF after oral administration.

We next further determined the antitubercular activities of DSF and DDC against clinical isolates of *M. tuberculosis in vitro*. As expected, these compounds exhibited potent antitubercular activities against more than 40 clinical isolates of *M. tuberculosis*, including MDR/XDR-TB strains (Table 2). The MIC_{90}s of DSF and DDC against clinical isolates were 1.56 μg/ml and 3.13 μg/ml, respectively (Table 2). Importantly, there was no cross-resistance of DSF or DDC to the currently available anti-TB drugs, including fluoroquinolones such as levofloxacin, sparfloxacin, and CIP (see Table S3 in the supplemental material). Thereby, DSF and DDC may be implemented in future pharmacological regimens against MDR/XDR-TB.

It has been reported that DDC enhances monocyte-induced antitubercular activity *ex vivo* (16). Therefore, in order to confirm whether DSF and its metabolites are effective within macrophages, we determined the bactericidal activities of these compounds against intracellular *M. tuberculosis* in differentiated THP-1 cells. As shown in Fig. 1, these compounds exhibited potent bactericidal activities at 6 to 30 μg/ml and 10 to 30 μg/ml, respectively, in a dose-dependent manner, unlike STR and the bacteriostatic drug EMB. Likewise, S-Me-DTC sulfone, the active metabolite of DSF, exhibited intracellular bactericidal activity at 30 μg/ml, but S-Me-DTC did not, in agreement with the results presented in Table 1 (Fig. 1). These data indicate that DSF, DDC, and *S*-oxidized metabolites, especially S-Me-DTC sulfone, are effective against intracellular *M. tuberculosis*.

Subsequently, we examined whether DSF and DDC exhibited bactericidal activity *in vivo* by means of a serum bactericidal test. The serum bactericidal activities of INH and RIF were significant at a titer of 1:32 compared to the activity of the vehicle control. As expected, the serum sample collected at 2 h after DSF administration orally exhibited bactericidal activity at a titer of 1:2, which was comparable to that of DDC administered intravenously, whereas the serum sample collected at 0.083 h after administration exhibited less activity (Table 3). Likewise, the serum sample collected at 2 h after DDC administration orally exhibited bactericidal activity, and the serum sample collected at 0.083 h exhibited less activity at a titer of 1:8; the serum sample from mice treated orally with DSF, but not DDC, exhibited bactericidal activity. Therefore, DSF has more potent bactericidal activity than DDC (Table 3). These results also indicated that DDC exhibits bactericidal activity *in vivo*.

Previously, the tuberculostatic effects of dithiocarbamates and thiuram disulfides have been examined in experimental tuberculosis of guinea pigs (n = 10) (17). Whereas this report is considerably important for the development of drugs with activity against TB, only evidence based on pathological findings without bacteriological examination of the lungs had been shown. This prompted us to evaluate the bactericidal activity of DSF *in vivo* in the mouse model of chronic TB. In the group administered DSF, significant reductions in the numbers of CFU were observed in both lungs and spleen at 80 to 160 mg/kg ($P < 0.01$) compared to the numbers of CFU for the vehicle control group (Fig. 2). The reduction in the numbers of pulmonary CFU by DSF at 80 mg/kg was similar to that by RIF at 10 mg/kg (Fig. 2). Whereas it has been

