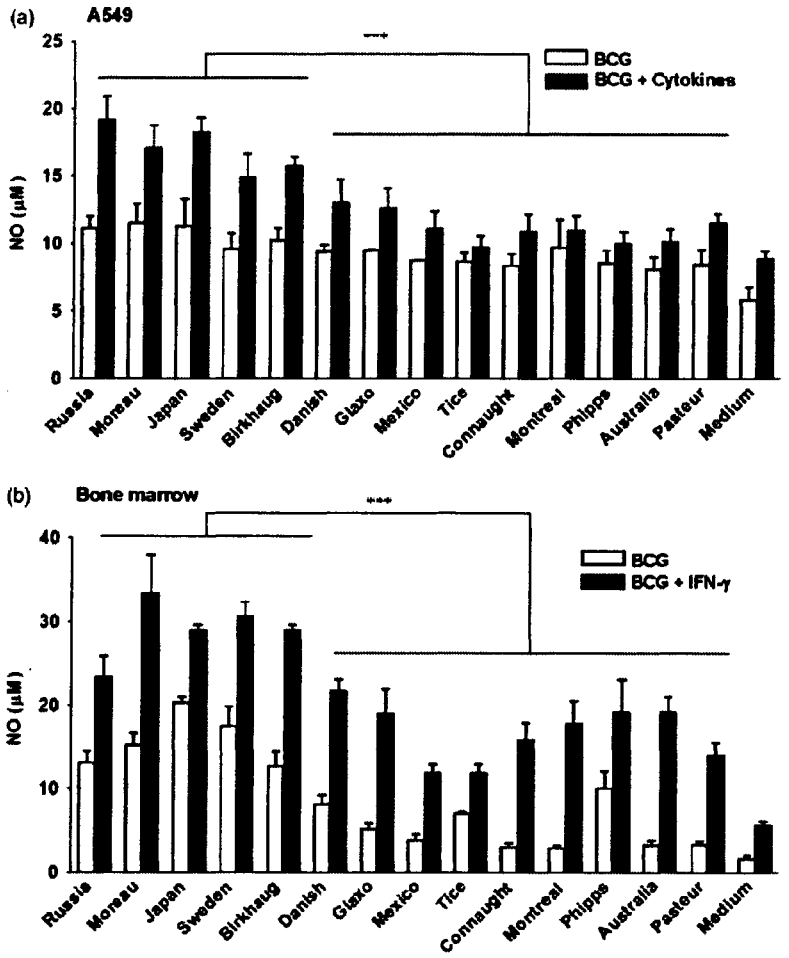

Fig. 1. Production of NO from human alveolar epithelial cells and mouse primary BMCs stimulated with BCG strains. (a) Human alveolar epithelial cell line A549 cells or (b) mouse primary BMCs were stimulated with different BCG strains at MOI 10 in the presence (solid columns) or absence (open columns) of cytokines; TNF-α (100 U mL $^{-1}$)+IL-1α (100 U mL $^{-1}$) mix for A549 (a), and IFN-γ (100 U mL $^{-1}$) for mouse BMC (b). The cells were incubated for 48 h and the amount of NO in culture supernatants was measured by NO assay. The NO production with cytokine stimulation in the 'early-shared strains' infected group (Russia, Moreau, Japan, Sweden, Birkhaug) was compared with that with cytokine stimulation in the 'late-shared strains' infected group (Danish, Glaxo, Mexico, Tice, Connaught, Phipps, Australia, Pasteur) (Student's *t*-test; $^{***}P < 0.001$). Results shown are representative of the results of three independent experiments.

to the identification of acid-fast bacilli. Behr *et al.* (2000) reported that genetic and phenotypic changes occurred in BCG-Pasteur after 1927 because of the mutation at *mma3*. Therefore, BCG strains that were passaged from 'authentic BCG-Pasteur' lacked methoxymycolate due to the mutation (Scheme 1 and Fig. 3). It is intriguing that not only the subclass of mycolate but also the composition ratio of different mycolates differ among BCG strains (Fig. 3). To investigate the NO-inducing ability of TDM in host cells, TDM was extracted from BCG strains and coated on polystyrene microbeads or the wells of a culture plate, and then cocultured with the host cells for 48 h. TDM from BCG-Japan induced higher NO production than that from BCG-Connaught in mouse macrophage cell line, RAW264.7, in a dose-dependent manner (Fig. 4a).

A synergistic effect between TDM and IFN-γ on the production of NO was observed and TDM from BCG-Japan exhibited higher activity than TDM from BCG-Connaught and *M. phlei* (Fig. 4b). These results were supported by the fact that heat-killed 'early-shared strains' of BCG, BCG-Russia, -Moreau, -Japan and –Sweden, exhibit higher NO production than the 'late-shared strains,' BCG-Danish, -Connaught and -Pasteur (Fig. 4c, solid column). TDM from 'early-shared strains' such as BCG-Japan has full sets of a subclass of mycolate, but TDM from BCG-Connaught lacks methoxymycolate, and *M. phlei* contains α-mycolate and wax. These data suggest that the composition and ratio of mycolate may play an important role in the induction of NO from host cells.

IFN-γ exhibits a synergistic effect with live 'early-shared strains' on the production of inflammatory cytokines, but reduces the production of IL-1β induced by the dead bacilli

The inflammatory cytokines IL-1β, IL-6, IL-8, IL-12 and TNF-α are induced by BCG-infected macrophage (Friedland *et al.*, 1993; Suzuki *et al.*, 1993; Lucey *et al.*, 1996).

Pretreatment or presence of IFN-γ augments the production of these cytokines. To evaluate the cytokine induction activity and synergistic effect between BCG strains and IFN-γ, human myelomonocytic cell line, THP-1, was infected with live or heat-killed BCG strains in the presence or absence of IFN-γ for 72 h, and the accumulation of cytokines was then determined by ELISA. The synergistic effect of IFN-γ and the bacilli of 'early-shared strains' of BCG, BCG-Russia, -Moreau, -Japan and -Sweden, on the production of inflammatory cytokines IL-1β, IL-6, IL-8, IL-12 and TNF-α was observed (Fig. 5). Similarly, the 'early shared strains' of BCG, BCG-Russia, -Moreau, -Japan and -Sweden exhibited strong inducing activity with or without IFN-γ, on the production of IL-12 and TNF-α from BMCs (Fig. 6).

In the absence of IFN-γ, these strains also induced higher production of IL-1β, IL-8 and IL-12 as compared with 'late-shared strains' of BCG, BCG-Danish, -Connaught and -Pasteur (Figs 5 and 6). Heat-killed bacilli of the 'early-shared strains' of BCG, BCG-Russia, -Moreau, -Japan and -Sweden, also have IL-8-, IL-12- and TNF-α-inducing ability, and a synergistic effect with IFN-γ was also observed (Fig. 7, solid column). However, IFN-γ reduced the production of IL-1β induced by heat-killed bacilli of the 'early-shared strains' of BCG, BCG-Russia, -Moreau, -Japan and -Sweden (Fig. 5a vs. Fig. 7a, solid column). IFN-γ reduced the IL-1β production by not only 'early', but also 'late-shared strains'. IL-1β, IL-8 and IL-12 were induced by not only live but also heat-killed bacilli (Figs 5a, c, e, 6a and 7a, c, d, open column). It is interesting to note that IL-6 and TNF-α were not induced by heat-killed bacilli; however, the synergistic effect of dead bacilli with IFN-γ on these cytokines was observed in THP-1 and BMCs (Figs 5b, e, 6b, 7b, d, solid column). The amount of cytokines induced by live bacilli was higher than those induced by dead ones (Figs 5 and 7). These results suggest that live 'early-shared strains' could be a better candidate for immune-stimulating agents, contributing to the induction of innate and acquired immunity in humans.

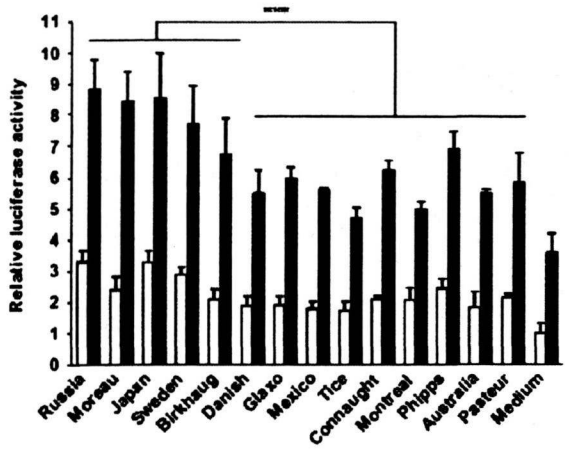

Fig. 2. NF-κB activation in BCG-infected A549 cells. A549 cells transfected with NF-κB/luciferase reporter plasmid were stimulated by MOI 10 of BCG strains with or without IL-1α (100 U mL $^{-1}$) and TNF-α (100 U mL $^{-1}$). After 48-h incubation, the cells were lysed and tested for luciferase activity. Luciferase activity was represented as fold-induction over the unstimulated control. Data are mean of values from three experiments (± SD) with assays in triplicate. Aspin–Welch's *t*-test was used to compare three groups (***$P < 0.001$): the cytokine-stimulated 'early-shared strains' infected group (Russia, Moreau, Japan, Sweden, Birkhaug), the cytokine-stimulated 'late-shared strains' infected group (Danish, Glaxo, Mexico, Tice, Connaught, Montreal, Phipps, Australia, Pasteur) and the cytokine-stimulated group. Results shown are representative of the results of three independent experiments.

TDM augments IFN-γ-induced inflammatory cytokine production

TDM extracted from BCG-Japan, -Connaught and *M. phlei* were incubated with THP-1 cells. TDM alone induced the

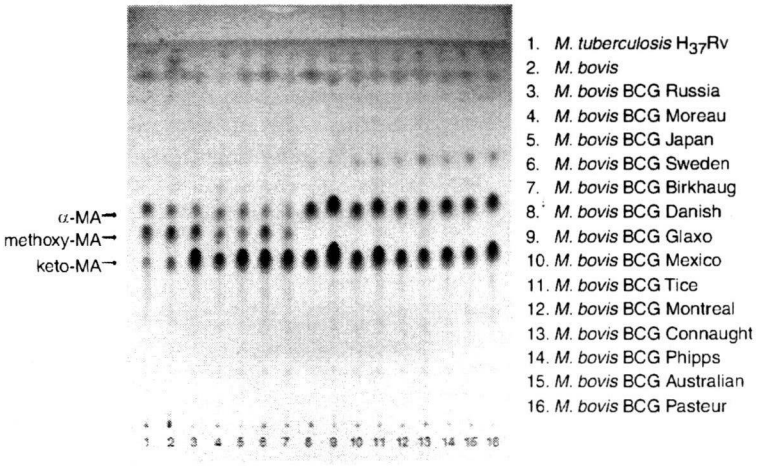

Fig. 3. Difference of the mycolic acid subsets among BCG substrains. TLC pattern of mycolic acid methyl ester subclasses derived from BCG strains. Lanes (1) *Mycobacterium tuberculosis* $H_{37}Rv$, (2) *Mycobacterium bovis*, and (3–15) *M. bovis* BCG strains; (3) Russia, (4) Moreau, (5) Japan, (6) Sweden, (7) Birkhaug, (8) Mexico, (9) Glaxo, (10) Danish, (11) Tice, (12) Montreal, (13) Connaught, (14) Phipps, (15) Australia and (16) Pasteur.

