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抗酸菌における菌体構成成分の動態解析

分担研究報告書

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研究要旨.
結核菌やらい菌を含む抗酸菌の菌体成分には、糖脂質成分をはじめとして様々な分子が含まれる。主に菌体表層或いは細胞膜・壁に存在する糖脂質やタンパク質については、病原性との関連がこれまでの多くの研究により指摘されている。一方、菌体内に存在する有機酸やアミノ酸などの基礎代謝系に関わる物質についてはその動態を含めほとんどが明らかになっていない。本研究では、抗酸菌の中でもハンセン病の原因細菌であり且つ生体内でのみ増殖可能ならい菌に注目し、菌体内に蓄積された基礎代謝産物の同定及び定量化を行った。その結果、同定された物質の種類・量の両面において、一部の化合物群が他の抗酸菌とは異なる動態を示した。このことは、らい菌が細胞内寄生等の病原性を発揮する上で、自らが保有する特異的な代謝機構を利用している可能性を示唆しており、らい菌を含む抗酸菌の基礎代謝系の一端が明らかとなった。

A. 研究目的

アジア等の発展途上国を中心に現在もなお年間数十万人の新患が発生しているハンセン病は、らい菌によって引き起こされる慢性抗酸菌感染症である。その主な病原性は、末梢神経障害等であるが、それに至る過程でらい菌は、慢性的に宿主内で増殖を続けるいわゆる細胞内寄生性を発揮する。しかしながら、その機構は未だ解明されておらずハンセン病の対策上、病態と関わるこの性質を解明することは重要である。結核菌やらい菌などの抗酸菌は、多種多様な糖脂質分子を豊富に含む細胞壁を有する。この特性は抗酸菌の重要な生化学的特徴であり、宿主免疫システムからの防御や逃避といった場面で役割を負うことが知られている。一方で、宿主の攻撃から逃れた抗酸菌が細胞内で増殖していくには、細胞内の極めて偏った栄養環境を利用し得る能力を有する必要がある。このことは、らい菌が宿主細胞内でのみで分裂増殖し *in vitro* では培養が不可能であることなどから示唆されるが、未だその仕組みについては不明である。そこで、本研究では、らい菌を含む抗酸菌の基礎代謝系ついて物質的な側面から網羅的な解析を行い、細胞内寄生性へ寄与する機構を明らかにすることを目的とする。

B. 研究方法

らい菌は、*Mycobacterium leprae* Thai53 株を使用し、BALB/c ヌードマウスの足蹠部位に接種して９カ月間かけて増殖させた。足蹠部位より 1.5×10^9 個の菌体を調製し、Milli-Q 水による洗浄後、内部標準物質を含むメタノールによって基礎代謝産物が含まれる菌体内部の成分を抽出した。クロロホルムによる脂質成分、さらに限外濾過フィルターによるタンパク質成分の除去をそれぞれ行い、分析用サンプルとした。解析には、イオン性化合物の検出に適した CE-MS (capillary electrophoresis-mass spectrometry) 法を採用し、サンプル中に含まれる全ての既知化合物を同定した。らい菌に対するコントロール群として、7H9+ADC 培地で培養した BCG-Tokyo 株を 1.8×10^9 個調製し、らい菌と同様に菌体内成分の抽出及び分析を行った。一方で、菌体内

物質の抽出を通して最終的に分析サンプルに含有される内部標準物質は、らい菌及びBCG の同一菌数当たり等量になるように添加した。内部標準物質及び各化合物の検出ピーク面積から算出された比率により半定量化を行った。

<u>倫理面への配慮</u>　倫理面を配慮すべきサンプル等は取り扱っていない。

C. 研究結果

らい菌及び BCG 由来サンプルより、合計 167 個の化合物が検出された。この内、68 個がカチオン（陽イオン性化合物）で、99 個がアニオン（陰イオン性化合物）であった。また、167 個の化合物の内、らい菌サンプルから 130 個が、BCG サンプルから 133 個がそれぞれ検出された。さらに、96 個の化合物が両方のサンプルに認められた（図 1）。