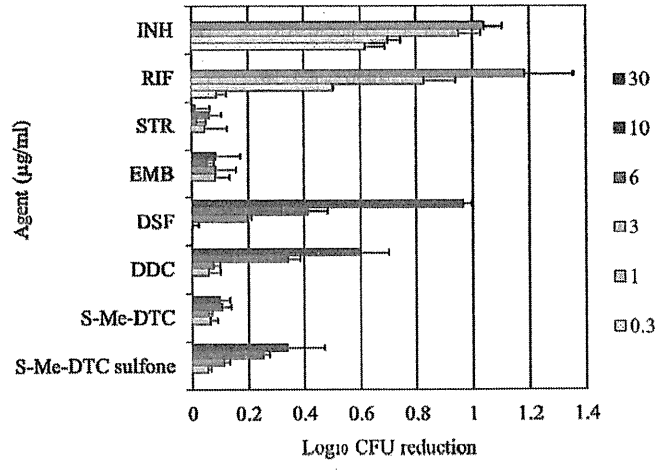

FIG 1 Intracellular antitubercular activities of each agent in differentiated THP-1 cells. The bactericidal activities of DSF, DDC, S-Me-DTC, and S-Me-DTC sulfone against intracellular *M. tuberculosis* $H_{37}Rv$ were assessed by the amount of CFU reduction compared to the amount achieved with antitubercular drugs, i.e., INH, RIF, STR, and EMB. Error bars represent means ± SDs (n = 3). Experiments were performed in triplicate and were carried out more than three times, and representative data are shown.

TABLE 3 Serum bactericidal activities of each agent compared to vehicle control[a]

	Log reduction ($\Delta\log_{10}$ CFU/ml)							
	DSF, p.o.		DDC, p.o.					
Titer	0.083 h	2 h	0.083 h	2 h	INH, p.o., 1 h	RIF, p.o., 1 h	DDC, i.v., 0.083 h	INH, i.v., 0.083 h
1:2	1.84 ± 0.03	2.66 ± 0.01	1.93 ± 0.05	2.46 ± 0.01	4.60 ± 0	ND	2.63 ± 0.03	ND
1:4	1.55 ± 0.003	1.79 ± 0.11	1.57 ± 0.04	1.58 ± 0.05	4.22 ± 0.12	4.600	1.90 ± 0.08	4.30 ± 0.43
1:8	1.41 ± 0.01	1.49 ± 0.1	—	—	3.57 ± 0.06	3.85 ± 0.21	1.36 ± 0.07	3.72 ± 0.16
1:16	—	—	—	—	2.83 ± 0.09	3.58 ± 0.03	—	3.12 ± 0.06
1:32	—	—	—	—	2.32 ± 0.02	2.68 ± 0.03	—	2.70 ± 0.12

[a] Each value (sample-vehicle control) represents the mean ± SD (n = 3). ND, not detected (>5.0 $\Delta\log_{10}$ CFU/ml); —, <1.0 $\Delta\log_{10}$ CFU/ml; DSF, disulfiram; DDC, diethyldithiocarbamate; INH, isoniazid; RIF, rifampin; p.o., *per os*; i.v., intravenous injection.

reported that DSF possessed a tuberculostatic effect at 20 mg/kg in the experimental model of tuberculosis in guinea pigs, there was no significant bactericidal activity in either the lungs or spleens of mice treated with DSF at 40 mg/kg (Fig. 2) (17). Taken together, these results suggest that DSF would exhibit a therapeutic effect against *M. tuberculosis* infection. According to previous reports, DSF could be administered at 6 g without considerable harm to humans, and intriguingly, it has been demonstrated that DSF and its metabolites preferentially transfer to lungs rather than to plasma, brain, or liver, suggesting that the use of DSF is feasible for the treatment of TB, especially for MDR/XDR-TB (8, 29).