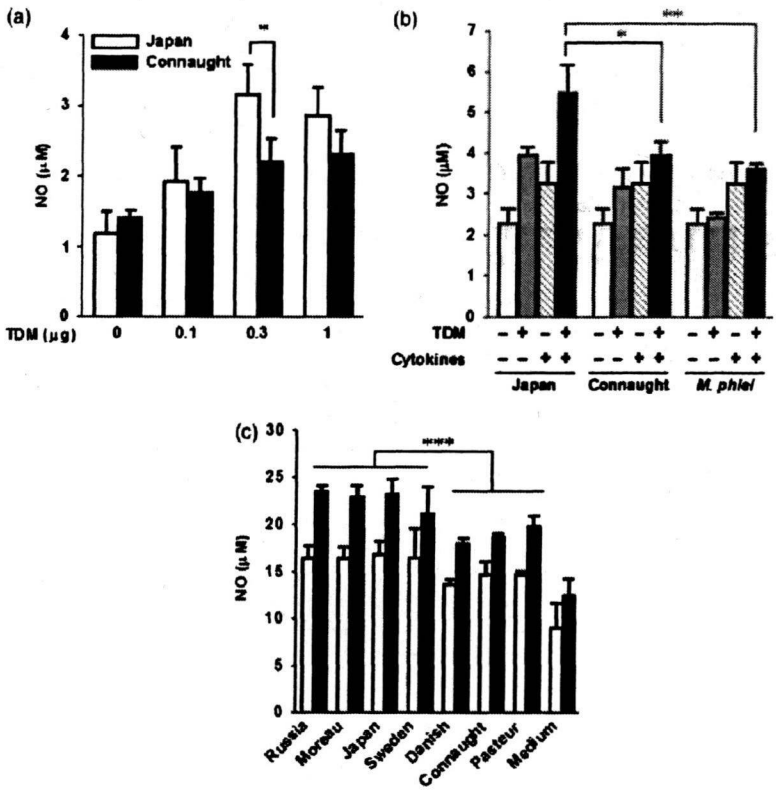

Fig. 4. NO production from host cells was induced differentially by TDM extracted from various BCG strains. (a) TDM (0.1–1 μg) from BCG-Japan and -Connaught was coated on polystyrene microbeads ($\varphi = 0.98$ μm). RAW 264.7 cells were incubated with microbeads. (b) TDM (1 μg) extracted from BCG-Tokyo, -Connaught and *Mycobacterium phlei* were coated on the microplates. RAW264.7 cells were cultured on the TDM-coated plates in the presence or absence of the cytokines (IL-1α and TNF-α). (c) RAW264.7 cells were stimulated with 10 μg mL^{-1} of heat-killed BCG strains. After 72 h incubation, culture supernatants were harvested, and the concentration of NO was determined by NO assay. (a) TDM stimulated from the BCG-Japan group was compared with that from the BCG-Connaught using Student's *t*-test ($^{*}P < 0.05$). (b) The TDM of three strains and cytokine-stimulated cells was compared using Tukey's multiple pairwise comparison test ($^{*}P < 0.05$, $^{**}P < 0.01$). (c) The 'early-shared strain' group (Russia, Moreau, Japan, Sweden) was compared with the 'late-shared strain' group (Danish, Connaught, Pasteur) using Student's *t*-test ($^{***}P < 0.001$). Values are the mean ± SD of triplicate samples and are representative of three independent experiments.

production of IL-8 compared with TDMs extracted from BCG-Connaught and *M. phlei*, but other cytokines such as IL-1β, IL-6, IL-12 and TNF-α were not induced (Fig. 8, open column). A synergistic effect between TDM and IFN-γ was observed in these three TDMs. TDM from BCG-Japan exhibited higher production of IFN-γ-induced IL-12 than TDM from BCG-Connaught and *M. phlei* (Fig. 8d and e). No significant difference in the synergistic effect of TDM vs. that of IFN-γ and TDM on the production of IL-1β, IL-6, IL-8 and TNF-α was observed between BCG-Japan and -Connaught (Fig. 8a–c).

Discussion

More than 14 strains of *M. bovis* BCG are subcultured and used widely as BCG vaccine (Scheme 1). To evaluate the immunomodulatory effect of various BCG strains, we used human myelomonocytic cell line THP-1, human lung epithelial cell line A549, mouse macrophage cell line, RAW264.7, and mouse primary BMCs as host cells. The advantages of using cell lines include reproducibility, easy handling and not having to use tedious animal models. The cell conditions can be kept constant, and therefore the biological differences among BCG strains would reflect the characteristics of BCG strains. Moreover, the lung epithelial cells are often used to investigate the role of host defense against tuberculosis infection (Wickremasinghe *et al.*, 1999; Chan *et al.*, 2001).

To compare the immunogenicity of BCG strains, the doses of live and dead bacilli and TDM were adjusted and the production of NO and cytokines in host cells was investigated. Both dead bacilli and their TDM exhibited weaker inducing activities than viable BCG (Figs 1, 5 and 6). Yang *et al.* (1995) reported that only viable BCG can induce NO production in splenocyte culture (Yang *et al.*, 1995). It is intriguing that the synergistic effect of IFN-γ on the production of IL-1β differed between live and dead bacilli (Figs 5a and 7a). Kubin *et al.* (1994) observed that lipopolysaccharide, but not *Mycobacterium*, reduces the production of IL-1β in the presence of IFN-γ, although the precise mechanism has not been elucidated (Kubin *et al.*, 1994). Differences in intracellular compartmentalization and interactions with phagocytes have been demonstrated in relation to patterns of cytokine induction, and it is probable that these factors have a profound influence on immunogenicity (Van der Laan *et al.*, 1999; Volpe *et al.*, 2006). Our results

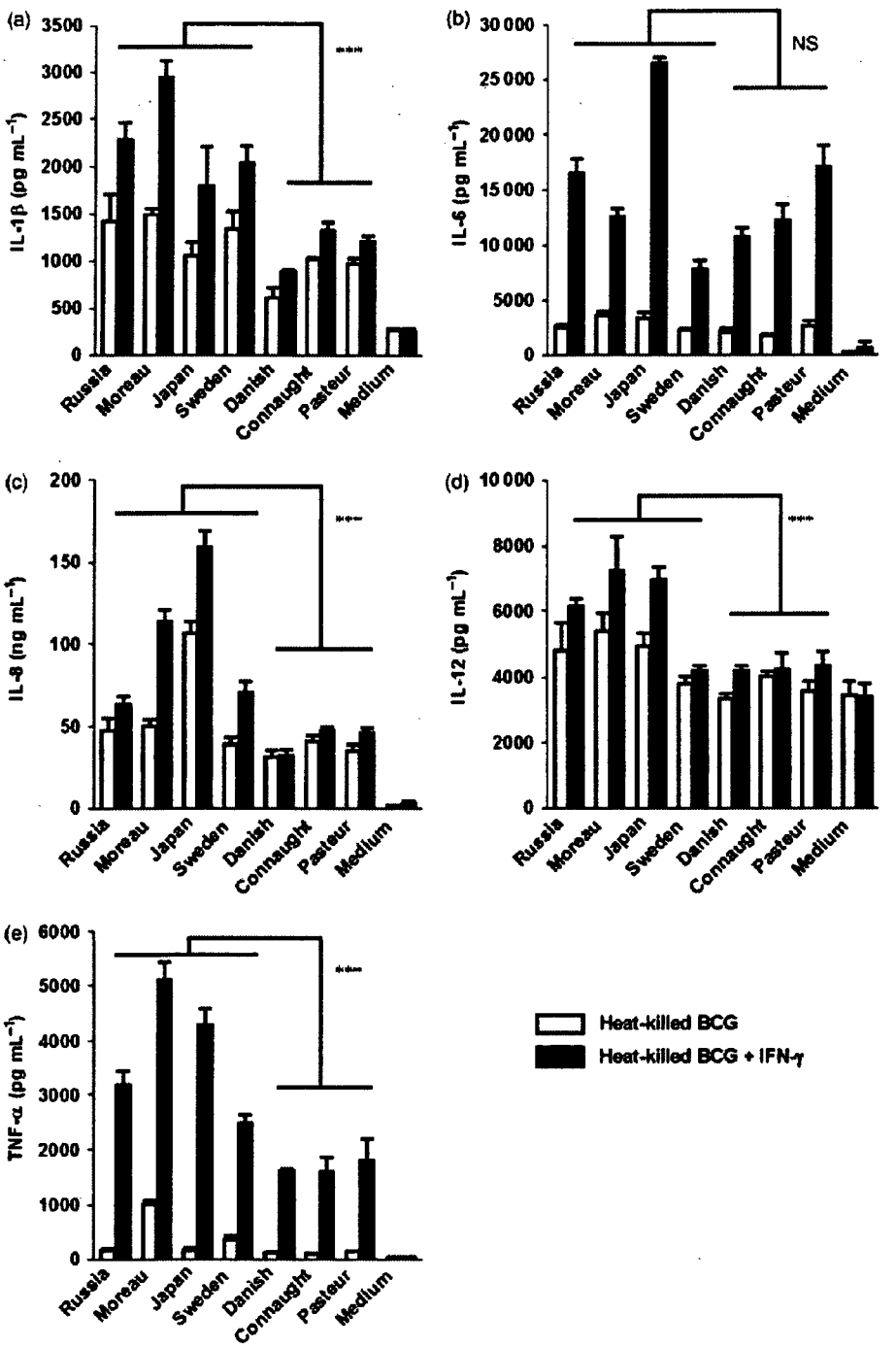

Fig. 5. 'Early-shared strains' of BCG more strongly induce the cytokines than 'late-shared strains' in THP-1 cells. THP-1 cells were incubated with live BCG (MOI 100) in the presence (solid columns) or absence (open columns) of IFN-γ (100 U mL $^{-1}$). Three days after infection, the culture supernatants were harvested and filtered. The concentrations of IL-1β (a), IL-6 (b), IL-8 (c), IL-12 (d) and TNF-α (e) in the supernatants were determined by ELISA. Values are the mean ± SD of triplicate samples and are representative of three independent experiments. Aspin–Welch's *t*-test was used to compare the IFN-γ-stimulated 'early-shared strain' group (Russia, Moreau, Japan, Sweden) with the 'late-shared strain' group (Danish, Connaught, Pasteur) (***$P < 0.001$). NS, nonsignificant.