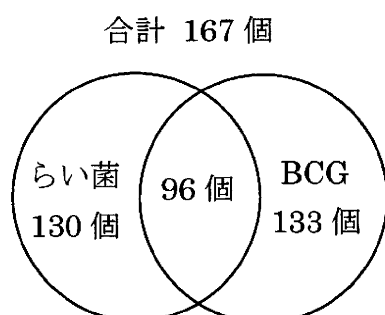

図 1. 検出化合物の内訳

検出されたピークエリアより、添加した内部標準物質に対する各化合物の検出比率を算出した。らい菌及び BCG の両方より検出された 96 個の化合物について、同一菌数当たりの検出比率を比較した結果、らい菌に多く認められたものは 73 個で、その内、一 1〜2 倍の比率で多く検出された化合物は 17 個で、さらに 2〜10 倍は 35 個、10 倍以上は 21 個であった。一方、96 個の内 23 個が BCG 側に多く認められた。BCG に検出されたが、らい菌に認められなかった化合物は 37 個であった。それらの主なものは ATP, ピルビン酸, マレイン酸, GTP, CTP 等であった。逆に、らい菌にのみ検出された化合物は 34 個であり、それらは Asparagine, Guanine, シキミ酸, グアノシン等であった。著しい検出比率の差やらい菌、BCG どちらか一方だけに認められた化合物の一部を基礎代謝経路に当てはめて比較すると、らい菌では、核酸合成及び解糖系・TCA サイクル経路上に位置する化合物の検出率が BCG に比べ著しく低下していた反面、アミノ酸の多くが蓄積していた（図 2）。

D. 考察

らい菌の大きな微生物学的特徴として、生体内でのみ分裂増殖し *in vitro* ではそれが不可能であることが挙げられる。この性質は、らい菌のゲノムは偽遺伝子が膨大な割合を占めることからエネルギー産生等に関与する基本的な遺伝子群が機能しなくなってしまっているという推測により説明できる。しかしながら、らい菌の全ゲノムシークエンス解析等により明らかになった解糖系や TCA サイクル等の基礎代謝系に関与する遺伝子群は、結核菌のものと比較した場合、各経路において一部が偽遺伝子化しているのみで、大規模な脱落等は観察できない。このことはゲノム解析からのみでは実際の基礎代謝系の機能を評価することは困難であることを示している。そこで、本研究ではらい菌内部で蓄積された基礎代謝系産物を解析することで実際の経路がどのように動いているのかについて解析した。その結果、らい菌においては、解糖系、TCA サイクル等のエネルギー産生に関与する化合物が著しく欠落していた。それらの多くがらい菌では未検出であったが、クエン酸、Succinic acid 等の一部の化合物は十分量蓄積されていた（図 2）。つまり、エネルギー産生へと至る生合成の流れは多くが遮断されている一方で、断片的であるが機能を保っているステップも存在し得ることを示している。また、逆の現象として、BCG では検出されなかった多くのアミノ酸類がらい菌に蓄積していることが観察された。これは、らい菌がアミノ酸代謝を活性化することで他の代謝系を補っている、若しくは自

らの代謝系に利用するため宿主由来のアミノ酸を過剰に取り込んでいる可能性を示唆するものである。このように、らい菌がBCGとは異なった代謝系化合物のプロファイルを示したことは、生体内でのみ分裂増殖できるらい菌の特性を物質面から反映するものであると考えられる。今後は、混入物等の確認や厳密な定量化等、さらなる詳細な解析を行うことによりこれまで未解明であったらい菌基礎代謝系の全容が明らかになり、病原性に関わる細胞内寄生性のメカニズム解明へと至ることが期待される。

E. 結論

らい菌及びBCGに存在する菌体内低分子化合物をCE-MSにより比較解析した結果、らい菌においては解糖系・TCAサイクル等のエネルギー産生に関わる代謝系化合物が著しく欠落している一方、アミノ酸類が過剰に蓄積していることが判明した。

G. 研究発表

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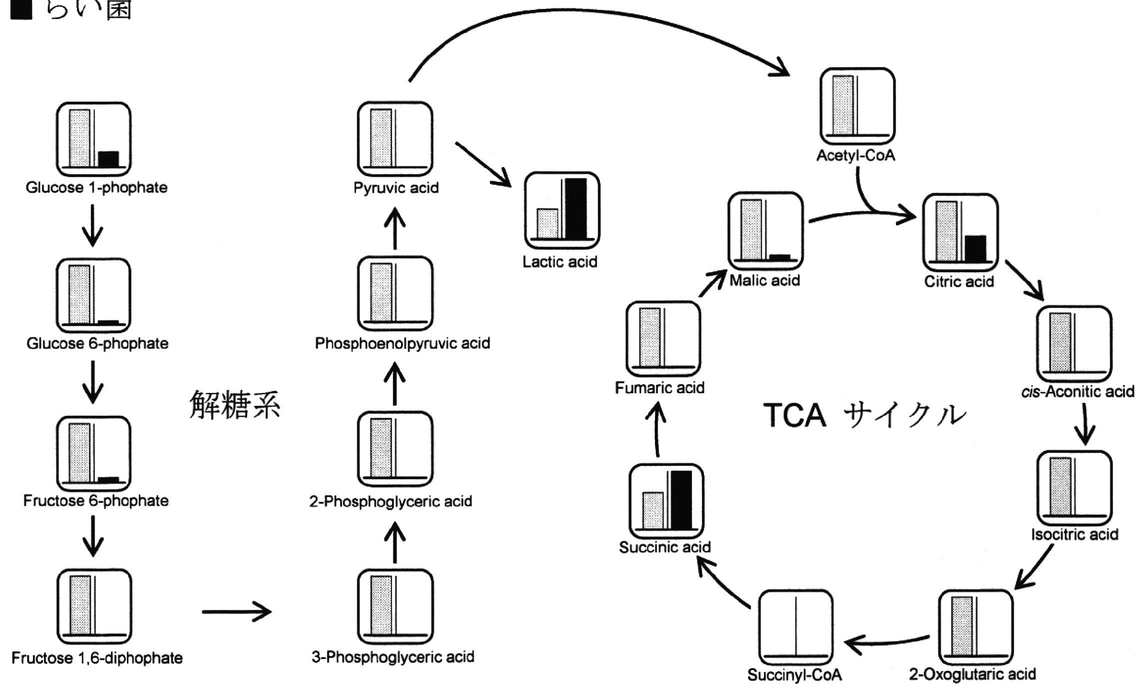
H. 知的財産権の出願・登録状況