The currently available anti-TB drugs, i.e., isoniazid, streptomycin, ethambutol, and *p*-aminosalicylic acid, have hitherto been known to be metal chelators (10, 11, 12). Intriguingly, for example, it has been reported that PAS may exert a synergistic effect with copper ion on antitubercular activity (11a). β-CAs, reported to be the drug target of dithiocarbamates, belong to the metalloenzyme family and include Zn ion at the active site when the enzymatic reaction is catalyzed (19). On the other hand, dithiocarbamates, e.g., DDC and PDTC, have been known to be metal chelators and have been reported to possess synergistic effects with copper ion on killing activity against protozoa, i.e., *Plasmodium falciparum* and *Toxoplasma gondii* (5, 21). Thus, to assess whether metal ions, namely, Zn^{2+}, Cu^{2+}, Co^{2+}, or Al^{3+}, affect the antitubercular activities of DSF and DDC, we prepared metal ion-containing Sauton broth medium and determined the MICs of these compounds. Increased antitubercular activities of these compounds were observed in $CuSO_4$-containing broth in a dose-dependent manner, unlike the findings for broth containing $ZnSO_4$, $CoCl_2$, or $Al_2(SO_4)_3$, indicating that the antitubercular activities of these compounds are dependent on small amounts of copper ion (see Table S4 in the supplemental material). Consequently, these results indicate that DSF and DDC exert synergistic effects with copper ion (>1 nM), unlike Zn^{2+}, Co^{2+}, or Al^{3+}. For this roundup, the antitubercular activities of these compounds were determined using the cupper ion chelator BCPS. BCPS was reported to inhibit the activation of signal transduction and the cell growth activated by DSF in melanoma via chelating copper ion (3). As expected, the antitubercular activities of these compounds were suppressed by BCPS in 7H9 broth, normally supplemented with 4 μM Cu^{2+} ion, and similar results were obtained for INH and ETH (Table 4). These results suggest that these compounds possess synergistic effects with the small amount of copper ion existing in the human body (30).

Based on our knowledge, β-CAs have been reported to be the drug targets of sulfonamides and sulfamates (25). Thereby, we considered that dithiocarbamates required sulfonation via intracellular enzymes, e.g., monooxygenase (EthA), for activation (1, 7). Meanwhile, DDC is catalyzed by the monooxygenase CYP2E1 in the human body. Hence, we hypothesized that DDC may possess a mechanism of activation that relies on the monooxygenase EthA and studied whether *ethA* expression was required for the

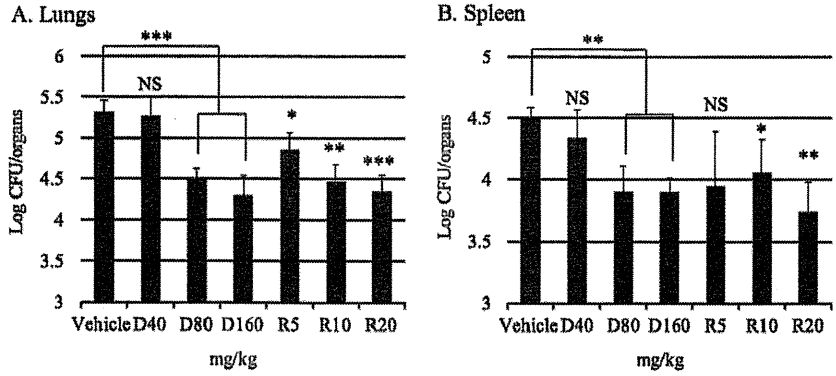

FIG 2 Therapeutic effects of rifampin (R) and disulfiram (D) in an experimental mouse model of chronic TB. ICR mice were inoculated intravenously with *M. tuberculosis* $H_{37}Rv$. After 28 days, test compounds were administered orally once daily for 28 days, and then lungs and spleen were extracted and the numbers of colonies in organs ($\log_{10}$ CFU/organ) were determined using 7H11 agar plates. Rifampin was used at concentrations from 5 to 20 mg/kg, disulfiram was used at concentrations from 40 to 160 mg/kg, and vehicle (5% arabic gum) alone was administered as a control. Error bars represent means ± SDs (n = 5). The Student t test was used to compare different treatment groups. Statistical significance was set at P values of <0.05 (*), <0.01 (**), and <0.001 (***) compared to the vehicle control group. NS, not significant.