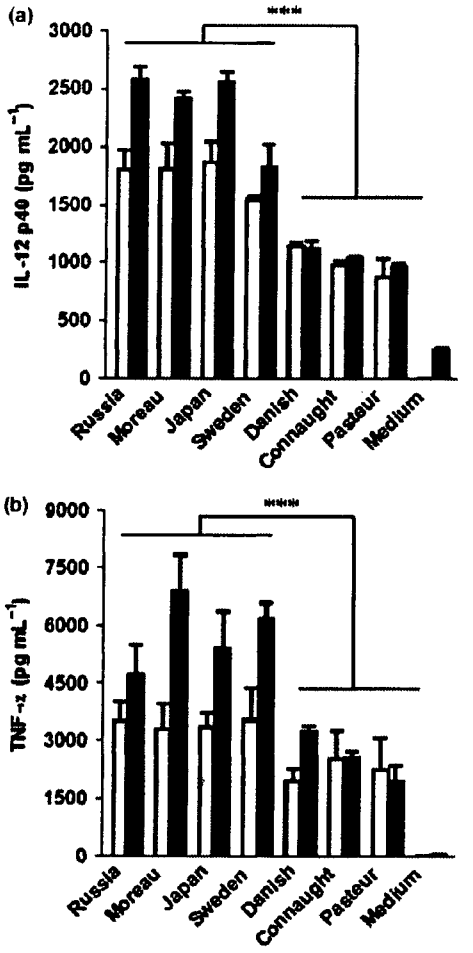

Fig. 6. Cytokine production from BCG-stimulated mouse BMCs. Mouse BMCs were incubated with MOI 10 of BCG strains in the presence (black bars) or absence (white bars) of IFN-γ (100 U mL $^{-1}$). Three days after infection, the culture supernatants were harvested and filtered. The concentrations of (a) IL-12 and (b) TNF-α in the supernatants were measured using ELISA. Aspin–Welch's *t*-test was used to compare the IFN-γ-stimulated 'early-shared strain' group (Russia, Moreau, Japan, Sweden) group with the 'late-shared strain' group (Danish, Connaught, Pasteur) (***$P < 0.001$).

may suggest that the difference in signals from live and dead bacilli may have an important influence on the differential response of host cells.

In our study, the 'early-shared strains,' BCG-Russia, -Moreau, -Japan, -Sweden and -Birkhaug, induce NF-κB activation and NO production from host cells in the presence of cytokines to a greater extent than the 'late-shared strains' BCG-Danish, -Glaxo, -Mexico, -Tice, -Connaught, -Montreal, -Phipps, -Australia and -Pasteur (Figs 1 and 2). TNF-α and IL-1 exhibit a synergistic effect on NO production with these strains in A549 cells (Fig. 1a, solid column). IFN-γ also exhibits a synergistic effect on the production with these strains in BMCs (Fig. 1b, solid column). *In vitro*, the expression of iNOS in macrophages is augmented by IFN-γ, TNF-α and IL-1, as well as infection with *M. tuberculosis* and *M. bovis* BCG (Wickremasinghe *et al.*, 1999). The expression of iNOS gene is well known to be regulated by NF-κB (Taylor *et al.*, 1998). The production of NO and activation of NF-κB was observed by stimulation of both live and dead bacilli (Figs 1 and 2). Therefore, we investigated whether the differences in the induction of NO among BCG strains would be reflected by the availability of heat-stable components, such as mycolic acid. Mycolic acid linked to trehalose was named 'cord factor' due to the cord-forming characteristics of the bacilli (Ryll *et al.*, 2001). In the host–parasite relationship, TDM has immunostimulatory (adjuvant) (Bekierkunst *et al.*, 1971), granulomagenic (Bekierkunst, 1968), antitumor (Azuma & Seya, 2001) and nonspecific infection prevention activities (Yarkoni & Bekierkunst, 1976) via cytokine-signaling processes. The synergistic effect of IFN-γ on the production of NO and cytokines was observed when the endothelial and monocytic/macrophage cell lines were stimulated with TDM-coated beads or plates (Figs 4 and 8). TDM extracted from BCG-Japan exhibited higher NO-inducing activity than TDM from BCG-Connaught (Fig. 4a and b). 'Early-shared strains' exhibited higher synergistic effect with IFN-γ than 'late-shared strains' (Fig. 4c). These results strongly suggest that compositions and moieties of mycolic acids play an important role in the induction of NO and the immunological responses in the presence of cytokines such as IFN-γ, IL-1 and TNF-α. Geisel *et al.* (2005) demonstrated that TDM-coated biodegradable microspheres, when embolized into murine lungs, induce intense inflammatory infiltrates and IL-4, IL-6, TNF-α, IL-12, IL-10 and IFN-γ production. BCG-Russia, -Moreau and -Japan exhibited higher synergistic effect with IFN-γ on the production of inflammatory cytokines (IL-1β, IL-6, IL-8, IL-12 and TNF-α) in THP-1 cells (Fig. 5). These synergistic effects on the production of IL-12 and TNF-α were observed in BMCs as well (Fig. 6). Therefore, these events were not limited to cultured cell lines. We have previously that reported the structure–activity relationships of TDM extracted from 11 species of mycobacteria and three (α-, methoxy-, keto-) or two (α-, keto-) types of mycolic acid were found in TDM among BCG strains (Fujita *et al.*, 2005a,b, 2007). The possibility that the length of mycolate affects the induction of NO remains. The composition and carbon length of mycolic acid of *M. phlei* was expected from our previous reports, in which the trehalose 6-monomycolate or TDM from *M. phlei* was analyzed with TOF/MS (Fujita *et al.*, 2005a, b). Variety in the number of mycolate between *M. phlei* and BCG is not so different (Fujita *et al.*, 2005a, b). Therefore, the effect of the length of mycolate is considered to be small, if any.

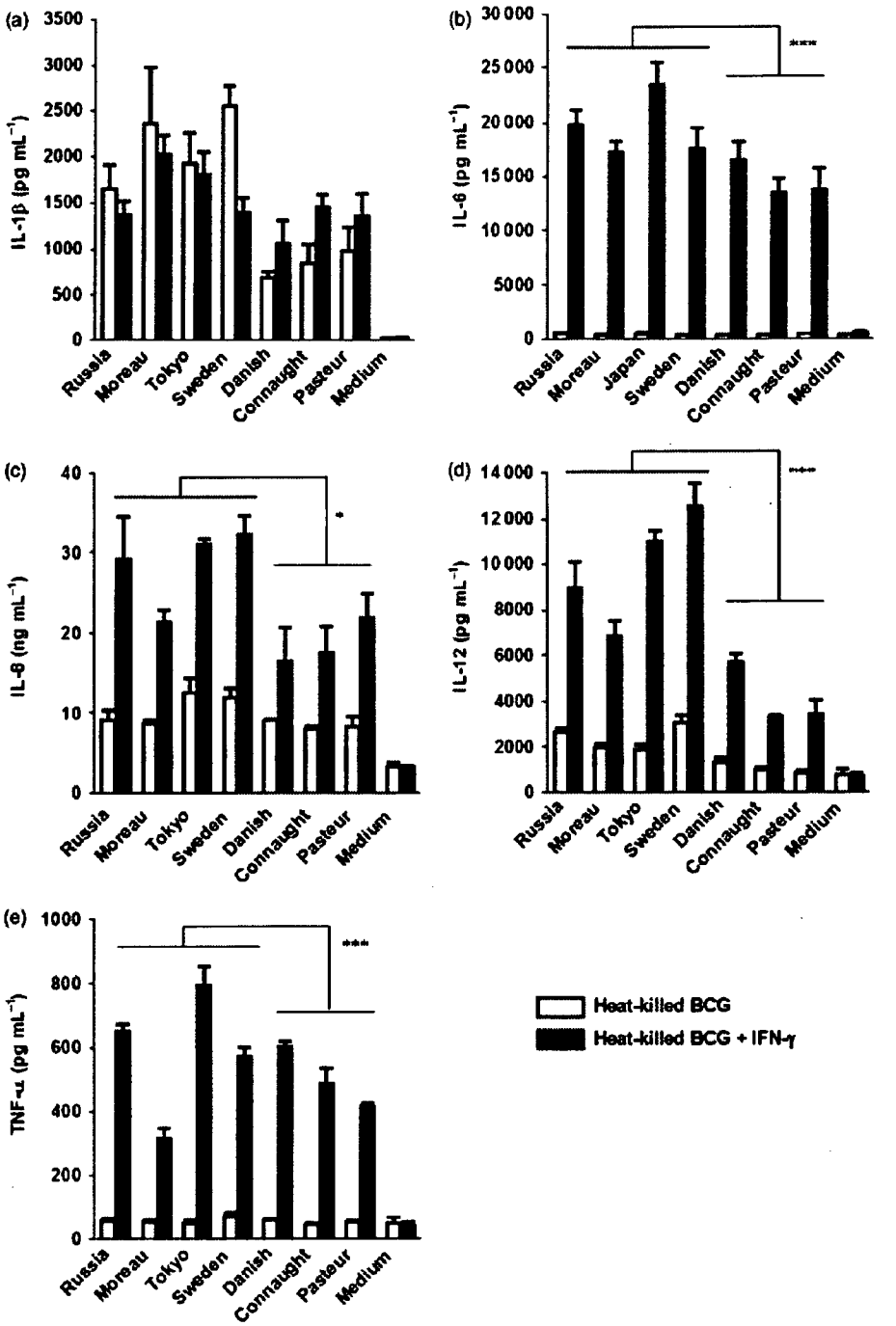

Fig. 7. Cytokine production from heat-killed BCG-stimulated THP-1 cells. THP-1 cells were incubated with 1 μg of heat-killed BCG in the presence (black bars) or absence (white bars) of IFN-γ (100 U mL $^{-1}$). Three days after infection, the culture supernatants were harvested and filtered. The concentrations of IL-1β (a), IL-6 (b), IL-8 (c), IL-12 (d) and TNF-α (e) in the supernatants were measured using ELISA. Values are the mean ± SD of triplicate samples and are representative of three independent experiments. Student's *t*-test was used to compare the IFN-γ-stimulated and 'early-shared strain'-stimulated group (Russia, Moreau, Japan, Sweden) with the 'late-shared strain' group (Danish, Connaught, Pasteur) ($^{*}P < 0.05$, $^{***}P < 0.001$).