1. 特許取得 なし
2. 実用新案登録 なし
3. その他 なし



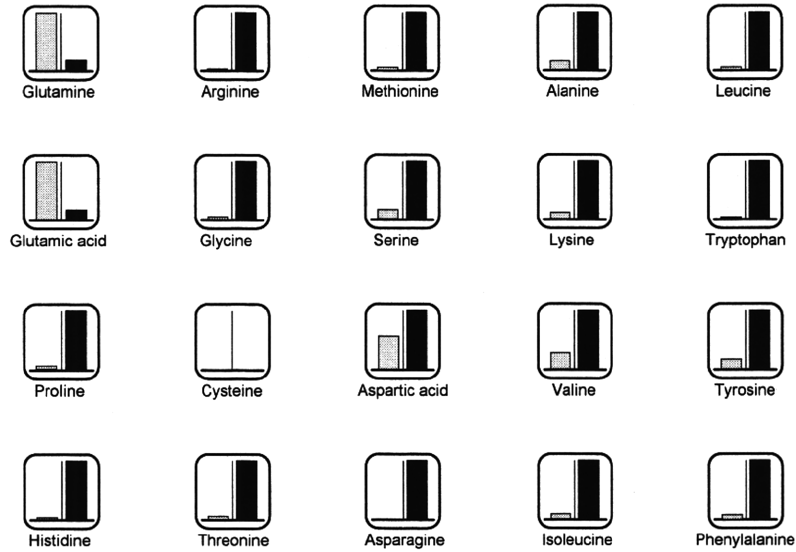


図 2. 解糖系-TCA サイクル関連化合物及びアミノ酸類の検出比率

研究成果の刊行に関する一覧表

別紙 5-1

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の 編集者名	書　籍　名	出版社名	出版地	出版年	ページ

別紙 5-1

雑誌

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RESEARCH LETTER

Biochemical characteristics among *Mycobacterium bovis* BCG substrains

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BCG; substrain; vaccine; biochemical characteristics; tuberculosis.

Abstract

In order to evaluate the biochemical characteristics of 14 substrains of *Mycobacterium bovis* bacillus Calmette Guérin (BCG) – Russia, Moreau, Japan, Sweden, Birkhaug, Danish, Glaxo, Mexico, Tice, Connaught, Montreal, Phipps, Australia and Pasteur – we performed eight different biochemical tests, including those for nitrate reduction, catalase, niacin accumulation, urease, Tween 80 hydrolysis, pyrazinamidase, *p*-amino salicylate degradation and resistance to thiophene 2-carboxylic acid hydrazide. Catalase activities of the substrains were all low. Data for nitrate reduction, niacin accumulation, Tween 80 hydrolysis, susceptibility to hydrogen peroxide and nitrate, and optimal pH for growth were all variable among these substrains. These findings suggest that the heterogeneities of biochemical characteristics are relevant to the differences in resistance of BCG substrains to environmental stress. The study also contributes to the re-evaluation of BCG substrains for use as vaccines.

Introduction

Biochemical tests are currently used as a technique for the identification of bacterial species. Recently, several studies have investigated the physiological meaning of the biochemical characters in the genus *Mycobacterium*. Sohaskey and colleagues reported variable nitrate production among *Mycobacterium bovis* bacillus Calmette Guérin (BCG) substrains in relation to survival in host cells (Sohaskey, 2008; Sohaskey & Modesti, 2009). Recycling of NAD and NAD-quinoline reductase relevant to the latent infection of *Mycobacterium tuberculosis* and resistance to oxidative stress, respectively, have also been reported (Boshoff *et al.*, 2008). Mycobacterial phospholipase A (MPLA) catalyses the hydrolysis of lipids including Tween 80 (Parker *et al.*, 2007), and this activity appears to contribute to survival under starvation at the dormant stage of growth (Jackson *et al.*, 1989; Deb *et al.*, 2009). Here, we analysed the biochemical characteristics and their relationship to susceptibility to environmental stress, such as oxidative stress, nitrosative stresses and pH changes, among BCG substrains.