TABLE 4 MICs of thiuram disulfides, dithiocarbamates, and antitubercular drugs against *M. tuberculosis* with or without copper chelator[a]

Agent	MIC (μg/ml) using 7H9 broth with:	
	No BCPS	BCPS at 50 mM
Thiuram disulfide		
Thiuram	0.78	6.25
DSF	1.56	12.5
Dithiocarbamate		
DMDC	1.56	6.25
DDC	3.13	25
PDTC	0.4	1.56
Anti-TB drugs		
INH	0.08	0.31
RIF	0.002	0.002
STR	0.39	0.39
EMB	1.56	1.56
ETH	3.13	12.5
CIP	0.39	0.39

[a] Experiments were performed in duplicate. These results are representative of three separate experiments. DSF, disulfiram; DMDC, dimethyldithiocarbamate; DDC, diethyldithiocarbamate; PDTC, pyrrolidine dithiocarbamate; INH, isoniazid; RIF, rifampin; STR, streptomycin; EMB, ethambutol; ETH, ethionamide; CIP, ciprofloxacin; BCPS, bathocuproinedisulfonic acid.

antimycobacterial activities of DSF and DDC. As control results, the *ethR*-overexpressing strain exhibited high levels of resistance to ETH, whereas the *ethA*-overexpressing strain was hypersusceptible to ETH (see Table S5 in the supplemental material). However, the MICs of both DSF and DDC against either the *ethR*- or *ethA*-overexpressing strain were equivalent to those against the wild-type strain (BCG pMV261), indicating that the antitubercular activities of these compounds do not depend on *ethA* expression (see Table S5 in the supplemental material). Albeit the mechanisms of sulfonation of these compounds are currently unknown, this mechanism via monooxygenase must be necessary to exert the antimycobacterial activities.

In conclusion, DSF is effective against MDR/XDR-TB, exhibits bactericidal activity within macrophages, and kills *M. tuberculosis* in mice, indicating that DSF might be a drug that may be repurposed for the treatment of MDR/XDR-TB. Further investigation on the mechanisms of action is now required to identify the potent drug targets, which will be important for the design of novel antitubercular drugs.

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Review

Current status of tuberculosis and recombinant bacillus Calmette-Guérin vaccines

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ABSTRACT

Tuberculosis (TB) is an infectious disease and a global concern of enormous proportions. One-third of the world's population is latently infected with *Mycobacterium tuberculosis*, and 2 million people die from TB annually. The only currently available vaccine—bacillus Calmette-Guerin (BCG)—is the most extensively used vaccine ever, with a record 3 billion doses administered during the last 4 decades. Because BCG is a live attenuated vaccine, it can be developed by genetic engineering to serve as a foreign antigen–producing multivalent vaccine. A new antibiotic-free host–vector system has been developed that expresses the foreign antigen from BCG by using a thymidylate synthase ThyX-deletion mutant of BCG and a plasmid harboring the *thyX* gene. This host–vector system is stable and can be useful for clinical purposes.

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1. Introduction

I started my study on the pathogenicity of mycobacterial species, including the causative agent of tuberculosis (TB), *Mycobacterium tuberculosis* (MTB), and bacillus Calmette-Guerin (BCG) vaccine in 1990. Several *Mycobacterium* species, especially pathogenic species, are slow-growing. MTB and BCG have a doubling time of at least 15 h, and it takes 3–6 weeks for their colonies to be visible on solid media. These species tend to aggregate. Even when bacteria is grown in liquid medium containing detergents, considerable clumping occurs. These properties have greatly hindered the understanding of the genetic makeup of mycobacteria. Genetic manipulation of mycobacterial species and the possibilities of the use of the BCG vaccine fascinated me.

2. Tuberculosis and the tubercle bacillus

Mycobacterial species, especially MTB, are very attractive microorganisms. TB is one of the oldest human plagues [1]. Signs of skeletal TB were evident in Europe in Neolithic times (8000 BC), in ancient Egypt (1000 BC), and in the pre-Columbian New World. TB is thought to be one of the microorganisms that have adapted most successfully to human beings and is the most common cause of infectious disease–related mortality worldwide. Despite its long history, MTB continues to cause more deaths than any other bacterial pathogen and is yet to be completely controlled.