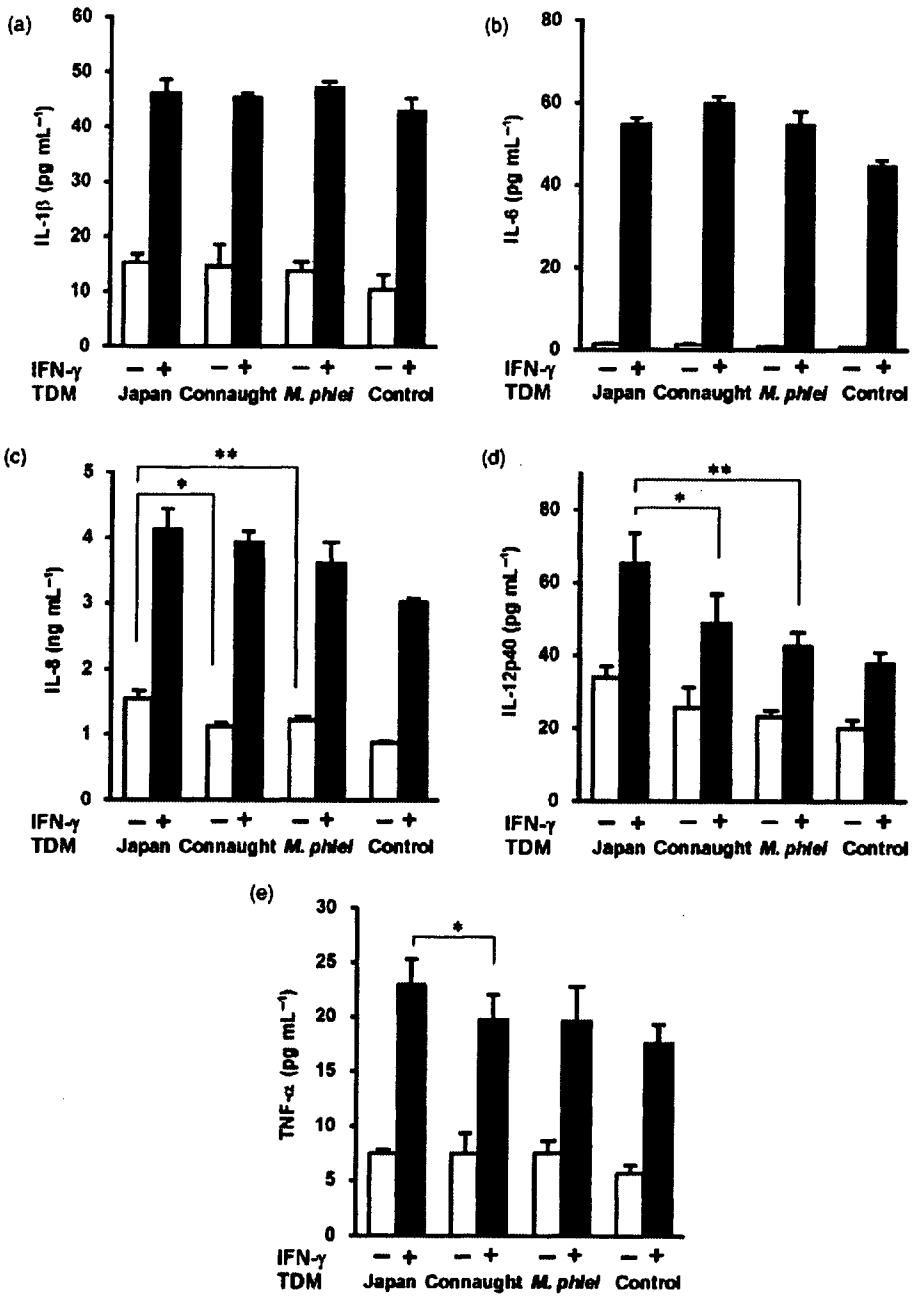

Fig. 8. TDM enhances the production of cytokines from THP-1 cells. TDM (1 μg) from BCG-Japan, -Connaught and *Mycobacterium phlei* were coated on the microplates. THP-1 cells were incubated on the TDM-coated plates with (solid column) or without (open column) 100 U mL ^{1} of IFN-γ. Following incubation for 72 h, culture supernatants were harvested, and the concentrations of IL-1β (a), IL-6 (b), IL-8 (c), IL-12 (d) and TNF-α (e) in the supernatants were determined by ELISA. Data are the mean of values from three experiments (± SD) with assays in triplicate. Tukey's multiple pairwise comparison test was used to compare the TDM from BCG-Japan, -Connaught and *M. phlei* with IFN-γ (d, e) and without IFN-γ (c). $^{*}P < 0.05$, $^{**}P < 0.01$.

Behr *et al.* (2000) reported that a point mutation at *mma3* gene in BCG strains obtained after 1927 is responsible for the impaired methoxymycolate production. The deficiency of methoxymycolate is considered to reflect the differences in immunogenicity among BCG strains. Recently, it has been reported that the mutation of *mma4*, which reflects the conformation of methoxymycolate, modulates the production of IL-12 from macrophage (Dao *et al.*, 2008). These

reports strongly support our findings that 'early-shared strains' with methoxymycolate (Fig. 3) exhibit higher synergistic effect with cytokines on the production of NO and inflammatory cytokines (Figs 4 and 8). Our study suggests that 'early-shared strains' may confer better protection against tuberculosis and could be the better vaccines among the various BCGs currently used.

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Synthesis of new sugar derivatives and evaluation of their antibacterial activities against *Mycobacterium tuberculosis*

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ABSTRACT

A series of sugar derivatives **(1–13)** were synthesized and evaluated for antibacterial activity against *Mycobacterium tuberculosis* (MTB), especially multi-drug resistant (MDR) MTB, and the structure–activity relationships of these compounds were studied. The results showed that the compound OCT313 (2-acetamido-2deoxy-β-D-glucopyranosyl *N,N*-dimethyldithiocarbamate) **(4)** exhibited significant in vitro bactericidal activity, and that the dithiocarbamate group at C-1 position of the glucopyranoside ring was requisite for the antibacterial activity.

Tuberculosis (TB) has become an important worldwide problem: about two million people die each year, particularly in developing countries. It is estimated that about one-third of the world population is currently infected with the bacillus in its latent form and that nearly nine million new cases develop each year.[1] According to WHO, multi-drug resistant tuberculosis is responsible for approximately 460 thousand new cases per year and for about 740 thousand new patients infected by both *Mycobacterium tuberculosis* and HIV/AIDS. Recent estimates show that 10% of all new TB infections are resistant to at least one anti-TB drug.[2] To treat an infection, a cocktail of drugs including, for example, isoniazid, rifampin, ethambutol and pyrazinamide are prescribed for two months followed by a continuation phase in which isoniazid and rifampin are taken. Long-term therapies lasting for between six and nine months have frequently led to patients' non-compliance and, in turn, contributed to the emergence of multi-drug resistant TB (MDR-TB).[3] The ever-increasing drug resistance, toxicity, and side effects of currently used anti-tuberculosis drugs, and the disappearance of their bactericidal activity necessitate new, safer, and more effective antimycobacterial compounds. In the last 10 years the research on *M. tuberculosis* and possible drug candidates have made much progress with the genome unrevealed and the discovery of different biological targets.[4,5]

Over 200 sugar derivatives were investigated for antibacterial activity by the broth dilution method. Two candidates were obtained after this random screening. One of them, OCT359 (allyl *O*-(2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl)-(1→6)-*O*-(2,3,4-tri-*O*-acetyl-α-D-galactopyranosyl)-(1→6)-*O*-2,3,4-tri-*O*-acetyl-β-D-glucopyranoside), has been reported previously.[6] In this study another compound, OCT313, 2-acetamido-2deoxy-β-D-glucopyranosyl *N,N*-dimethyldithiocarbamate **(4)**, was investigated for structure–activity relationships and antibacterial activities against MTB, including multi-drug resistant (MDR) MTB. OCT313 **(4)** consists *N*-acetyl-D-glucosamine and dimethyldithiocarbamate. This compound was prepared from acetylated glucosamine **(1)**, followed by chlorination,[7] substitution of the dimethyldithiocarbamate group and de-O-acetylation (Scheme 1).[8–10] OCT313 **(4)** was white crystals, mp 184–185 °C, and obtained in a 22.5% yield from **(1)**. In its NMR spectrum one proton doublet of H-1 appeared at δ 5.67 ($J_{1,2}$ = 11.0 Hz), indicative of β-configuration. The *N*-acetyl group appeared at δ 1.95 as a three proton singlet. Dimethyl signals of the dimethyldithiocarbonate group appeared at δ 3.37, 3.51 as each 3H singlet.

The isomers of OCT313 **(4)** were synthesized (Scheme 2). The general methods of synthesis of 2-acetamido-2-deoxy-α-D-mannopyranosyl *N,N*-dimethyldithiocarbamate **(5)** and 2-acetamido-2-deoxy-β-D-galactopyranosyl *N,N*-dimethyldithiocarbamate **(6)** are as follows. Instead of *N*-acetyl-D-glucosamine, *N*-acetyl-D-mannosamine or *N*-acetyl-D-galactosamine was used as a starting material. The reaction steps were the same as the synthesis of OCT313

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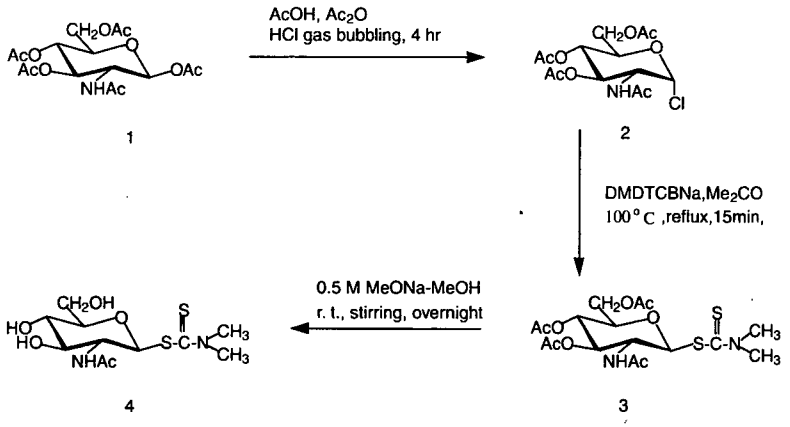
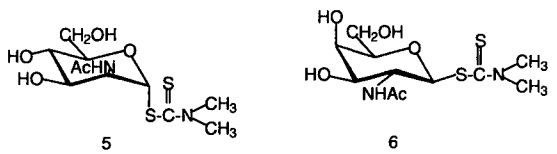


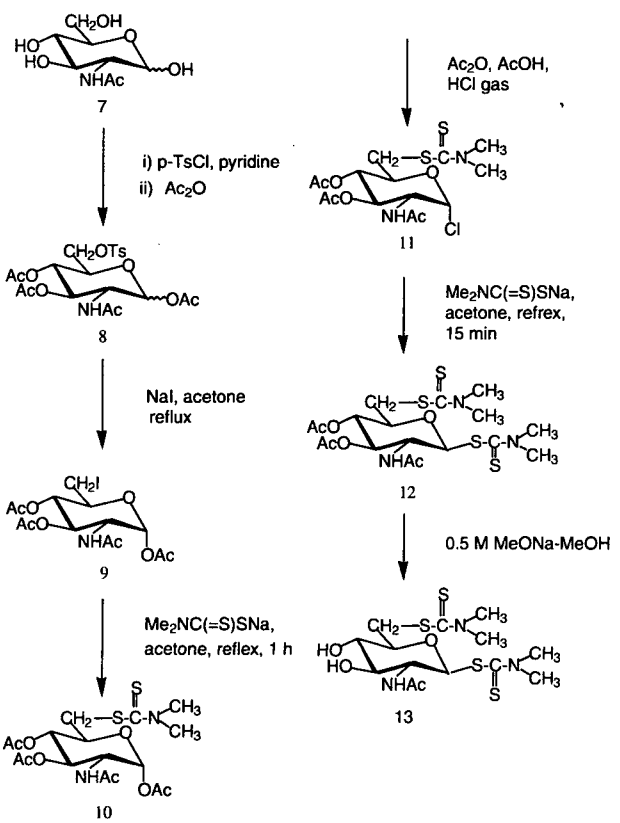
Scheme 1. Synthesis of 2-acetamido-2-deoxy-β-D-glucopyranosyl *N,N*-dimethyldithiocarbamate (OCT313) (**4**).