Materials and methods

Bacterial strains

Mycobacterium bovis BCG strains Australia (ATCC 35739), Birkhaug (ATCC 35731), Connaught (ATCC 35745), Danish (ATCC 35733), Glaxo (ATCC 35741), Mexico (ATCC 35738), Montreal (ATCC 35735), Pasteur (ATCC 35734), Phipps (ATCC 35744), Tice (ATCC 35743), Russia (ATCC 35740) and *M. tuberculosis* strain $H_{37}Rv$ (ATCC 25618) were purchased from American Type Culture Collection (ATCC, Manassas, VA). BCG-Moreau, *M. bovis* (JATA) and *Mycobacterium smegmatis* were provided by Dr M. Takahashi (The Research Institute of Tuberculosis Japan Anti-tuberculosis Association, Kiyose, Tokyo, Japan). BCG-Japan (Tokyo 172) was purchased from Japan BCG Laboratory (Kiyose, Tokyo, Japan). BCG-Sweden (vaccine seed) was provided by Dr S. Yamamoto (Japan BCG Laboratory). *Mycobacterium avium* strains 724S and 2151SmO were kindly provided by Drs J. Inamine and E. Torsten (Colorado State University, Fort Collins, CO).

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Bacterial culture and freeze stock

Bacterial culture and freeze stocking were performed as reported by Hayashi *et al.* (2009).

Biochemical tests

Tests for nitrate reduction, catalase, Tween 80 hydrolysis, urease, pyrazinamidase and resistance to thiophene 2-carboxylic acid hydrazide (TCH) were performed by standard procedures except as described below (Gangadharam & Jenkins, 1998). Nitrate reduction was performed by the classical procedure with liquid reagent. Pyrazinamidase activity was tested on Middlebrook 7H11 broth (BD, Franklin Lakes, NJ) instead of Dubos broth. Resistance to TCH was determined on solid Ogawa medium containing 1 or $10\,\mu g\,mL^{-1}$ TCH. Niacin accumulation was detected using the Kyokuto Niacin Test (Kyokuto Pharmaceutical Industries, Tokyo, Japan) in accordance with the manufacturer's instruction. Degradation of *p*-amino salicylate (PAS) was determined according to Tsukamura (1961). *Mycobacterium tuberculosis*, *M. bovis*, *M. avium* and *M. smegmatis* were used as controls. In the urease test, urease-deficient recombinant BCG (Mukai *et al.*, 2008) was used as a negative control.

Culture and differentiation of THP-1 cells

The human monocytic cell line THP-1 (ATCC TIB202) was purchased from ATCC and maintained in RPMI 1640 medium containing $100\,U\,mL^{-1}$ penicillin G and 5% heat-inactivated fetal bovine serum (FBS). THP-1 cells were stimulated with 10 nM phorbol 12-myristate 13-acetate (PMA; Wako Pure Chemical Industries, Osaka, Japan) for 24 h to be differentiated to macrophages. Cells were washed three times with culture medium and used for the assays.

Isolation and culture of bone marrow-derived macrophages (BMMs)

Bone marrow was isolated from the tibias and femurs of C57BL/6J female mice at 4–8 weeks of age. Bone marrow cells haemolysed in 0.83% NH_4Cl–Tris buffer were cultured in RPMI 1640 supplemented with 10% FBS, $100\,U\,mL^{-1}$ penicillin G, $50\,\mu M$ 2-mercaptoethanol and $10\,ng\,mL^{-1}$ granulocyte-macrophage colony-stimulating factor (Wako) in 24-well culture plates; the culture medium was refreshed every 2 days. On day 7, adherent cells were collected and used for the assays.

Macrophage infection

Macrophages infected with bacilli at a multiplicity of infection (MOI) of 20 were incubated at 37 °C for 6 h. Extracellular bacilli were washed out three times and killed by $100\,\mu g\,mL^{-1}$ amikacin treatment for 6 h. Interferon (IFN)-γ (final concentration of $100\,U\,mL^{-1}$) was added to some of the wells as a stimulator. Following incubation, cells were washed three times and ruptured with $100\,\mu L$ of sterile distilled water. To determine the number of intracellular live bacteria, the lysates were diluted and plated on 7H11 agar in triplicate. Colonies were counted after 3 weeks' incubation.

Tolerance test for hydrogen peroxide and nitric oxide

Bacilli (2×10^6 CFU) were incubated in 7H9 broth containing albumin, dextrose (without catalase) and 0–10 mM H_2O_2 for 6 h. In the same manner, bacilli were incubated in 7H9 broth supplemented with ADC (albumin, dextrose, catarase) and containing 0–10 mM $NaNO_2$, as an NO donor, at pH 6.6, 6.0 or 5.5 for 3 days. Following incubation, bacilli were washed with 7H9 medium three times, diluted and plated on 7H11 agar. Plates were incubated for 3 weeks and the percentage of live bacilli relative to control (0 mM H_2O_2 or $NaNO_2$) was calculated.