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TB is a pandemic infectious disease and a major economic and public health concern worldwide. It is estimated that one-third of the world's population is latently infected with MTB, and approximately 9.4 million new cases of TB are reported annually, with 1.7 million people dying of the disease each year [2]. MTB usually enters the host on inhalation of an infected aerosol; the bacilli are then phagocytosed by alveolar macrophages. Intracellular replication of the bacterium results in a primary lesion, which is followed by lymphohematogenous dissemination and the formation of secondary lesions in the lungs and other organs [3]. Uncontrolled MTB growth at the site of infection is associated with extensive lung damage, ultimately leading to the death of the host. However, in most individuals, disease progression is arrested at this stage by an acquired immune response, resulting in the formation of granulomatous lesions, and a clinically latent state ensues. Post-primary disease state results from the subsequent reactivation of dormant bacilli [3,4].

3. BCG

The only currently available vaccine against TB—*Mycobacterium bovis* BCG—is the live attenuated strain of *M. bovis* obtained by Albert Calmette and Camille Guérin at the Institute Pasteur in Lille, France. They subcultured a virulent strain of *M. bovis* isolated from a cow with tuberculosis every 3 weeks on potato slices cooked in beef bile supplemented with glycerol. In 1921, after 230 passages over the course of 13 years, they found the culture was attenuated without reverting to the virulent phenotype but retained limited invasiveness in experimental animals [5].

BCG is thought to be effective against disseminated forms of TB in children, such as military TB and tuberculous meningitis, although most of the supporting data were derived from observational and case-control studies [6]. BCG vaccination was also shown to protect against leprosy in a controlled trial [7]. However, the protective efficacy conferred by BCG vaccination against pulmonary TB in adults has been variable in clinical trials (in the range of 0–80%), depending on the population, country, and BCG sub-strain used [8,9]. Therefore, a more effective vaccination strategy against TB is urgently needed.

4. Recombinant BCG vaccines

Although the protective efficacy of BCG is variable in humans, it remains the sole available vaccine for TB. BCG has many properties of an ideal vaccine: it has excellent adjuvant activity, it can be given at birth or any time thereafter, and a single inoculum can produce long-lasting immunity for several days to 10 years. It also has a long-standing safety profile. Since 1921, more than 3 billion doses of BCG have been administrated worldwide, with a remarkably low incidence of adverse effects in immunocompetent individuals. BCG is stable and inexpensive to produce. It has also been licensed for use in bladder cancer treatment. Because of these attributes, many investigators have focused their efforts on developing BCG into a novel vaccine vehicle that is capable of simultaneously expressing recombinant antigens of multiple pathogens [10].

In the mid-1980s, many advances in the field helped provide an understanding of the development of the mycobacterial genetic makeup and the molecular biology of mycobacteria. Jacob et al. first reported the development of the *Escherichia coli–Mycobacterium* shuttle phasmid [11]. In 1990, Yamada's group established a foreign antigen secretion system in mycobacteria in which the alpha antigen secreted by *Mycobacterium kansasii* was used as a carrier [12]. In this system, a B-cell epitope of human immunodeficiency virus type-1 (HIV-1) $p17^{gag}$ was secreted by BCG along with the alpha antigen. This was the first report on the expression and secretion of a foreign virus antigen from BCG. Two later reports first demonstrated the augmentation of immune responses by recombinant BCG (rBCG) [13,14]. At that time, the editor of Nature (London) evaluated these rBCGs as "rebirth of a star performer" [15]. Within 2 decades, many rBCGs were constructed, and animal experiments were performed using these rBCGs.

Many foreign antigens, including viral antigens, bacterial antigens, parasitic antigens, allergens, and cytokines have been used to express BCG [10,16]. I focused on rBCG for mycobacterial diseases.