Scheme 2. Isomer of OCT313 2-acetamido-2-deoxy-α-D-mannopyranosyl *N,N*-dimethyldithiocarbamate (**5**) and 2-acetamido-2-deoxy-β-D-galactopyranosyl *N,N*-dimethyldithiocarbamate (**6**).

(**4**). Their yields were 53.6% and 29.7% from their peracetates, respectively.

Two dimethyldithiocarbamate groups were substituted at C-1 and C-6 position of *N*-acetyl-D-glucosamine (**13**) (Scheme 3). The general method of synthesis of 2-acetamido-6-*N,N*-dimethyldithiocarbamyl-2,6-dideoxy-β-D-glucopyranosyl *N,N*-dimethyldithiocarbamate (**13**) is as follows. *N*-Acetyl-D-glucosamine (**7**) was selectively tosylated, and then acetylated to give the tosylated peracetate (**8**). Treatment of the tosylated peracetate with sodium iodite in boiling acetone caused replacement of the sulfonyloxy group by iodine and crystalline 2-acetamido-1,3,4-tri-*O*-acetyl-2,6-dideoxy-6-iodo-α-D-glucopyranose (**9**) was obtained in a yield of 87.8%. This indicated that the sulfonyl group in the tosylated peracetate was located on the primary alcohol group. A mixture of (**9**) and sodium dimethyldithiocabamate in acetone was refluxed for 15 min. After work-up, 6-dimethyldithiocabamate (**10**) was obtained as an amorphous powder in a 92.3% yield. Compound **10** was similarly treated as described for the preparation of **4** to afford 13 in a 30.9% yield from **9**.



Scheme 3. Synthesis of 2-acetamido-6-*N,N*-dimethyldithiocarbamyl-2,6-dideoxy-β-D-glucopyranosyl *N,N*-dimethyldithiocarbamate (**13**).

The antibacterial activity of compounds **1–13** was investigated by using *M. tuberculosis* $H_{37}Rv$, *Mycobacterium bovis* BCG (Tokyo-172), *Mycobacterium avium* 724S, *M. avium* SmO, *Mycobacterium smegmatis*, *Staphylococcus aureus*, and *Escherichia coli* as target bacteria (Table 1). OCT313 (**4**) was specifically effective to slow the grows of *Mycobacterium* species, such as *M. tuberculosis* and *M. bovis* BCG (Table 1). The character of a narrow spectrum of anti-bacterial activity is appropriate to anti-tuberculosis drugs, because the therapeutic term should be at least six months. The MIC of OCT313 (**4**) to *M. tuberculosis* and *M. bovis* BCG was between 25 and 32 μg/ml (Table 1). The MIC of C-4 isomer of OCT313 (**4**), 2-acetamido-2-deoxy-β-D-galactopyranosyl *N,N*-dimethyldithiocarbamate (**6**) was 2 times lower than that of OCT313 (**4**). The MIC of 2-acetamido-6-*N,N*-dimethyldithiocarbamyl-2,6-dideoxy-β-D-glucopyranosyl *N,N*-dimethyldithiocarbamate (**13**), a derivative of OCT313 with two dimethyldithiocarbamate groups at C-1 and C-6 position was the same as that of OCT 313 (**4**). The acetylated compounds, **3**, lost the antibacterial activity. A finding worthy of note was that sodium dimethyldithiocarbamate (DMSTCA.SS) exhibited strong anti-bacterial activity (Table 1).

The dimethyldithiocarbamate group at C-1 position of OCT313 (**4**) was responsible for the bactericidal effect. Sodium dimethyldithiocarbamate exhibited toxicity to human cell lines; however, the sugar bound to the dimethyldithiocarbamate compound, OCT313, reduced the cytotoxicity (Supplementary Fig. 1 and Table 1). The ratio of the toxic to effective dose of OCT 313 was from 28 to 242 in OCT313, however that of the sodium dimethyldithiocarbamate was from 14 to 318 (Supplementary Table 1). Degradation of OCT 313 (**4**) in the assay medium of anti-bacterial activity was not observed during assay period (data not shown).

In conclusion, the dimethyldithiocarbamate group at C-1 of *N*-acetyl-D-glucosamine is critical for antibacterial activity (Table 1).

Table 1
Antibacterial activities of glucosamine derivatives (MIC, μg/ml)[a]

	Organisms						
	M. tuberculosis $H_{37}Rv$	*M. bovis* BCG (Tokyo)	*M. avium* 724S	*M. avium* SmO	*M. smegmatis*	*S. aureus*	*E. coli*
Compounds							
1 Glc-N-Ac free	>100	>100	ne	ne	>100	ne	ne
3 OCT313-peracetate	>100	>100	>100	>100	>100	>100	>100
4 OCT313 (Glc-N-Ac DMDTCB)	25	31.3	>100	>100	>100	>100	>100
5 Man-*N*-Ac DMDTCB	>100	>100	>100	>100	>100	>100	>100
6 Gal-*N*-Ac DMDTCB	12.5	25	>100	>100	>100	>100	>100
13 Glc-*N*-Ac 1,6 DMDTCB	25	>100	>100	>100	>100	>100	>100
Raw materials							
DMDTCA.SS	0.78	0.78	ne	ne	>100	>100	>100
Dimethylamine	>100	>100	ne	ne	>100	ne	ne
Dimethylamine HCl	>100	>100	ne	ne	>100	ne	ne
anti-MTB Antibiotics							
INH	0.04	0.04	50	3.13	6.25	>100	>100
RFP	0.004	0.004	<0.05	0.004	1.56	0.002	50
SM	0.39	0.20	0.39	0.39	0.39	50	50
EB	2.5	1.5	3.13	1.56	12.5	>100	>100
PAS	0.16	50	100	100	1000	>100	>100
AMK	1.75	0.78	>100	1.56	1.25	100	50
KM	1.56	0.3	>100	1.56	3.13	12.5	12.5
GM	3.13	1.56	25	1.56	1.56	25	6.25

[a] Broth dilution methods using MiddleBrook 7H9 broth containing albumin, dextrose, and catalase for derivatives (ne, not examined). For *Staphylococcus aureus*, we used the heat-infusion broth. INH, isoniazid; RFP, rifampicin; SM, streptomycin; EB, ethambutol; AMK, amikacin; KM, kanamycin; GM, gentamycin.

Table 2
Antimycobacterial activity of OCT313 on drug-sensitive and resistant clinical isolates of *M. tuberculosis*

Clinical isolates	Resistance to	MIC for OCT313 (μg/ml)
Drug-susceptive strains		
A-1-1		6.25
A-1-2		6.25
A-3-5		6.25
A-3-11		6.25
A-3-12		6.25
A-3-13		6.25
A-3-15		6.25
A-3-16		6.25
A-3-20		6.25
A-3-21		6.25
A-3-22		6.25
A-3-9		6.25
A-3-22		6.25
A-1-3		6.25
A-2-5		6.25
A-3-1		6.25
A-3-2		6.25
A-3-6		6.25
A-3-17		12.5
A-3-19		6.25
Drug-resistant strains		
A-3-47	SM	6.25
A-4-8	SM	6.25
A-2-1	INH	6.25
A-2-3	INH, RFP	3.12
J-1-19	INH, RFP	6.25
K-3-6	INH, SM, EB	6.25
M-1-32	INH, RFP, SM, EB	6.25
N-4-11	INH, RFP, EB	6.25
N-5-2	INH, RFP, SM, EB	1.56
P-1-50	INH, RFP, SM, EB	6.25
P-4-11	INH, RFP, SM, EB	6.25
Q-4-1	INH, RFP, SM, EB	6.25
R-1-38	INH, RFP, SM, EB	6.25
U-2-15	INH, RFP, SM, EB	3.12
U-4-6	INH, RFP, SM, EB	6.25
Z-1-4	INH, RFP, SM, EB	6.25
A-2-4	RFP	6.25
A-2-6	INH, RFP, SM, EB	6.25
A-4-25	INH, SM	6.25

Proportion methods using Middlebrook 7H11 agar plates for INH, RFP, SM, EB and 7H9 broth for OCT313. Cut off concentrations of each antibiotic were 10, 10, 100 and 100 μg/ml, respectively.

Compounds having dimethyldithiocarbamate groups have been used for pesticides and their toxicity for humans was due to inhibition of choline esterase. However, the inhibitory effect of OCT313 on choline esterase was undetectable (Supplementary Fig. 2). The precise mechanism of the anti-tuberculosis effect of dimethyldithiocarbamate is unknown; however, OCT313 **(4)** exhibits bactericidal and lytic activities against *M. tuberculosis* and *M. bovis* BCG (Supplementary Fig. 3), strongly suggesting that OCT313 **(4)** exerts antibacterial activity by the mechanism distinct from that of dimethyldithiocarbamate. Furthermore, 25 clinical isolates of drug-resistant MTB and 19 drug-sensitive MTB were sensitive to OCT313 **(4)** (Table 2). The MICs of OCT313 **(4)** to these clinical isolates were from 1.56 to 12.5 μg/ml. Cross-resistance of OCT313 **(4)** to currently used anti-TB drugs was not observed (Table 2). These results strongly indicate that OCT359 possesses novel drug targets and may be a useful lead compound for MDB, especially MDR-MTB.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.09.095.