Determination of permissive pH range for growth of bacilli

Bacterial log-phase cultures in Middlebrook 7H9 (BD) supplemented with 10% ADC (BD) were adjusted to an OD of 0.1 at 530 nm and mixed with 100-fold volume of various pH-adjusted broths (pH 3, 4, 5, 5.4, 5.7, 6.2, 6.6, 7, 8, 9, 10, 11 and 12, adjusted with HCl or NaOH). Following incubation at 37 °C for 21 days, bacterial growth was evaluated by measuring OD at 530 nm.

Statistical analysis

Each experiment was repeated three times. Statistically significant differences between two series were assessed by Student's *t*-test or Aspin–Welch's *t*-test following an *F*-test assessment of variance.

Results and discussion

Eight different biochemical tests, nitrate reduction, niacin, catalase, Tween 80 hydrolysis, urease, pyrazinamidase, PAS degradation and resistance to TCH, were applied to 14 substrains of BCG, BCG-Russia, -Moreau, -Japan, -Sweden, -Birkhaug, -Danish, -Glaxo, -Mexico, -Tice, -Connaught, -Montreal, -Phipps, -Australia and -Pasteur (Table 1). BCG-Birkhaug was positive for nitrate reduction whereas BCG-Mexico, -Australia and -Pasteur were negative; the other BCG strains were weakly positive, although *M. bovis*, the parental strain of BCG, was negative. The nitrate respiration system may be responsible for the survival of *M. tuberculosis* under anaerobic conditions (Sohaskey, 2008), and the nitrate reductase gene *narGHJI* contributes to the virulence of BCG in immunodeficient mice (Weber *et al.*, 2000). BCG-Russia and -Japan survived better both in THP-1 and in mouse BMMs than other substrains (Fig. 1 and Table 1). Although host *M. bovis* was negative for nitrate reduction,

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Table 1. Summary of characteristics of BCG substrains *in vitro**

Organism	Nitrate reduction	Niacin accumulation	Tween 80 hydrolysis	Urease	Pyrazinamidase Day 4	Pyrazinamidase Day 7	PAS degradation	Resistance to TCH ($\mu g\ mL^{-1}$) 1	Resistance to TCH ($\mu g\ mL^{-1}$) 10	Catalase (room temperature) Bubble column (mm)	Catalase (room temperature) Activity	68 °C catalase	H_2O_2 tolerance	NO tolerance	Optimal pH	Viability in THP-1 −IFN-γ	Viability in THP-1 +IFN-γ	Viability in BMM −IFN-γ	Viability in BMM +IFN-γ
BCG[‡]																			
Russia	±	+	–	+	–	–	–	+	–	9.3 ± 2.4	Low	–	+	–	6.6	+	+	+	+
Moreau	±	+	±	+	–	–	–	+	–	7.1 ± 1.8	Low	–	ND	ND	ND	ND	ND	ND	ND
Japan	±	+	–	+	–	–	–	+	–	14.8 ± 2.3	Low	–	+	+	6.6	+	+	+	+
Sweden	±	+	±	+	–	–	–	+	–	6.7 ± 1.7	Low	–	–	–	8–9	–	–	+	+
Birkhaug	+	+	–	+	–	–	–	+	–	11.8 ± 2.3	Low	–	+	–	8–9	+	+	–	–
Danish	±	–	±	+	–	–	–	+	–	9.4 ± 2.4	Low	–	–	+	7–8	+	+	–	+
Glaxo	±	–	–	+	–	–	–	+	–	7.4 ± 1.1	Low	–	–	+	7–8	–	–	–	–
Mexico	–	–	–	+	–	–	–	+	–	6.4 ± 1.8	Low	–	ND	ND	ND	ND	ND	ND	ND
Tice	±	–	–	+	–	–	–	+	–	6.3 ± 1.6	Low	–	ND	ND	ND	ND	ND	ND	ND
Connaught	±	–	±	+	–	–	–	+	–	7.9 ± 1.9	Low	–	+	–	7–8	–	–	–	+
Montreal	±	+	±	+	–	–	–	+	–	6.0 ± 2.3	Low	–	ND	ND	ND	ND	ND	ND	ND
Phipps	±	+	±	+	–	–	–	+	–	6.0 ± 2.2	Low	–	–	–	6.6	–	–	–	–
Australia	–	–	±	+	–	–	–	+	–	6.1 ± 2.1	Low	–	ND	ND	ND	ND	ND	ND	ND
Pasteur	–	–	±	+	–	–	–	+	–	7.3 ± 2.6	Low	–	–	+	6.6	+	+	–	+
M. bovis	–	–	–	+	–	–	–	+	–	5.4 ± 0.7	Low	–	+	+	6.6	+	+	+	+
M. tuberclosis																			
H37Rv	+	+	±	+	+	+	–	+	+	8.4 ± 1.1	Low	–	ND	ND	ND	ND	ND	ND	ND
H37Ra	+	+	±	+	+	+	–	+	+	10.0 ± 1.6	Low	–	ND	ND	ND	ND	ND	ND	ND
M. avium																			
724S	–	–	–	–	+	+	–	+	+	35.8 ± 13.0	Low	–	ND	ND	ND	ND	ND	ND	ND
2151SmO	–	–	–	–	+	+	–	+	+	27.6 ± 3.5	Low	+	ND	ND	ND	ND	ND	ND	ND
M. smegmatis	+	–	+	–	+	+	–	+	+	14.0 ± 1.3	Low	–	ND	ND	ND	ND	ND	ND	ND