5. rBCG producing large amounts of protective antigens

Several different types of TB vaccines have been developed using different technological platforms, including subunit vaccines, mycobacterial proteins and peptides in adjuvants, attenuated vectors expressing mycobacterial antigens, attenuated mutants of *MTB*, DNA vaccines, rBCG strains, and live attenuated vaccines. BCGs are among the most potent candidates.

Several strategies have been developed to produce effective rBCGs against mycobacterial diseases. One approach to increase the efficacy of BCG is to construct rBCG that overproduces autologous protective antigens. Baumgart et al. first constructed rBCG expressing the 18-kDa protein of *Mycobacterium leprae*. It stimulated the production of an antibody against the 18-kDa protein and lymphocyte proliferative responses in mice [17].

We have shown that immunization of mice with rBCG overproducing and secreting Ag85A rBCG/85A reduced *M. leprae* multiplication in the footpads of mice [18]. Immunization with rBCG/BA51, which results in the overproduction and secretion of 3 components of the Ag85 complex, i.e., Ag85A, Ag85B (also called alpha antigen), and MPB51, was more effective than that with rBCG/85A in inhibiting *M. leprae* multiplication in the footpads of mice [19]. Immunization with these rBCGs, especially rBCG/BA51, also induced greater protection against MTB challenge by aerosol, both in mice and guinea pigs relative to parent BCG (published elsewhere).

rBCG that overproduced and secreted the Ag85 protein was also constructed and reported by other investigators. Horwitz et al., reported that use of rBCG that expressed and secreted the Ag85B protein (rBCG30) improved protection in a guinea pig model of TB [20]. Sugawara et al. reported that rBCG with an Ag85A gene insert (rBCG-Ag85A[Tokyo]) had better protective efficacy against *M. tuberculosis* infection than did that with Ag85A DNA [21]. The higher protective efficacy induced by rBCG-Ag85A[Tokyo] was also shown in cynomolgus monkeys [22]. The antigens that were present in MTB but not in BCG were also present in rBCG. The RD1 locus of MTB contains genes for the protective ESAT-6 and CFP-10 antigens and is not present in BCG. Compared to BCG, rBCG that expressed the RD1 locus was reported to induce increased protection in mice and guinea pigs [23]. However, this strain has also been reported to have increased virulence.

6. rBCG-expressing immunomodulating molecules

BCG can also produce molecules that enhance host immune systems. Hess et al. constructed rBCG that secretes listeriolysin (Hly) of *Listeria monocytogenes* [24]. Hly is a pore-forming sulfhydryl-activated cytolysin that alters the membrane permeability of phagosomes in host cells. The rBCG expressing Hly improved major histocompatibility complex (MHC) class I presentation of co-phagocytosed soluble proteins. They also showed that a urease

C-deficient *hly*$^+$ rBCG (Δ*ureC hly*$^+$ rBCG) vaccine providing an intraphagosomal pH closer to the acidic pH optimum for Hly activity exhibited higher vaccine efficacy than did the parental BCG in mice [25].

rBCG vaccines expressing cytokines, including interleukin (IL)-2, IL-4, IL-6, IL-15, IL-18, granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon-γ, and monocyte chemotactic protein 3, were also constructed and their protective activities against MTB infection were evaluated [26–29]. IL-15 plays an important function in the maintenance of memory CD8$^+$ T cells. Mice immunized with rBCG expressing IL-15 fused with the dominant antigen Ag85B (rBCG-Ag85B-IL15) exhibited CD8$^+$ and CD4$^+$ T cell responses that were stronger than those in mice immunized with rBCG-Ag85B. Further, it provided robust protection in the lungs against intratracheal MTB challenge [29].