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8. *General procedures*—Melting points were determined with a Yamagimoto MP-S2 micro melting point apparatus and uncorrected. Solutions were concentrated in a rotary evaporator below 50 °C under vacuum. Optical rotations were measured with a JASCO P-1020 automatic digital polarimeter in a 0.1 dm tube. IR spectra were recorded with a JASCO FT/IR-4100 Spectrometer. ^{1}H NMR spectra were recorded at 500 MHz with a JNM-α500 spectrometer and JNM-ECA500/KJ, at 600 MHz with a BRUKER-AV600. ^{13}C NMR spectra were recorded at 125 MHz with a JNM-α500 spectrometer. Tetramethylsilane was used as an internal standard. Chemical shift are given on the δ scale. TLC was performed on precoated silica gel plates 0.25 mm thick (Kieselgel 60F_{254}, Merck). Detection was effected with H_2SO_4 or by UV irradiation at 254 nm. Column chromatography was performed on Silica Gel BW-820MH (Fuji-Silysia Chemical Ltd, Nagoya, Japan).
9. *2-Acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl N,N-dimethyldithiocarbamate* (**3**)—To a solution of 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl chloride[7] (26.77 g, 73.2 mmol) in dry acetone (200 ml) was added sodium *N,N*-dimethyldithiocarbamate dihydrate (21 g, 146.6 mmol) and the mixture was refluxed for 15 min. After checked the disappearance of the starting materials by TLC ($CHCl_3$/acetone 6:1, v/v), the reaction mixture was evaporated to afford a syrup which was dissolved in water and $CHCl_3$. The organic layer was separated, washed with H_2O, dried ($MgSO_4$), and evaporated to afford a syrup. The syrup was dissolved in a small amount of $CHCl_3$ and chromatographed on a column with $CHCl_3$/acetone (10:1–3:1, v/v). Evaporation of the solvent gave (**1**) (12.4 g, 37.6 %) as an amorphous powder, $[\alpha]_D^{20}$ +49.6 (*c* 1.36, $CHCl_3$), IR (KBr) cm^{-1}: 3282 (NH), 1749 (C=O), 1666 (amide I), 1547 (amide II). ^{1}H NMR ($CDCl_3$) δ: 1.92,2.05 (×2), 2.08 (s, 12H, Ac × 4), 3.37, 3.54 (each s, 6H, NCH_3 × 2), 3.85 (m, 1H, H-5), 4.13 (dd, 1H, $J_{5,6a}$ = 2.1 Hz, $J_{6a,6b}$ = 12.5 Hz, H-6a), 4.25 (dd, 1H, $J_{5,6b}$ = 4.6 Hz,,H-6b), 4.57 (ddd, 1H, $J_{1,2}$ = 11.0 Hz, $J_{2,NH}$ = 9.8 Hz, $J_{2,3}$ = 9.8 Hz, H-2), 5.16 (dd, 1H, $J_{3,4}$ = 9.2 Hz, $J_{4,5}$ = 9.8 Hz, H-4),5.20 (dd, 1H, H-3), 5.79 (d, 1H, H-1), and 6.13 (d, 1H, NH). ^{13}C NMR ($CDCl_3$) δ: 20.8, 20.9, 21.0, 23.3 ($COCH_3$ × 4), 42.1, 45.8 (NCH_3 × 2), 52.2 (C-2), 62.1 (C-6), 68.1 (C-4), 74.8 (C-3), 76.7 (C-5), 89.3 (C-1), 169.5, 170.3, 170.9, 171.4 ($COCH_3$ × 4), and 193.7 (C=S).
10. *2-Acetamido-2-deoxy-β-D-glucopyranosyl N,N-dimethyldithiocarbamate* [(**4**), OCT313]—A 0.5 M methanolic MeONa (1 ml) was added to a suspension of (**3**) (9.3 g, 20.6 mmol) in dry MeOH (93 ml), and the mixture was stirred at room temperature for 2 h under exclusion of moisture. After neutralization with Amberlite IR-120B(H^+) resin and removal of the resin by filtration, the filtrate was evaporated to syrup which was crystallized from EtOH. Recrystallization from EtOH gave pure (**4**) (4.0 g, 59.7 %) as white crystals, mp 184–185°, $[\alpha]_D^{24}$ +50.7 (*c* 1.16, H_2O), IR(KBr) cm^{-1}: 3600–3100 (br OH), 1656 (amide I), 1562 (amide II). ^{1}H NMR (CD_3OD) δ: 1.95 (s, 3H, $NCOCH_3$), 3.37, 3.51 (each s, 6H, NCH_3 × 2), 3.39 (m, 1H, H-5), 3.44 (dd, 1H, $J_{3,4}$ = 8.6 Hz, $J_{4,5}$ = 9.5 Hz, H-4), 3.56 (dd, 1H, $J_{2,3}$ = 9.8 Hz, H-3), 3.69 (dd, 1H, $J_{5,6a}$ = 2.1 Hz, $J_{6a,6b}$ = 12.2 Hz, H-6a), 3.83 (dd, 1H, $J_{5,6b}$ = 4.9 Hz, H-6b), 4.07 (dd, 1H, $J_{1,2}$ = 11.0 Hz,H-2), and 5.67 (d, 1H, H-1). ^{13}C NMR (CD_3OD) δ: 22.9 ($COCH_3$), 42.0, 45.7 (NCH_3 × 2), 54.6 (C-2), 62.6 (C-6), 71.6 (C-4), 77.6 (C-3), 82.3 (C-5), 90.4 (C-1), 173.7 (C=O), and 195.4 (C=S).

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Population Structure Dynamics of *Mycobacterium tuberculosis* Beijing Strains during Past Decades in Japan▽†

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We used 909 strains to compare the population structures of the *Mycobacterium tuberculosis* Beijing family between different birth-year cohorts in Japan. The results revealed that the spread of a modern sublineage that has high transmissibility is currently increasing, while the spread of an ancient sublineage, STK, has significantly decreased in younger generations.

Comparative studies of the *Mycobacterium tuberculosis* population structure have generated interest in understanding its epidemiological relevance to human disease (2, 3, 9, 15, 21). One of the most extensively studied clades with respect to host-pathogen compatibility is the Beijing family (5, 7, 8, 16, 23), which is highly prevalent in East Asia (26). Recent studies have demonstrated the worldwide dissemination of the modern Beijing family strains (having IS*6110* insertions in the NTF region) (1, 14, 17, 18) and have led to speculation about the hypervirulent features of this sublineage (4, 7, 13, 16). In contrast to the worldwide prevalence of modern Beijing strains, the ancient Beijing strains (having an intact NTF region) are highly diverse and dominant in Japan (11, 27). Studying this singularity of the Beijing strains in Japan would expand our understanding of the heterogeneity in the fitness of different sublineages. In this study, we used data from a population-based study lasting 5 years and 8 months to investigate the probable shift in the *M. tuberculosis* population structure during the previous decades and determine the future trend in Japan, with special attention to the modern Beijing strains.

We obtained 909 *M. tuberculosis* isolates from newly diagnosed pulmonary tuberculosis (TB) patients between April 2002 and December 2007. These isolates accounted for approximately 70% of the new culture-positive pulmonary TB cases detected during the collection period in Kobe City. The isolates were phylogenetically classified as belonging to the Beijing clade or other clades by spoligotyping (12) and to the ancient and modern Beijing sublineages on the basis of the presence of IS*6110* in the NTF region (19, 27). The ancient and modern Beijing strains were subclassified by analyzing 10 synonymous single-nucleotide polymorphisms (6, 8, 11). Molecular typing based on the variable number of tandem repeats (VNTR) method with 19 loci, which comprised the 15 loci of Supply et al. (24) and 4 hypervariable loci (QUB-11a, QUB-3232, VNTR-3820, and VNTR-4120) (10), was performed for all strains. Recent transmission was suggested by the clustering of identical VNTR profiles. The presence or absence of region of difference 181 (25) in all the Beijing strains was analyzed.

Of the 909 isolates, 714 (78.5%) were classified as Beijing family strains (Table 1), and these included 44 Beijing-like strains. Except for the sequence type 11 (ST11) and ST26 sublineages, all the Beijing strains contained deletions in region of difference 181. All the modern strains harbored only one IS*6110* insertion element. The non-Beijing family strains comprised 32 spoligotypes. The details of the genetic data of all isolates are summarized in Table S1 in the supplemental material.

The average patient age, patient gender, cluster rate (number of clustered isolates/total number of isolates), and proportion of multidrug-resistant strains did not differ significantly between the Beijing and non-Beijing strains (Table 1). However, further classification of the Beijing family strains revealed a significantly high cluster rate (42.9% versus 31.3%, $P = 0.022$, Pearson's chi-square test) in the case of the modern Beijing strains. This indicates that the transmissibility of the modern Beijing strains is higher than that of the non-Beijing strains. Further, the cluster rate of the STK strains was observed to be low (12.6% versus 31.3%, $P < 0.01$). Moreover, the average age of patients affected by the modern strains was significantly younger than that of those affected by the non-Beijing strains (58.7 versus 64.9 years, $P < 0.01$, Welch's t test) (Table 1). These results imply that the transmission of various sublineages is different, which in turn implies that the population structure of the Beijing *M. tuberculosis* strains that are prevalent in Japan is more dynamic than stable. Human immunodeficiency virus infection and introduction by foreigners remain minor factors in the epidemiology of TB in Japan (22), and these factors have negligible effects on the population dynamics of *M. tuberculosis* strains there.

We attempted to determine the shift that had occurred in the population of the Beijing family strains during previous decades by comparing the population structures of strains iso-

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TABLE 1. Genotypic characteristics of *M. tuberculosis* isolates from 909 tuberculosis patients

Genotypic sublineage	Total no. (%) of isolates	No. (%) of patients that were:		Avg age (yrs)	No. of clusters[b]	No. (%) of isolates that clustered[b]	No. of MDR strains[d]
		Male	Female				
Non-Beijing	195 (21.5)	133 (68.2)	62 (31.8)	64.9	22	61 (31.3)	2
Total Beijing	714 (78.5)	509 (71.3)	205 (28.7)	63.0	60	228 (31.9)	10
Beijing sublineages[a]							
ST11	3 (0.4)	3 (100)	0 (0)	76.3	0	0 (0)	0
ST26	50 (7.0)	38 (76.0)	12 (24.0)	58.5	5	20 (40.0)	1
STK	111 (15.5)	74 (66.7)	37 (33.3)	68.1	4	14 (12.6)	2
ST3	182 (25.5)	122 (67.0)	60 (33.0)	63.4	13	59 (32.4)	4
ST25	6 (0.8)	2 (33.3)	4 (66.7)	58.2	0	0 (0)	0
ST19	194 (27.2)	140 (72.2)	54 (27.8)	64.5	17	63 (32.5)	2
Modern[c]	168 (23.5)	130 (77.4)	38 (22.6)	58.7	21	72 (42.9)	1

[a] Sequence type designations are from Filliol et al. (6) and Iwamoto et al. (11).
[b] Clusters consisted of isolates having identical 19-locus VNTR profiles.
[c] Modern Beijing strains are ST10 (135 isolates), ST22 (32 isolates), and ST19 (1 isolate).
[d] MDR, multidrug resistant.

lated from elderly TB patients (these strains represent the population structure that existed decades ago) and young TB patients (these strains reflect the population structure of currently prevalent strains) (Table 2). The major cause of TB in the elderly is the reactivation of the *M. tuberculosis* strains acquired prior to World War II, when TB was highly prevalent in Japan (20, 22). The fact that the cluster rate in the cohorts born in or before 1925 is lower than that of cohorts born later (Table 2) indicates that the elderly are not actively involved in recent TB transmission. A comparison of the *M. tuberculosis* populations isolated from cohorts born in different years (Table 2) suggested that the population structure of the *M. tuberculosis* Beijing family strains in Japan before World War II—when TB was highly prevalent—was significantly different from that of the currently prevalent strains. A probable artifact bias from an unrecognized large-scale outbreak could be ruled out since no large cluster formation was found in a particular generation (see Table S2 in the supplemental material). Notably, the incidence of disease due to the modern Beijing strains was low in the elderly (18% in the cohort born in 1925 or earlier and 15.3% in the cohort born from 1926 to 1935) but highest in the young (31.1% in the cohort born in 1965 or later) (Table 2) ($P = 0.016$, Z test for the proportions between the cohort born in 1925 or earlier and the cohort born in 1965 or later).