*Summarizing the data from biochemical tests, tolerance to oxidative stress (Fig. 1) and survival activities in host cells (Fig. 2).
[†]Scores indicate the numbers that are positive (+) and slightly positive (±).
[‡]BCG substrains, which were historically distributed from the Pasteur Institute, are given in chronological order.
Methods for conventional biochemical tests for mycobacteria are described in Materials and methods. Experiments were conducted more than three times. Representative results are indicated.
ND, no data

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the viability in host cells was higher than BCG (Table 1 and Fig. 1). According to the standard method for the nitrate reductase test, the assay period was 2 h. Under different conditions, for example longer incubation times and anaerobic conditions, nitrite production has been found in some BCG strains (Weber *et al.*, 2000; Sohaskey & Wayne, 2003; Stermann *et al.*, 2003; Sohaskey & Modesti, 2009). Therefore, different incubation times could explain the discrepancy observed between nitrate reductase test results and intercellular survival. Nitrate reductase activity is not the sole explanation, but we believe it is partly responsible for the survival in host cells, as shown in previous reports (Weber *et al.*, 2000; Sohaskey, 2008) and the present study.

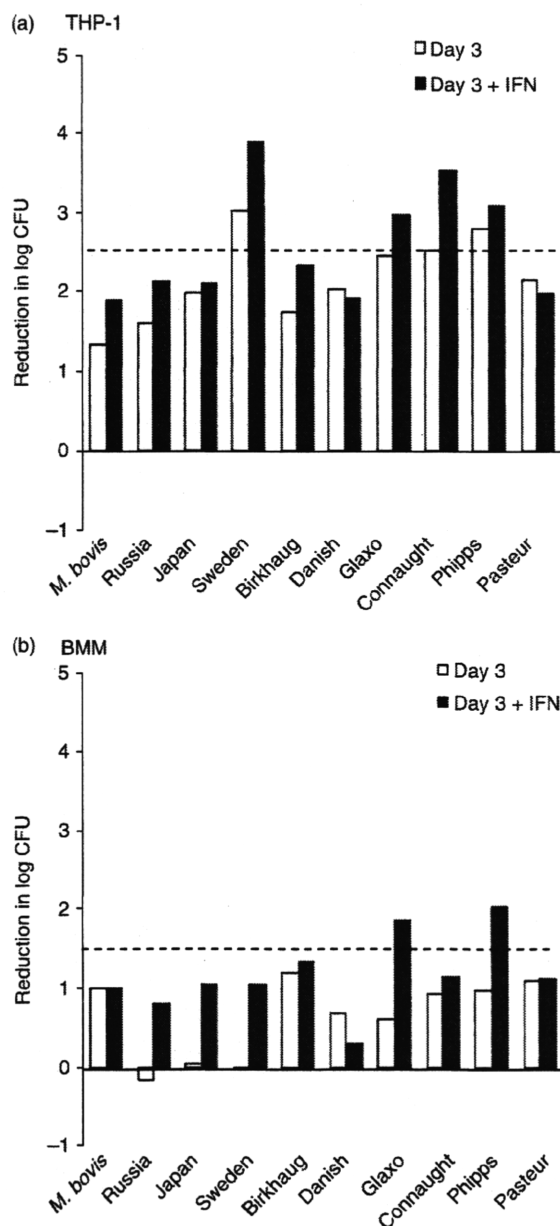

Fig. 1. Viability of BCG strains in THP-1 and mouse BMMs. PMA-differentiated THP-1 (a) or mouse BMMs (b) were infected with BCG at an MOI of 20 with (solid) or without (open) 100 U mL^{-1} of IFN-γ as a stimulator. After 6 h of infection, BCG CFU counts were determined from infected cell lysates and were monitored on days 0, 3 and 7. The data are expressed as the reduction in $\log_{10}$ CFU compared with control at day 0. Error bars represent means+SD for triplicate results from one of two similar experiments. Statistically significant differences between BCG group Russia, Japan, Birkhaug, Danish and Pasteur and BCG group Sweden, Glaxo, Connaught and Phipps were observed in (a) (Student's *t*-test, $P < 0.05$). In (b) there were statistically significant differences between BCG group Russia, Japan and Sweden and BCG group Birkhaug, Danish, Glaxo, Connaught, Phipps and Pasteur in the absence of IFN-γ (open column) (Aspin–Welch's *t*-test, $P < 0.05$). In the presence of IFN-γ (solid column) there were statistically significant differences between BCG group Russia, Japan, Sweden, Birkhaug, Danish, Connaught and Pasteur and BCG group Glaxo and Phipps (b) (Aspin–Welch's *t*-test, $P < 0.05$).

Heterogeneity of niacin accumulation was also observed among BCG substrains (Table 1). Recycling of NAD favours the latent infection of *M. tuberculosis* (Boshoff *et al.*, 2008), and NAD-quinoline reductase is responsible for resistance to oxidative stress (Akhtar *et al.*, 2006). These reports suggest that the activity of NAD metabolism is associated with the survival of BCG in macrophages or host cells. Whether the long or short survival of BCG in host cells favours the effectiveness of BCG has not been determined. However, the different characteristics of BCG substrains as reported here provide the basic information for further investigation of immunological characteristics and evaluation.