7. Construction of a new Host–vector system for rBCG

A large number of *E. coli–Mycobacterium* shuttle vectors have been developed to introduce foreign genes into BCG. These genes are maintained in the mycobacteria episomally or are integrated into the genome. Most plasmid vectors contain antibiotic resistance genes as selectable markers. These vectors would not be suitable in the clinical application of recombinant vaccines because there is a possibility of spreading of those genes to the microbial flora. Moreover, these gene product markers cannot work as selectable markers for the vaccine in vivo.

Thymidine synthase has been recognized as an essential enzyme in all free-living organisms. Organisms possess the canonical thymidylate synthase ThyA (EC 2.1.1.45), which is commonly found in eukaryotes and many eubacteria, and/or a novel FAD-dependent thymidylate synthase, ThyX (EC 2.1.1.148) [30]. ThyX also catalyzes the oxidation of NADPH. Only a few members of the suborder *Corynebacterineae* possess both ThyA and ThyX.

We constructed a ThyX gene (*thyX*)-deletion mutant (ΔthyX) by allelic exchange using a 2-step selection method [31] and found that ΔthyX could grow on 7H10-ADC (albumin-dextrose-catalase) agar plates but not on 7H10-ADS (albumin-dextrose-sodium chloride) agar plates that did not contain catalase. This result suggested that *thyX* on the mycobacterial plasmid could possibly be used as a selectable marker in ΔthyX mutants using the specified medium. To evaluate the usefulness of complementation as a marker, the *E. coli–Mycobacterium* shuttle vector containing *thyX* was constructed (Fig. 1). ΔthyX possessing the plasmid harboring *thyX* could grow on 7H10-ADS plates. To test the stability of this shuttle vector in ΔthyX, the green fluorescent protein (GFP) gene was inserted into it as the foreign antigen gene. The transformants expressing GFP retained the plasmid for over 6 months (more than 86% of bacterial cells expressed GFP); this suggests that this host–vector system is highly stable without the use of antibiotic resistance genes (published elsewhere). Therefore, the combination of the *thyX*-null mutant ΔthyX and the plasmid harboring *thyX* is a new host–vector system in BCG that abolishes the need for an antibiotic resistance gene as a vector component.

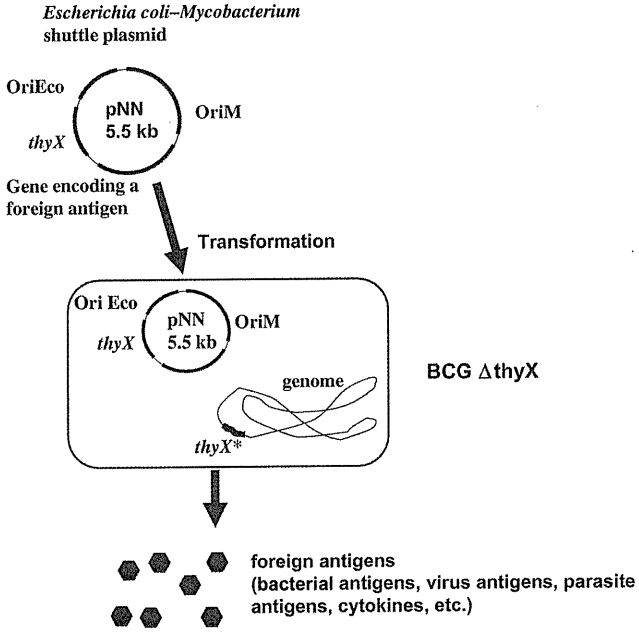

Fig. 1. Scheme of the new host–vector system in bacillus Calmette-Guerin (BCG) based on the combination of the *thyX*-null mutant ΔthyX and the plasmid harboring *thyX*. *thyX** indicates deletion of *thyX* from the BCG genome. OriEco, replication origin for *Escherichia coli*; OriM, replication origin for *Mycobacterium*.