The same trend was observed when we rechecked the data from our previous study using 355 Beijing family strains obtained from Osaka (175 isolates) and Kobe (180 isolates) (27). The data from Osaka showed that 2 out of 21 (9.5%) isolates from patients more than 75 years old were modern strains, while this number was 9 out of 35 (26.5%) in patients less than 35 years old. These data, which were collected from different cities in Japan, strengthened the conclusion of this study that the modern Beijing strains show a higher rate of occurrence in the young. On the other hand, the strains belonging to the STK sublineage, which accounted for 23.3% of the strains isolated from the cohorts born in 1925 or earlier, exhibited a significantly low incidence in the cohorts born later (Table 2). The cluster rates of these two sublineages were quite different (42.9% versus 12.6%) (Table 1). From these results, we assumed that the modern Beijing strains, with a high degree of transmissibility, are currently spreading in Japan, while there is a continuous shrinkage of the STK strains, with low transmissibility. Multidrug resistance does not appear to be the reason

TABLE 2. Distribution of *M. tuberculosis* Beijing sublineages among different birth-year cohorts

Birth-year cohort	Total no. of strains	No. (%) of isolates of indicated sublineage in birth-year cohort							No. (%) of isolates that clustered[a]		
		ST11	ST26	STK	ST3	ST25	ST19	Modern	Total	Ancient	Modern
1925 or before	133	1 (0.8)	6 (4.5)	31 (23.3)	33 (24.8)	1 (0.8)	37 (27.8)	24 (18.0)	21 (15.8)	17 (15.6)	4 (16.7)
1926–1935	202	1 (0.8)	11 (5.4)	36 (17.8)	57 (28.2)	3 (1.5)	63 (31.2)	31 (15.3)	50 (24.8)	39 (22.8)	11 (35.5)
1936–1945	119	1 (0.8)	10 (8.4)	17 (14.3)	30 (25.2)	0 (0)	29 (24.4)	32 (26.9)	39 (32.8)	28 (32.2)	11 (34.4)
1946–1964	154	0	11 (7.1)	17 (11.0)[b]	35 (22.7)	0 (0)	43 (27.9)	48 (31.2)[d]	68 (44.2)	40 (37.7)	28 (58.3)
1965 or after	106	0	12 (11.3)	10 (9.4)[c]	27 (25.5)	2 (1.9)	22 (20.8)	33 (31.1)[e]	50 (47.2)	32 (43.8)	18 (54.5)
Total	714	3 (0.4)	50 (7.0)	111 (15.5)	182 (25.5)	6 (0.8)	194 (27.2)	168 (23.5)	228 (31.9)	156 (28.5)	72 (42.9)

[a] Clusters consisted of isolates having identical 19-locus VNTR profiles.
[b] $P = 0.009$, Z test for proportionality (two-tailed) between cohorts with a birth year of 1925 or earlier and a birth year from 1946 to 1964.
[c] $P = 0.008$, Z test for proportionality (two-tailed) between cohorts with a birth year of 1925 or earlier and a birth year of 1965 or later.
[d] $P = 0.027$, Z test for proportionality (two-tailed) between cohorts with a birth year of 1925 or earlier and a birth year from 1946 to 1964.
[e] $P = 0.016$, Z test for proportionality (two-tailed) between cohorts with a birth year of 1925 or earlier and a birth year of 1965 or later.

(Table 1) for the prevalence of the modern strains, as reported in other studies (13, 16). The main reason for the occurrence of STK would be the endogenous reactivation of TB in elderly individuals. There were no significant differences in the incidence of the other ancient Beijing sublineages among the various cohorts (Table 2). It was reported that the modern Beijing sublineage shows a significantly higher transmissibility than the ancient Beijing sublineage among homeless people in Japan (28). Taken together, these data indicate that the population structure of *M. tuberculosis* in Japan may undergo changes and eventually resemble the typical worldwide situation, with a predominance of the modern Beijing sublineage. Further studies analyzing paleopathological samples, such as paraffin-embedded lung biopsy specimens or old cultures preserved for several decades, would be required to prove this hypothesis.

It is interesting to assume that the observed trends in the case of the modern and STK strains may be associated with the *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) vaccination. In Japan, mass vaccination with BCG was initiated in 1942. The individuals in the cohort born in the year 1925 and earlier were mostly infected with TB without receiving the BCG vaccination since 81% of the people in the cohort born from 1921 to 1925 were infected before they reached the age of 30 (22). On the other hand, it is highly probable that the cohort born in and after the year 1965 were administered BCG vaccines. It has been previously reported that the BCG vaccination favors the positive selection of modern Beijing strains (13). Our results support this finding, and we further demonstrate the possible negative selection of STK strains.

In conclusion, the population structures of the Beijing family strains in Japan were different for different birth cohorts. We believe that the modern Beijing strains, with a high degree of transmissibility, are currently spreading in Japan, while the spread of the STK strains, with low transmissibility, will decrease in the future. It is essential to continuously monitor the population shift for a long period in order to evaluate the effectiveness of current TB control measures and achieve better TB control in Japan.

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Allelic diversity of variable number of tandem repeats provides phylogenetic clues regarding the *Mycobacterium tuberculosis* Beijing family

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ABSTRACT

The Beijing family is the putative hypervirulent lineage of *Mycobacterium tuberculosis* that has been endemic in East Asia and has disseminated worldwide. The genetic population structure of Beijing family strains with regard to Japan is notable in its high diversity and dominance of the ancestral sublineage, in contrast to the modern sublineage found worldwide. Therefore, it is expected to be a suitable population model for investigating the microevolutionary process of the lineage. Variable number of tandem repeats (VNTR) has become a reliable genotyping method for *M. tuberculosis*, but its dynamics in the phylogenetic process remains unclear. Using 355 clinical Beijing family isolates in Japan, genetic traits, including VNTR, were analyzed and subjected to minimum spanning tree (MST) reconstruction. In the results, the topology of the tree was tightly related to other genotypic characters. We also found that some VNTR alleles were specific to each sublineage and provided clues for reconstructing a valid MST topology for the population. It is suggested that VNTR typing can elucidate genetic markers representing the phylogenetic classification in the lineage.

1. Introduction

With the progress of genotyping methods for bacterial species, variable number of tandem repeats (VNTR) analysis has been developed as a high-throughput and reproducible technique (Lindstedt, 2005). Its polymorphism is derived from genomic loci, including repetitive structures (units) of nucleotide sequences, in a genome. The number of repetitive units on each VNTR locus is enumerated as a molecular marker. With the advances in whole-genome sequencing of various bacterial species, VNTR loci have been increasingly identified and utilized for epidemiological purposes (Moxon et al., 1994; van Belkum, 1999; Lindstedt, 2005). In contrast to its successful introduction in epidemiology, the dynamics of VNTR alleles is generally so variable and reversible that they were considered not to be applicable for phylogenetic use. However, recently it has been considered that the rapid variation in VNTRs can coincide with short timescale of microevolution and can become phylogenetic traits in a population with high clonality. Actually, it has been reported that VNTR can provide valuable information for phylogenetic analysis of *Shigella sonnei* (Chiou et al., 2009). Grant et al. (2008) mathematically proposed the possibility of its phylogenetic use for *Mycobacterium tuberculosis*.

M. tuberculosis is the pathogen of tuberculosis (TB) which remains a major public health threat to the world. VNTR analysis has become a popular genotyping method for *M. tuberculosis*. Numerous VNTR loci have been identified systematically from the genome sequence of a reference strain, H37Rv (Cole et al., 1998; Supply et al., 2000). They have been analyzed and evaluated epidemiologically in various populations in order to improve VNTR analysis for a TB genotyping tool. Recently, Supply et al. (2006) proposed 15- or 24-MIRU-VNTR as an international standard set for *M. tuberculosis*. These VNTR sets were constructed and improved by analyses of strains with worldwide cosmopolitan origins.

Structure analysis of the global population of *M. tuberculosis* has highlighted the phylogeographic distribution of its genotypic lineages (Filliol et al., 2002, 2003, 2006). They appear to evolve in each endemic area and increase their adaptability by gaining protection against natural selections via the immunity of geographically or ethnically distinct human hosts (Hirsh et al., 2004; Gagneux et al., 2006). Linkage disequilibrium among VNTR loci has been observed in high-incidence areas of *M. tuberculosis*; this implies that the pathogen has gained its properties through clonal evolutions (Supply et al., 2003). Therefore, phylogenetic analysis of *M. tuberculosis* could be important to approaching genetic causes and determining a strategy for epidemiological

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trace-back studies. The Beijing family is one of the phylogenetic lineages of *M. tuberculosis* that is predominant throughout East Asia (van Soolingen et al., 1995; Kremer et al., 2004). Worldwide dissemination of the strains belonging to the lineage is also observed (Bifani et al., 2002; Glynn et al., 2002). Although their hypervirulence has been speculated upon, the causes and characteristics of this widespread distribution remain unknown. Previous studies have identified the genotypic traits and variations in this lineage. Mokrousov et al. (2002, 2005) reported that it could be divided phylogenetically into the ancient and modern (atypical and typical) subfamilies based on the absence or presence of a transposon IS*6110* in the NTF region. The latter sublineage is predominant in the world (Bifani et al., 2002; Mokrousov et al., 2005, 2006). Filliol et al. (2006) identified at least 11 types of sequences (STs) of the Beijing family by 212 single nucleotide polymorphisms (SNPs) in the structure analysis of their global population. Tsolaki et al. (2005) reported that large sequence polymorphisms (LSPs) in the genome could be observed in the lineage. These genotypic data may not only function as molecular markers of Beijing family strains, but also provide their micro-evolutionary process within the lineage.

In our previous study, we reported that the Beijing family strains in Japan mainly belonged to the ancient subfamily; this suggested that they became endemic independently from the evolutionary stream that led to the dominant modern Beijing subfamily (Wada et al., 2009). Phylogenetic investigation of Beijing family strains in Japan will be useful for understanding how they acquired hypervirulent phenotypes and adapted to the Japanese human host population. We also found that the clustering analysis based on 15-MIRU-VNTR was concordant with ST classification (Wada et al., 2009). It was suggested that VNTR alleles were phylogenetically informative in the population of the *M. tuberculosis* Beijing family.