Parker *et al.* (2007) purified and characterized MPLA. MPLA is associated with cutinase, a serine esterase and catalyses the hydrolysis of lipids including Tween 80. MPLA activity was observed not only in pathogenic *M. tuberculosis*, but also in BCG-Pasteur. BCG-Pasteur was weakly positive for Tween 80 hydrolysis (Table 1). In fact, eight of the 14 substrains, namely BCG-Moreau, -Sweden, -Danish, -Connaught, -Montreal, -Phipps, -Australia and -Pasteur, were weakly positive. Mycobacteria are known to use this fatty acid as carbon source at the dormant stage. Therefore, this activity could contribute to survival under starvation conditions during dormancy (Jackson *et al.*, 1989; Deb *et al.*, 2009).

All BCG strains belong to the low-catalase group, although there were variations in the height of bubble column among them (Table 1). It was over 10 mm in BCG-Japan (14.8 mm) and -Birkhaug (11.8 mm) (Table 1). No mutation in the coding region of the *ahpC* gene among was observed among the substrains (data not shown). The

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differences between transcription of the genes and the activities have not yet been analysed. Catalase (*katG*) and peroxidase (*ahpC*) activities of *M. tuberculosis* are related to resistance to oxidative killing in human monocytes *in vitro* (Manca *et al.*, 1999). The expression of *katG* is partially regulated by ferric uptake regulators (*fur*), and contributes to the virulence of *M. tuberculosis* (Lucarelli *et al.*, 2008). Resistance to hydrogen peroxide of *M. bovis*, BCG-Russia and -Japan was higher than that of other BCG substrains (Fig. 1). This resistance relates well to survival in host cells, THP-1 and BMMs (Fig. 1). These findings suggest that resistance to H_2O_2 contributes to survival of BCG substrains in host cells and that enzyme activities other than of catalase could be relevant to the resistance to oxidative stress from host cells.

We next investigated the susceptibility of BCG substrains to nitrosative stress by exposing them to sodium nitrite for 3 days (Fig. 2b). BCG-Pasteur was tolerant to nitric oxide, and moderate susceptibility was observed in BCG-Japan, -Danish and -Glaxo. BCG-Russia, -Sweden, -Birkhaug, -Connaught and -Phipps were sensitive to NO. The parental strain of BCG, *M. bovis*, was able to tolerate NO. To assess NO production from the bacilli, reduction of pH of the media is required to generate NO from sodium nitrate (Darwin *et al.*, 2003; MacMicking *et al.*, 2003). Intriguingly, optimal pH levels were found to be different among the BCG substrains (Table 2). The optimal pH of BCG-Russia, -Moreau, -Japan, -Phipps, -Pasteur and *M. bovis* was 6.6. Optimal pH of BCG-Sweden and -Birkhaug was 8–9, and that of BCG-Danish, -Glaxo and -Connaught was 7–8. According to maturation state, pH in phagosomes decreases from about 6 to 4. All BCG strains were positive for urease (Table 1). The changes in pH of the culture broths for each BCG strain were not significantly different (data not shown). Therefore, these data indicate that the increasing pH of the culture broth, such as by generating ammonium, is not responsible for the tolerance of BCG strains to a reduction of pH. The precise mechanisms of adaptability to pH changes have not been elucidated.