8. Conclusions

The development of a novel vaccine for TB, an exciting but time-consuming task, is important for the control of TB. Once a vaccine candidate is constructed, the evaluation of its efficacy in humans requires several decades. However, vaccines have the potential to save lives of many people at once and thus have great impact. We hope that individuals worldwide can be saved through the use of novel vaccines.

Conflict of interest

No potential conflicts of interest are disclosed.

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Coronin-1a inhibits autophagosome formation around *Mycobacterium tuberculosis*-containing phagosomes and assists mycobacterial survival in macrophages

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Summary

Mycobacterium tuberculosis **is an intracellular bacterium that can survive within macrophages. Such survival is potentially associated with Coronin-1a (Coro1a). We investigated the mechanism by which Coro1a promotes the survival of *M. tuberculosis* in macrophages and found that autophagy was involved in the inhibition of mycobacterial survival in Coro1a knock-down (KD) macrophages. Fluorescence microscopy and immunoblot analyses revealed that LC3, a representative autophagic protein, was recruited to *M. tuberculosis*-containing phagosomes in Coro1a KD macrophages. Thin-section electron microscopy demonstrated that bacilli were surrounded by the multiple membrane structures in Coro1a KD macrophages. The proportion of LC3-positive mycobacterial phagosomes colocalized with p62/SQSTM1, ubiquitin or LAMP1 increased in Coro1a KD macrophages during infection. These results demonstrate the formation of autophagosomes around *M. tuberculosis* in Coro1a KD macrophages. Phosphorylation of p38 mitogen-activated protein kinase (MAPK) was induced in response to *M. tuberculosis* infection in Coro1a KD macrophages, suggesting that Coro1a blocks the activation of the p38 MAPK pathway involved in autophagosome formation. LC3 recruitment to *M. tuberculosis*-containing phagosomes was also observed in Coro1a KD alveolar or bone marrow-derived macrophages. These results suggest that Coro1a inhibits autophagosome formation in alveolar macrophages, thereby facilitating *M. tuberculosis* survival within the lung.**

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Introduction

Mycobacterium tuberculosis, a causative bacterium of tuberculosis, infects one-third of the world population and causes approximately 1.7 million deaths per year (World Health Organization, 2010). The ability to survive and persist in host macrophages is a major determinant of *M. tuberculosis* pathogenicity (Hingley-Wilson *et al.*, 2003). Studies suggest that this bacterium gains the ability to proliferate within infected macrophages by blocking phagolysosome biogenesis, because mycobacterial phagosomes do not fuse with lysosomal vesicles (Armstrong and Hart, 1971; Clemens and Horwitz, 1995). *M. tuberculosis* can interfere with intracellular membrane trafficking and subsequently cause phagosome maturation arrest in infected macrophages (Vergne *et al.*, 2003; 2004; Deretic *et al.*, 2004; 2006). We recently demonstrated that *M. tuberculosis* modulated the trafficking of Rab GTPases regulating phagosome maturation and that this modulation is relevant to the maturation arrest of mycobacterial phagosomes in macrophages (Seto *et al.*, 2009; 2010; 2011; Sugaya *et al.*, 2011). However, there is currently a lack of direct evidence that phagosome maturation arrest facilitates *M. tuberculosis* survival in macrophages. For example, some mutants generated by transposon mutagenesis survived and persisted within infected macrophages despite the progression of their phagosome maturation (Pethe *et al.*, 2004).

Autophagy is a unique lysosomal degradation pathway for cytoplasmic materials. This process is involved in the innate and adaptive immune systems (Deretic and Levine, 2009). The same process is also involved in the selection and exclusion of intracellular parasites especially in innate immunity and is termed xenophagy (Levine, 2005). *M. tuberculosis* is eliminated from infected macrophages by the induction of autophagy as a consequence of nutrient starvation, drug inducer or interferon-γ (Gutierrez *et al.*, 2004; Singh *et al.*, 2006). Autophagy also potentially controls the intracellular burdens of *M. tuberculosis* in macrophages (Kumar *et al.*, 2010).