In this study, we examined the genetic diversity and distribution of VNTR alleles of *M. tuberculosis* Beijing family strains in Japan to investigate the microevolutionary dynamics of VNTR. Our analyses will promote a better understanding of the population genetic structure of the Beijing family and extend the information derived from the VNTR genotyping method.

2. Materials and methods

2.1. Bacterial strains

A total of 355 *M. tuberculosis* Beijing strains were analyzed in this study. They were the same population as in our previous report (Wada et al., 2009). Briefly, they were isolated from individual patients with pulmonary TB in 3 hospitals and 2 public medical centres in Osaka and Kobe, Japan, between 2001 and 2004. They were entirely conveyed to our laboratories, and then identified as Beijing strains based on their spoligotyping patterns (Kremer et al., 2004). The trends of the population were similar to those of the whole population in Japan (Hirano et al., 1996; Ohmori et al., 2002). The genomic DNA of each strain was prepared from bacteria grown on Ogawa medium by using an ISOPLANT Kit (Wako, Osaka, Japan), according to the user's manual.

2.2. VNTR analyses

The genotypic data of 26 loci that comprised three sets of VNTR: classical MIRU-VNTR (Supply et al., 2000), 15-MIRU-VNTR (Supply et al., 2006), and JATA-VNTR (Murase et al., 2008; Maeda et al., 2008), and hypervariable locus VNTR 3232 (QUB-3232) were used in this study. The profiles were listed on a supplementary data in our previous study (Wada et al., 2009) and Table S1. Their copy number was calculated from their size and assigned according to the number of repeats for each locus, and in agreement with published allelic tables (Iwamoto et al., 2007). The accuracy of the size of amplified PCR fragments was confirmed by using a capillary electrophoresis system SV1210 (Hitachi High Technologies, Tokyo, Japan) or the ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA) (Iwamoto et al., 2007; Wada et al., 2007).

2.3. Phylogenetic characteristics of Beijing family isolates

The classification of the ancient and modern Beijing subfamilies and the STs of all isolates were determined in previous reports (Iwamoto et al., 2008; Wada et al., 2009). The presence or absence of three LSPs (RD181, RD150, and RD142) was examined by PCR. The length of each locus and the set of primer sequences were described in the previous study (Tsolaki et al., 2004). They were designed to detect deletions of LSP regions since shorter DNA fragments were amplified.

2.4. Minimum spanning tree

For the clustering analysis of VNTR profiles, a graphing algorithm termed minimum spanning tree (MST) has been introduced (Schouls et al., 2004, 2005; Liao et al., 2006; Wada et al., 2009). MST, similar to maximum-parsimony phylogenetic tree reconstruction methods, constructs a tree that connects all the genetic profiles in such a way that the summed genetic distance of all branches is minimized. It is particularly useful for analyzing genetic population structures with relatively high similarity. We performed MST construction by using the Bionumerics software version 4.6 (Applied Maths, St. Martens-Latem, Belgium) for the 355 Beijing family strains based on the VNTR genotyping. The rules for constructing trees were almost the same as in our previous report (Wada et al., 2009). The categorical coefficient was selected, and the priority rule was set such that the type that had the highest number of single-locus variants (SLVs) would be linked first. The creation of hypothetical types was disallowed.

3. Results

3.1. Phylogenetic classification of Beijing family isolates in Japan

All 355 Beijing family strains in this study were classified based on STs, two RDs, and IS*6110* insertion in the NTF region. Ten sublineages were identified (Table 1). They were defined phylogenetically as G1 to G9, according to the putative evolutionary order of their STs and deletions of RD. The phylogenetic classification was projected into clustering analysis of 15-MIRU-VNTR by MST (Fig. 1). Each of the major ancient Beijing sublineages in the population (G2, G3, G4 and G6a) was found to cluster topologically in the tree. On the other hand, modern Beijing sublineages (G6m, G7, G8 and G9) were observed to merge into a single branch indiscriminately.

3.2. Allelic diversity of each VNTR locus

To verify whether certain VNTR alleles are responsible for the phylogenetic topology in the MST analysis, we analyzed the allelic diversity and distribution patterns of 26 VNTR loci of five major phylogenetic sublineages (G2, G3, G4, G6a, and modern), respectively. Allelic diversity and distribution of all VNTR loci in each sublineage are listed in Table S2. Of these 26 VNTR loci, specific and exclusive alleles were detected in the case of seven VNTR loci in the corresponding phylogenetic groups (Table 2). Fig. 2 illustrates the allelic distribution of three VNTR loci (VNTR 2163b (QUB-11b), VNTR 4156 (QUB-4156), and VNTR 3232 (QUB-3232)) in each sublineage as examples. The number of repetitive

Table 1
Definition of phylogenetic sublineages of 355 Beijing family *M. tuberculosis* isolated in Japan.

Sublineage	No. of isolates (%)	Type of sequence	Large sequence			IS*6110* insertion polymorphism in the NTF (subfamily)
			RD181	RD150	RD142	
G1	4 (1.13)	ST11	+	+	+	Ancient
G2	28 (7.89)	ST26	+	+	+	Ancient
G3	51 (14.37)	STK	–	+	+	Ancient
G4	84 (23.66)	ST3	–	+	+	Ancient
G5	2 (0.56)	ST25	–	+	+	Ancient
G6a	110 (31.0)	ST19	–	+	+	Ancient
G6m	1 (0.28)	ST19	–	+	+	Modern
G7	50 (14.08)	ST10	–	+	+	Modern
G8	11 (3.10)	ST10	–	–	+	Modern
G9	14 (3.94)	ST22	–	+	+	Modern

units of QUB-11b was converged into three in G3, although the allelic distribution in the other sublineages mainly ranged from five to seven (Fig. 2A). The alleles in the case of QUB-4156 ranged mainly from three to five in the total population, but the alleles in each group were almost constant (Fig. 2B). On the other hand, the hypervariable VNTR, QUB-3232, exhibited a broad allelic variability in all the sublineages (Fig. 2C).

3.3. Phylogenetic VNTR loci and MST topology

An MST tree was constructed by using the seven loci listed in Table 2 (Fig. 3). Although the number of genotypes drastically decreased, the topology of the tree was almost concordant with the phylogenetic classification of the strains. We noticed that 15-MIRU-VNTR did not include the VNTR loci that provided putative phylogenetic information specific to G2 (Table 2). When VNTR 3155 (QUB-15), a specific allele of which is present in G2, was included along with the 15-MIRU-VNTR genotypes, a branch of the sublineage was reinforced in the MST construction (Fig. S1) and was more distinct than that of the original tree (Fig. 1). In contrast, exclusion of phylogenetically informative loci from the 15-MIRU-VNTR genotypes resulted in the disappearance of the MST branches corresponding to the respective sublineages (Fig. S2).

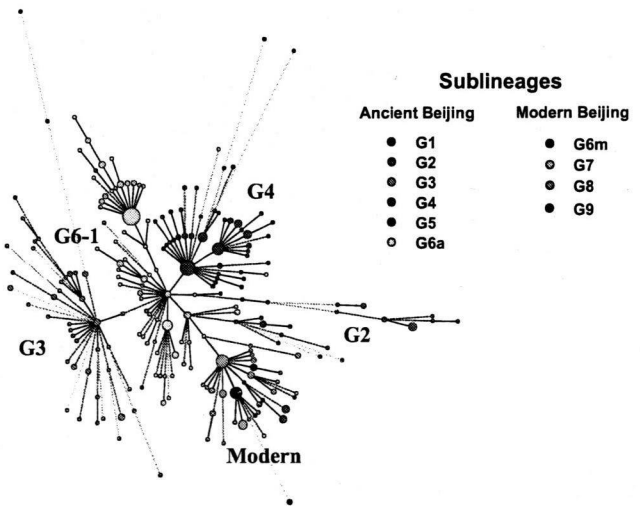

Fig. 1. A minimum spanning tree based on 15-MIRU-VNTR genotyping of 355 *M. tuberculosis* Beijing isolates. The 202 circles depicted correspond to the different types discriminated by 15-MIRU-VNTR genotypes. Their sizes correspond to the number of isolates with a particular genotype. They were coloured according to the phylogenetic sublineages listed in Table 1. Heavy lines connecting two types denote single-locus variants; thin lines connect double-locus variants; and dotted lines (black), triple-locus variants. The grey dotted lines indicate the most likely connection between two types differing by more than three VNTR loci.

4. Discussion

In this study, we have investigated the phylogeny of the *M. tuberculosis* Beijing family in Japan and estimated the phylogenetic potentiality of VNTR analysis. The results showed that 7 of 26 VNTR loci could provide phylogenetic clues regarding the Beijing strain population analyzed in this study. Previously, successful phylogenetic analyses of *M. tuberculosis* have been conducted by analysis of minor variations in the DNA sequence, such as SNPs and RDs, and spoligotyping (Sola et al., 2001; Tsolaki et al., 2004; Filliol et al., 2006; Gutacker et al., 2006). VNTR genotyping was considered to be a promising tool for discriminating between clinical isolates, but not for phylogenetic interpretation. In general, it is not possible to distinguish between identical VNTR alleles in a broad genetic population due to VNTR homoplasy. In this study, we targeted Beijing family strains obtained in Japan; these strains constitute a major part of the ancient Beijing subfamily. The genetic diversity of the singular population might be so consistent with the timescale of variation of seven VNTR loci (Table 2) that the phylogenetic traits could be observed.

The modern Beijing subfamily could be divided into four sublineages (G6m, G7, G8, and G9) on the basis of SNPs and RD150 analysis in our population (Table 1). However, these sublineages could not be identified in the MST construction based on the VNTR analysis (Fig. 1). Although there appears to be a specific VNTR 2372 allele in G8 (Table 2), we were unable to obtain a sufficient number of strains to determine any obvious MST branches for this sublineage in this study (Fig. 3 and Fig. S1). The scale of phylogenetic diversity of the modern Beijing subfamily seems to be too small to be estimated by the timescale of VNTR variation. This is contrary to the successful classification of the ancient

Table 2
Specific VNTR alleles observed in each phylogenetic sublineage of Beijing family *M. tuberculosis* isolates.

VNTR locus	Specific allele	Corresponding sublineage(s)	Specificity to corresponding sublineage(s) (%)	Occupation in corresponding sublineage(s) (%)
0960 (MIRU10)	1	G4	100	95.2
1955 (Mtub21)	4	Modern[a]	80.7	94.7
2163(QUB-11b)	3	G3	95.3	80.4
2372 (JATA06)	4	G4	85.7	88.9
	1	G8	84.6	100
2401 (Mtub30)	2	G3	98.0	78.1
3155(QUB-15)	2	G2	100	96.6
4156 (QUB-4156)	3	Modern[a]	86.9	96.2
	4	G2, G3	94.9	94.9
	5	G4, G5, G6a	98.4	92.9

[a] Modern subfamily including four sublineages: G6m, G7, G8, and G9.