In summary, we have evaluated the usefulness of various biochemical tests currently used for identifying mycobacterial species. Surprisingly, there were differences in the results of these tests among BCG substrains. These differences could be generated during the long time of passage of BCG vaccine strains. Their characteristics are quality controlled by lyophilizing techniques. A good correlation between oxidative and nitrosative stress and survival in host cells were observed among BCG substrains. The relationship between antigen presentation and viability in host cells is not clear. The longer persistence of the bacilli in the host cells may favour antigen presentation by continuous supply of the antigens, while short persistent bacilli may stimulate antigen presentation through a different pathway (Grode L

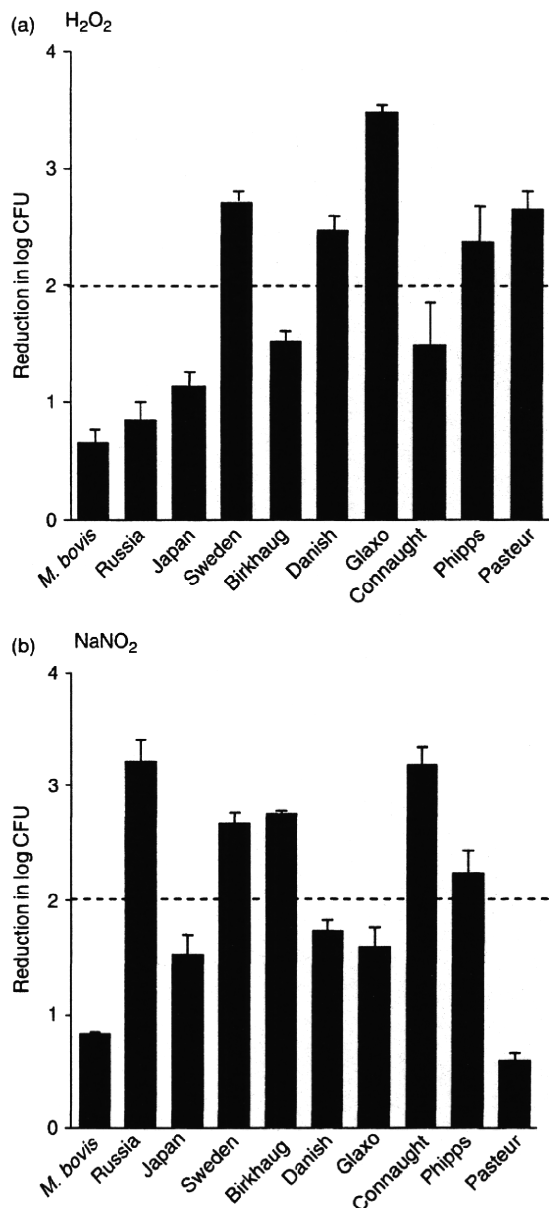

Fig. 2. Survival of BCG substrains in H_2O_2 and $NaNO_2$. In total, 2×10^6 CFU of *Mycobacterium bovis* or BCG substrains were treated with (a) 10 mM H_2O_2 for 6 h or (b) 10 mM $NaNO_2$ for 3 days. Treated and washed cells were serially diluted, and aliquots from four serial dilutions were plated in duplicate on 7H11 agar. The results are expressed as the reduction in $\log_{10}$ CFU compared with control at day 0. Error bars show means+SD of triplicate results from one of three similar experiments. BCG substrains, which were historically distributed from the Pasteur Institute, are aligned in chronicle order. In (a), statistically significant differences were found between BCG group Russia, Japan, Birkhaug and Connaught and BCG group Sweden, Danish, Glaxo, Phipps and Pasteur (Student's *t*-test, $P < 0.05$). In (b), statistically significant differences were found between BCG group Japan, Danish, Glaxo and Pasteur and BCG group Russia, Sweden, Birkhaug, Connaught and Phipps (Student's *t*-test, $P < 0.05$).

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Table 2. The range of pH permissive for growth of BCG and other mycobacteria

Organisms / broth pH	3	4	5	5.4	5.7	6.2	6.6	7	8	9	10	11	12
BCG													
Russia													
Moreau													
Japan													
Sweden													
Birkhaug													
Danish													
Glaxo													
Connaught													
Phipps													
Pasteur													
M. bovis													
M. tuberculosis $H_{37}Rv$													
M. avium TMC724S													
M. avium 2151SmO													
M. smegmatis													

BCG substrains, *Mycobacterium bovis*, *Mycobacterium tuberculosis*, *Mycobacterium avium* and *Mycobacterium smegmatis* were cultured in 7H9 broth at the indicated pH for 21 days and OD at 530 nm was monitored every 3 days.
Grey, pH ranges that the broth OD was above 0.1; black, maximal pH.

et al., 2005). Comparative analysis of BCG substrains on acquired immunity should be undertaken. This and our previous studies provide basic information on the biological characteristics and the effect on the innate immunological characteristics of BCG substrains, and these studies could contribute to the re-evaluation of BCG vaccine.

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